Screening of One-Bead-One-Peptide Combinatorial Library Using Red Fluorescent Dyes. Presence of Positive and False Positive Beads

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To screen one-bead-one-compound (OBOC) combinatorial libraries, tens of thousands to millions of compound beads are first mixed with a target molecule. The beads that interact with this molecule are then identified and isolated for compound structure determination. Here we describe an OBOC peptide library screening using streptavidin (SA) as probe protein, labeled with a red fluorescent dye and using the COPAS BIO-BEAD flow sorting equipment to separate fluorescent from nonfluorescent beads. The red dyes used were ATTO 590 and Texas Red. After incubating the library with the SA-red fluorescent dye conjugate, we isolated positive beads caused by peptide-SA interaction and false positive beads produced by peptide-fluorescent dye interaction. These false positives were a drawback when sorting beads by COPAS. However, an in depth analysis of both kinds of beads allowed the differentiation of positives from false positives. The false positive beads showed bright homogeneous fluorescence, while positive beads had a heterogeneous fluorescence, exhibiting a characteristic halo appearance, with fluorescence intensity greatest at the bead surface and lowest in the core. The difference was more evident when using Texas Red instead of ATTO 590. Thus, positive beads could be manually separated from false positive ones. The beads were analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). Most of the sequences obtained from positive beads had the His-Pro-Gln motif. Peptides from false positive beads were rich in Leu/Ileu, His, Phe, and Tyr.

Introduction

Combinatorial peptide libraries using the one-bead-one-peptide method were introduced by Lam et al.[2] and have proven to be a powerful approach for the discovery of binding peptides for various macromolecular targets. The one-bead-one-compound (OBOC) synthetic combinatorial library method, also called the "selectide process", involves the synthesis of millions of peptides on beads so that each bead displays only one peptide entity. With the OBOC method, ligands with pharmacological and analytical uses[3−5] and protein capture agents for purification or detection of proteins in complex mixtures[6,7] have been described.

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To screen these combinatorial libraries, tens of thousands to millions of compound beads are first mixed with the probe molecule. The beads that interact with it are then identified and isolated for compound structure determination. For probe molecules that cannot be detected directly, a reporter group, such as an enzyme or a fluorescent dye, is conjugated to them. If an antibody to the target molecule is readily available, an alternative method uses this antibody conjugated to a reporter group.[8]

In its native conception, the selectide process had some bottlenecks: (1) manual isolation of positive beads, (2) discarding of false positives, and (3) analysis of each bead by Edman microsequencing.

To reduce the cost and time of Edman microsequencing, in previous studies, we have reported a rapid and inexpensive strategy based on mass spectrometry analysis of peptide beads.[9,10] We used streptavidin (SA) as probe protein, labeled with peroxidase. SA was used as the probe protein because

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the peptides that interact with it with high affinity have been characterized in detail.[11]

The use of fluorescent dyes, instead of enzymes, to conjugate the probe protein allows the use of Complex Object Parametric Analyzer and Sorter (COPAS) BIO-BEAD flow sorting equipment (Union Biometrica) to facilitate the screening of OBOC libraries.[8,12] Furthermore, compared to enzymes, dyes are less costly, the coupling reaction to label the proteins is straightforward, and yields are high.[13,14] COPAS sorts at a rate of 50−100 thousand beads per hour on the basis of bead fluorescence intensity.

Here we assessed COPAS screening using SA conjugated with two red fluorescent dyes (ATTO 590 (SA-ATTO) and Texas Red (SA-TR)) and compared it with the screening process using SA conjugated with peroxidase (SA-POD).

The model library was the one-bead-one-peptide combinatorial library of the nonapeptide XXXXGGGGG, where X= Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Tyr and Val. The solid support was ChemMatrix, a poly(ethyleneglycol) (PEG) resin-based material with higher loading and higher chemical stability than other PEG resins.[15] We chose dyes with emission maximum in the red because resin beads exhibit a significant intrinsic fluorescence with an emission maximum in the green. This is a problem when screening against fluorescein-labeled proteins.[12]

Results and Discussion

The COPAS BIO-BEAD flow sorting instrument has the capacity to analyze and sort large objects (120−300 μm) at a high rate (up to 50 objects per second) on the basis of the physical characteristics of size, density, and fluorescence signals. To test the feasibility of this instrument for high-throughput screening, positive and negative control peptide beads were synthesized and screened using SA-ATTO 590 conjugate as described in the Materials and Methods. Lam and Lebl[11] reported that peptides containing the HPQ sequence interact with SA with high affinity. The positive control beads with the peptide FHPQGGGGG (FHPQ beads) showed red fluorescence and the negative control beads, with VGLVGGGGG immobilized (VGLV beads), were not fluorescent. The gate region for sorting was set so as to collect beads with high red fluorescence intensity (Figure 1).

A portion of the one-bead-one-peptide library was screened with SA-POD, and positive violet beads were selected manually and analyzed by MALDI-TOF MS. All the sequences contained the HPQ motif. Complete sequence data are included as Supporting Information (Table S1) from which a complete frequency for all amino acids was calculated (Figure S3, Supporting Information).

A new portion of the peptide library was poured into the COPAS without exposure to SA-ATTO, and no beads were sorted. Another portion of the library was exposed to SA-ATTO and poured into the COPAS to identify hits (Figure 2).

Hits were transferred into a Petri dish and examined under a fluorescence microscope. Those with the brightest fluorescent were isolated manually and analyzed by MALDI-TOF MS.[16] Table S1 (Supporting Information) shows the sequences obtained. None of the sequences contained the HPQ motif with high affinity for SA described by Lam et al.[11] Most of the peptides had Leu/Ileu, His, Phe, and Tyr, but no consensus sequence was found. An example of the MALDI-TOF MS analysis of a selected bead is included as Supporting Information (Figure S1). Another portion of the library was sorted manually, without pouring into the COPAS, and peptides with similar characteristics were obtained (Table S1, Supporting Information). When a portion of the library was incubated with ATTO 590, some beads became highly fluorescent. The fluorescent beads were isolated and analyzed by MALDI-TOF MS (Table S1, Supporting Information). The position frequency for all the amino acids of the peptide sequences identified by MALDI-TOF MS was calculated. The results are shown in Figure S1 (Supporting Information). The frequencies obtained were similar to those found in the screening with SA-ATTO in exception to the first position where His appeared more frequently. These findings evidence that the beads selected with SA-ATTO were false positives because of the interac-

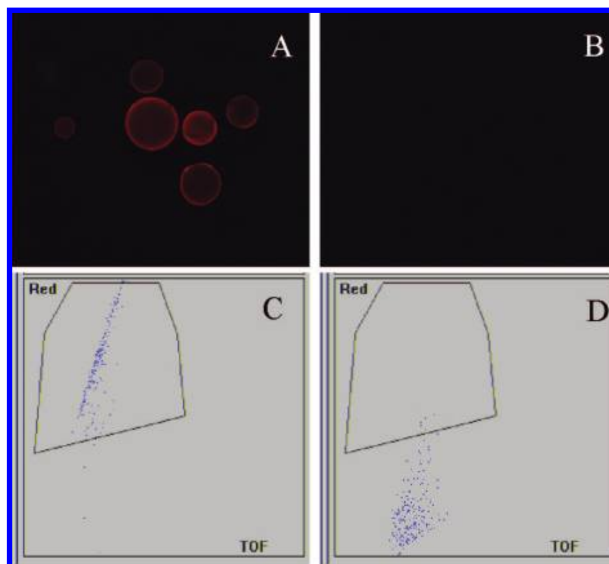

Figure 1. Fluorescence microscope images of beads after incubation with SA-ATTO taken with a 2.5× objective: (A) FHPQ and (B) VGLV. Gating and sorting regions defined for sorting beads on COPAS on the basis of their time-off-flight (TOF) to sort uniform sized beads and red fluorescence intensity (RED): (C) FHPQ and (D) VGLV beads after incubation with SA-ATTO.

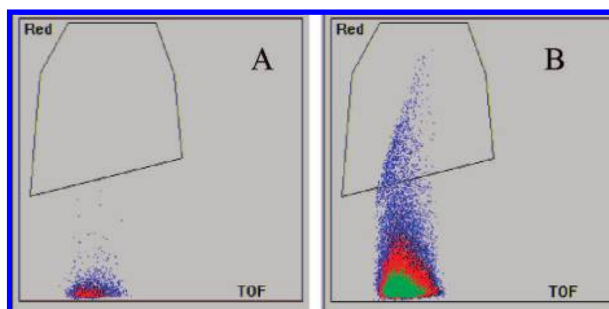

Figure 2. Gating and sorting regions defined for sorting beads on COPAS on the basis of their time-of-flight (TOF) and red fluorescence intensity (RED): a portion of the one-bead one-peptide library (A) without incubation and (B) after incubation with SA-ATTO.

tion of the ATTO 590 dye with some peptides. To evaluate whether these beads had affinity for the fluorescent dye and not for SA, some of them were resynthesized and incubated with SA-POD, SA-ATTO, and ATTO 590. All of the beads showed a positive reaction with SA-ATTO and ATTO 590; however, no positive reaction was observed with SA-POD, confirming that the peptides selected had affinity to ATTO dye and not to SA. The high content of hydrophobic amino acids in the false positive beads is in accordance with the overall fluorophore dye hydrophobicity. Rozinov and Nolan[17] described fluorescent dye-binding peptides with a high content of hydrophobic amino acids and high affinity for fluorescent dyes. Peptides interacting with ATTO 590 are a drawback for the OBOC screening.

We then evaluated Texas-Red. First, control positive beads (FHPQ beads) were incubated with SA-TR, and all the beads became fluorescent with a characteristic halo appearance, with highest fluorescence intensity at the bead surface and lowest in the core. Hu and co-workers found similar results performing an on-resin screening of a combinatorial peptide library with rhodamine B-streptavidin. They hypothesize that the halo effect is caused by the inability of the streptavidin to fully penetrate the resin.[18]

During the screening process, the beads were always incubated with an excess of BSA, thus to evaluate false positives, one portion of the library was incubated with BSA-TR 83 nM (0.083 μM) in the presence of 2% BSA (333 μM). Some beads became brightly and homogeneously fluorescent. The great molar excess of BSA respect to BSA-TR (approximately 4000×) allowed selection of peptide-beads that interacted with BSA-TR conjugate through the Texas Red and not via the BSA. Hence, these beads were false positives because of the interaction of the Texas Red with some peptides in the library.

When a portion of the library was incubated with SA-TR, two distinct groups of fluorescent beads were observed. One group had bright homogeneous fluorescence and the other had a heterogeneous fluorescence exhibiting a characteristic halo appearance, with fluorescence intensity highest at the bead surface and lowest in the core. The heterogeneously fluorescent beads were isolated manually and analyzed by MALDI-TOF MS. In this case, most of the sequences contained the HPQ motif described by Lam et al.[11] (Table S1 and Figure S4, Supporting Information).

Some of the peptide beads obtained with and without the HPQ motif were resynthesized and incubated with SA-TR and BSA-TR. Beads with the HPQ motif peptides showed heterogeneous fluorescence after incubation with SA-TR and no fluorescence after incubation with BSA-TR, while beads without the HPQ motif showed bright homogeneous fluorescence after incubation with SA-TR, as well as with BSA-TR. These results confirmed that the sequences obtained without the HPQ motif were false positives due to the interaction of the fluorescent dye with those peptides.

Figure 3 shows the difference between positive and false positive beads incubated with SA-ATTO and SA-TR. When using the latter, positive and false positive beads were clearly differentiated, and the former were successfully isolated manually from the library. When analyzed by MALDI-TOF MS, most of their peptides contained the HPQ motif (Table S1, Supporting Information). In contrast, when using SA-ATTO, although positive beads also showed heterogeneous fluorescence (halo appearance), there was little difference between positives and false positives, and we were unable to isolate positive beads from false positives.

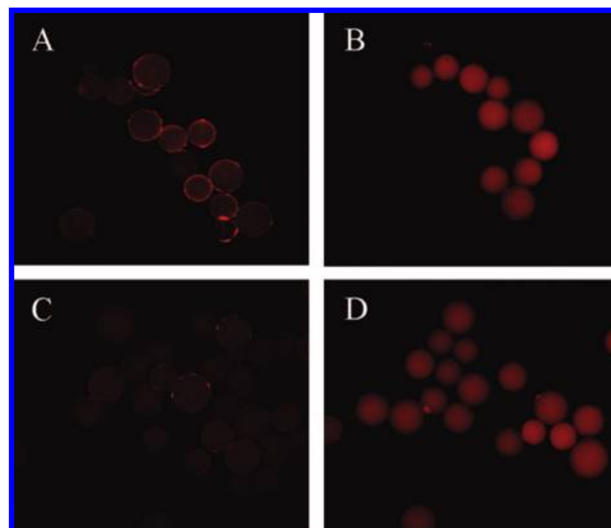

Figure 3. Fluorescence microscope images of beads taken with a 2.5× objective. (A) True positive beads after incubation with SA-TR, (B) false positive beads after incubation with SA-TR, (C) true positive beads after incubation with SA-ATTO, and (D) false positive beads after incubation with SA-ATTO.

Conclusions

The use of fluorescent dyes, instead of enzymes, to conjugate the probe protein allowed the use of COPAS to facilitate the screening of OBOC libraries.[8,12] COPAS sorted beads on the basis of their fluorescence intensity, and two groups of beads were discriminated: positive beads, as a result of the interaction of the proteins with the peptides, and false positives, caused by the interaction of the fluorescent dyes with the peptides. Peptides interacting with the fluorescent dyes are a drawback for the OBOC screening. The high content of hydrophobic amino acids in the false positive beads was in accordance with the overall fluorophore dye hydrophobicity. A manual inspection of the fluorescent beads sorted by the COPAS showed that false positives presented bright homogeneous fluorescence, while positive ones had a characteristic halo appearance.

ChemMatrix resin, as other PEG resins, is a porous gel-type matrix in the form of spherical particles. Small molecules like amino acids and peptides have full access to the pores, this allowing peptide synthesis with high loadings. High molecular weight proteins like streptavidin do not reach the interior of the bead, and therefore protein adsorption only takes place on the bead surface. Although resin swelling depends on the solvent used the screening process can be performed only in aqueous media.[19] We hypothesize that the difference between true positive and false positive beads is caused by the orientation of the complex on the bead surface. If the protein-dye complex binds to the peptide bead via peptide−protein interaction there is a space between the

bead and the emitter (fluorescent dye), thus resulting in beads with halo appearance. Alternatively, if the protein-dye complex binds by means of peptide-dye interaction, there is not any distance between the emitter and the bead and so the coat is more compact.[20] Thus, the possibility of differentiate true from false positive beads would depend on the target molecular weight and the resin pore diameters. If the target had full access to the interior of the bead, true from false positives beads could not be differentiated and fluorescent dyes would not be a good option for on-bead screening specially when screening peptide libraries with hydrophobic amino acids. Hu and co-workers also found beads with halo appearance performing a two stage on-resin screening of a combinatorial peptide library with streptavidin-coated magnetic microparticles and rhodamine B-streptavidin. They hypothesize that the halo effect is caused by the inability of the streptavidin to fully penetrate the resin.[18] The protocol herein described involves one-step fluorescence on-resin screening with streptavidin conjugated with a red fluorescent dye. Only those beads with the characteristic halo appearance (positive beads) were isolated and analyzed.

With Texas Red the difference between true and false positives was more evident than with ATTO 590, thereby facilitating the manual isolation of the positive beads. Thus Texas Red was the method of choice.

Experimental Section

Materials. HMBA-ChemMatrix resin was from Matrix Innovation, Inc. (Montreal, Québec, Canada). Fluorenylmethoxycarbonylamino acids (Fmoc-amino acids), 1-hydroxybenzotriazole (HOBt), and 1-[bis(dimethylamino)methylene]-1*H*-benzotriazolium hexafluorophosphate 3-oxide (TBTU) were from Luxembourg Industries (Tel Aviv, Israel). HMBA, 4-(*N*,*N*-dimethylamino)pyridine (DMAP), guanidine hydrochloride, α-cyano-4-hydroxycinnamic acid (CHCA), and 2,5-dihydroxybenzoic acid (2,5-DHB) were from Sigma-Aldrich (St. Louis, MO). 1,3-Diisopropylcarbodiimide (DIPCDI), *N*,*N*-diisopropylethylamine (DIPEA), streptavidin-ATTO 590 conjugate, and ATTO 590 were from Fluka Chemie AG (Buchs, Switzerland). Streptavidin-peroxidase conjugate (SA-POD) was from Roche (Basel, Switzerland). Streptavidin-Texas Red (SA-TR), and bovine serum albumin-Texas Red (BSA-TR) conjugates were from Molecular Probes, Invitrogen (U.K.). All other reagents were AR grade.

Previous Washings of HMBA-ChemMatrix Resin. HMBA-ChemMatrix resin (1 g, 0.64 mmol/g) was washed before use as follows: dichloromethane (DCM) (3 × 1 min), dimethylformamide (DMF) (3 × 1 min), DCM (1 × 15 min + 3 × 1 min), DMF (1 × 15 min + 3 × 1min).

Combinatorial Peptide Library and Control Peptides Synthesis. A one-bead-one-peptide library of the nonapeptide XXXXGGGGG, where X = Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Tyr, Val, was synthesized by using the divide-couple-recombine method following Lam et al.[2] The peptide library was synthesized using the Fmoc strategy with 5-fold excess of the amino acids at each coupling step. HMBA-ChemMatrix resin (100−200 mesh and 0.64 mmol/g substitution) was used. The library had 83 521 distinct peptides (17^4). A positive control peptide with the HPQ motif (FHPQGGGGG), a negative control peptide without the HPQ motif (VGLVGGGGG), and some hits founds during the screening were also synthesized manually. The C-termini Gly was incorporated with DIPCDI in the presence of DMAP in DMF as described by Mellor et al.[21] The remaining protected amino acids were incorporated with DIPCDI/HOBt. Randomization was carried out in the last four coupling steps. Fmoc removal was performed with piperidine/DMF (1:4). The five Gly residues were introduced as a spacer arm to increase the molecular weight of the peptides to facilitate mass spectrometry analysis, as described previously.[9,10] This also overcame the poor cleavage efficiency of esters of Ile and Val.[22]

Side-Chain Deprotection of the Peptide-Resin. Following elongation completion, the side-chain protecting groups were removed from the peptide-linker-resin by treatment with a mixture of trifluoroacetic acid (TFA)/triisopropyl silane (TIS)/H_2O (95:2.5:2.5) for 2 h, leaving the unprotected peptide anchored to the resin.

Library Screening. Screening was carried out at room temperature in syringes, each fitted with a polyethylene porous disk. The one-bead-one-peptide library was first soaked in DCM (5 × 1 min), DMF (5 × 1 min), DMF/H_2O (7:3, 5:5, 3:7) (5 × 1 min each one), and H_2O (5 × 1 min). Subsequently, they were blocked with 10% skim milk, 2% BSA, 0.1% Tween 20 in phosphate buffered saline (PBS), pH 7.2. The blocking solution coats on the bead surface that would bind protein nonspecifically. The beads were then washed 5 × 1 min with 0.1% Tween 20 in PBS (PBS-Tween) and incubated with the target protein in PBS with 10% skim milk, 2% BSA, and 0.1% Tween 20 for 1 h. Bead incubation steps were also done in the presence of blocking protein to minimize nonspecific binding of the marker (fluorescent dye) and the target molecule. The concentrations used were 83 nM for SA-ATTO, ATTO, SA-TR, and BSA-TR, and 1 U/mL for SA-POD. The beads were thoroughly washed with PBS-Tween (10 × 1 min); PBS (5 × 1 min), and Tris buffer saline, pH 8.0 (TBS) (3 × 1 min).

When using SA-POD, beads were revealed with a mixture of 1 mL solution A (3 mg of 4-Cl-naphtol in 1 mL of CH_3OH) with 4 mL of solution B (4 mL of PBS containing 20 μL of 30 vol H_2O_2). After 5 min, positive beads turned violet color.

Violet beads were isolated manually with needles. Red fluorescent beads were detected using a stereoscopic microscope Leica MZ FLIII (Leica Microsystems GmbH, Wetzlar, Germany) and isolated manually with needles or using COPAS BIO-BEAD flow sorting equipment.[23,24] In the latter, the library beads were suspended in a COPAS GP Sheath Reagent. They were poured into the sample cup at a density of about 50 beads/mL. Gating and sorting regions were defined for sorting beads on COPAS on the basis of their time-off-flight (TOF) to sort uniform sized beads and red fluorescence intensity (RED). All the sorted beads were then analyzed under a fluorescence microscope. Positive beads were treated with 8 M guanidine hydrochloride, pH 2.0, during 20 min, washed with water, DMF, and DCM. They were then transferred onto the stainless-steel MALDI sample plate for cleavage of the peptide and MS analysis.

Cleavage of the Peptide from the Resin Using Ammonia Vapor. Each bead was deposited manually onto the stainless steel target plate, which was placed in a desiccator together with a flask containing NH_4OH. Cleaved peptides were eluted from the bead with 1 μL of acetic acid (AcOH)/acetonitrile (ACN)/H_2O (3:4:3) and air-dried, and then they were eluted with ACN/H_2O (1:1) (1 μL) with 0.5% TFA and air-dried again.

MALDI-TOF-MS Analysis of Peptide Beads Selected from the One-Bead-One-Peptide Combinatorial Library Screening. MALDI-TOF-MS were recorded in a 4700 Proteomics Analyzer instrument (Applied Biosystems, Foster City, CA) or in an Ultraflex II TOF/TOF Bruker Daltonics GmbH (Leipzig, Germany). The CHCA MALDI matrix concentrations used were 2 or 5 mg/mL, respectively. A solution of CHCA in 50:50 H_2O/ACN with 0.1% TFA was overlaid on each spot of the plate. Mass spectra were acquired in the MS reflector positive-ion mode. Tandem mass spectra were obtained using a MSMS positive acquisition method.

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Supporting Information Available. Complete sequence data of all peptides selected during the library screening, examples of MALDI-TOF MS of selected peptide library beads, and analysis of the position frequency for amino acids of the peptide sequences identified by MALDI-TOF MS. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) The following abbreviations are used: ACN, acetonitrile; AcOH, acetic acid; BSA, bovine serum albumin; BSA-TR, bovine serum albumin-Texas Red; CHCA, α-cyano-4-hydroxycinnamic acid; DCM, dichloromethane; DIPCDI, *N*,*N*′-1,3-diisopropylcarbodiimide; DIPEA, *N*,*N*-diisopropylethylamine; DMAP, 4-(*N*,*N*-dimethylamino)pyridine; DMF, *N*,*N*-dimethylformamide; FITC, fluorescein isothiocyanate; Fmoc-amino acids, fluorenylmethoxycarbonylamino acids; HMBA, 4-hydroxymethylbenzoic acid; HOBt, 1-hydroxybenzotriazole; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; OBOC, one-bead one-compound; PBS, phosphate-buffered saline; PEG, poly(ethyleneglycol); SA, streptavidin; SA-ATTO, streptavidin ATTO 590 conjugate; SA-POD, streptavidin peroxidase conjugate; SA-TR, streptavidin Texas red conjugate; TBS, Tris buffer saline; TBTU, 1-[bis(dimethylamino)methylene]-1*H*-benzotriazolium hexafluorophosphate *N*-oxide; TFA, triflouroacetic acid; THF, tretrahydrofuran; TIS, triisopropylsilane; TOF, time of flight.

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