From the One-Bead-One-Compound Concept to One-Bead-One-Reactor

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The one-bead-one-compound method gives access to millions of compounds that can be screened directly on the bead. Although characterization techniques are increasingly potent and reliable, problems can still be encountered in deciphering the sequence of the active compound because of sensitiveness or manipulation of the bead. ChemMatrix, a totally PEG-based resin, has resolved the synthesis of peptides of outstanding difficulty. Like other PEG-based resins, it permits on-bead screening because of its compatibility in aqueous media and has the further advantage of having a high loading, comparable to polystyrene resins. ChemMatrix beads previously swelled in water can be nicely divided into four parts that can be characterized using different analytical techniques or just stored for safety or for further testing. The four bead parts show high homogeneity and can thus be considered to be replicas.

Introduction

The one-bead-one-compound (OBOC) method[2,3,4] has widespread applications in the search for new bioactive compounds. This methodology allows millions of compounds to be obtained quickly and cheaply by following a series of split and mix cycles, whereby each bead ultimately bears a single compound. Peptide- and small-molecule-based libraries can both be constructed via OBOC. In the latter case, however, the number of steps should be minimized to obtain compounds with good purities.[5] In contrast, larger molecules can be obtained for peptides because solid-phase peptide synthesis is firmly established.[6] Completed libraries are screened against the target of interest, and the biologically active beads are pooled and characterized. It is in the characterization of active beads where the limitations of OBOC arise. There are two general strategies for the analysis of active beads: indirect characterization, known as "encoded libraries",[7] and direct characterization.[8,9,10] For peptides, the second approach has been preferred. In the past, Edman microsequencing was the technique of choice for characterization, but it suffers from various drawbacks: it is time-consuming, rather expensive and does not allow characterization of N-blocked amino acids. In addition, because the number of cycles is limited, it is often impossible to complete the sequence of long peptides. Finally, peptides with non-natural amino acids can only be sequenced when a standard is both available and well separated from the other amino acids. Recent advances in the sensitivity and precision of MS have reduced the amount of material required for analysis to picomolar quantities. Nevertheless, MS characterization is highly dependent on the ionizability of the resin-bound compound. As such, different beads (i.e., different compounds) from the same library often demand different MS techniques. Moreover, MS analysis of beads can be further complicated by interference from the matrix. Because of the uniqueness of the single-bead, it is extremely important to minimize these potential drawbacks. Alternatively, we envisioned that dividing the active bead into different portions would allow independent analysis of each portion via different techniques, or storage of one or more portions for later screening. Thus, we have extended the OBOC concept to the one-bead-one-reactor (OBOR) concept, in which a reactor is defined as a platform containing a substance that can be removed piecemeal.

Results and Discussion

ChemMatrix,[11] a totally PEG-based resin, has shown excellent results for the synthesis of difficult peptide targets,[12] such as very hydrophobic peptides (e.g., poly-Arg) and structurally complex peptides (e.g., β-amyloid (1−42) peptide). Its hydrophilic nature minimizes aggregation of the growing peptide chain, permitting stepwise standard solid-phase synthesis of long peptides, as well as small proteins.[13,14,15] Compared to other PEG-based resins, ChemMatrix exhibits higher loadings, comparable to those of PS resins, which allows a single bead to contain enough material for several manipulations. As with other PEG-based resins,[16,17] ChemMatrix swells well in biocompatible solvents

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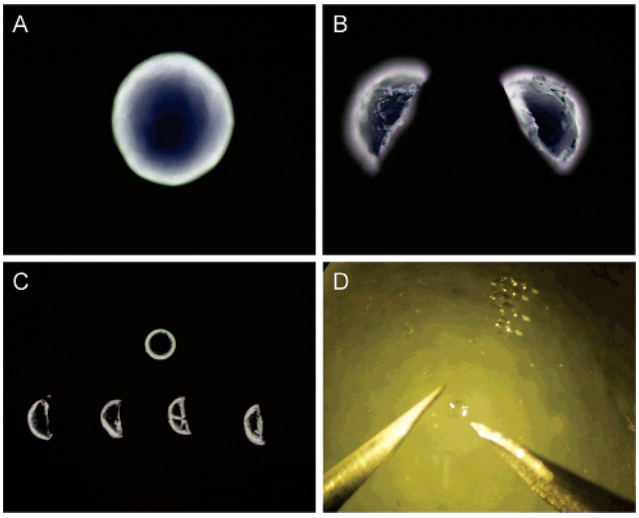

Figure 1. (A) one bead, (B) two halves of a bead, (C) four quarters of a bead, and (D) scission of a bead with two injection needles.

such as water, allowing on-bead screening.[18] Because of their high level of hydration in water (swelling, 11 mL/g of resin in water vs less than 2 mL/g for PS resins),[12] ChemMatrix beads can be smoothly sliced into four quarters (Figure 1).[19] The high loading of the resin permits that even a tiny quarter of a bead can be used as a microreactor for HPLC and MALDI-TOF analysis.

In this work, single beads bearing a decapeptide have been sliced into four quarters and characterized both by HPLC and MS. The peptide YGGFLGGGGG-NH_2 was used as a model. This peptide corresponds to Leu-enkephalin, to which five Gly have been added to separate the target molecule from the solid support and to increase the molecular weight beyond the matrix region. To facilitate visualization of the product by HPLC, the N-terminus has been derivatized with 4-(dimethylamino)azobenzene-4′-sulfonyl chloride (dabsyl chloride)[20,21] (absorbance at 425 nm, $\epsilon = 2.4 \times 10^{-4}$). Because of its high MW (288.08), this residue, which was incorporated just after screening, can be used instead of the 5 Gly to raise the MW of the target compound for MS purposes.

Two approaches have been followed: in the first, with the aim of obtaining HPLC profiles of each quarter, the acid-labile linker PAL (peptide amide linker),[22] was used in conjunction with the ChemMatrix resin. In the second, the HMBA linker,[23] which also yields peptide amides when cleaved with ammonia, was used. In this case, cleavage of each bead quarter can be performed directly on the MALDI plate by subjecting the plate to ammonia vapor.

The decapeptide was manually synthesized on PAL-ChemMatrix using Fmoc-protected amino acids (3 equiv) and TBTU (3 equiv)/DIEA (6 equiv) as coupling system. Elongation of the peptide onto HMBA-CM was identical, but the first amino acid was incorporated onto the resin with Fmoc-Gly-OH (3 equiv)/DIPCDI (4 equiv)/DMAP (0.1 equiv). After the last Fmoc removal, dabsyl chloride was coupled to the N-terminus: dabsyl chloride (5 equiv) was first dissolved in DMF, and then Et_3N was added until a clear solution was observed (20 equiv). The mixture was added to the resin, and the resulting slurry was allowed to react for 5 h.

Following attachment of the chromophore, a single bead was removed and swelled in water. Under a microscope, with the help of two needles (Figure 1D), the single-bead was sliced into four pieces. For the peptide synthesized with the acid-labile linker PAL, the four-quarters were then each introduced into a different Eppendorf tube, cleaved using TFA/H_2O (9:1) for 90 min, redissolved in 10 μL of ACN/H_2O (1:1), and then injected into the analytical HPLC (Figure 2).

The resulting four chromatograms (Figure 2), recorded at 425 nm, were basically equivalent, with the main peak corresponding to the desired peptide and with only very narrow differences in impurities (2.3%). On the basis of this level of homogenity among the quarters, we considered them to be replicas for screening and characterization.

For the peptide synthesized with the HMBA linker, the protecting groups were removed with TFA/TIS/H_2O (95:2.5:2.5) prior to coupling of the chromophore. Once the coupling was complete, each quarter was deposited onto the MALDI plate, and the plate was subjected to ammonia vapor inside of a desiccator overnight. Then, to each spot was added 1 μL of ACN/H_2O/TFA (49.75:49.75:0.5). After evaporation, each spot was treated with 1 μL of AcOH/H_2O/ACN (3:4:3), and the solvent was evaporated again. Finally, a solution of ACN/H_2O/TFA/α-cyano-4-hydroxycinnamic acid (2 mg/mL) (49.5:49.5:0.5:0.5) was deposited onto each spot, and the plate was analyzed by MALDI-TOF (Figure 3). For each quarter, four peaks were observed, corresponding to $[M + H]^+$, $[M + Na]^+$, $[M + K]^+$, and $[M + Na + K]^+$. As in the case of the HPLC samples, there was a high degree of homogeneity among the MS bead quarters.

For analytical characterization, at least 5 pmol of resin-bound peptide are needed for sequencing on a bead. An estimation of the capacity per bead for ChemMatrix resins can be derived based on the loading and number of beads per gram. This gives approximately 300 and 500 pmol/bead for HMBA-CM and PAL-CM, respectively. This is comparable to other PEG resins such as Tentagel, where for the same particle size (between 90 and 130 μm) with a loading of 0.25−0.30 mmol/g, the capacity per bead is 80 to 330 pmol/bead.

Conclusions

In conclusion, we have developed the one-bead-one-reactor (OBOR) concept using the PEG-based ChemMatrix resin, which allows division of a single bead into four portions for characterization, screening, or storage. For the HPLC analysis portion, a dabsyl moiety is incorporated onto the product to increase its UV absorbance. The chromophore also facilitates visualization of the product in MS, because it increases the molecular weight beyond the matrix region.

Experimental Section

General. Unless otherwise stated, all reagents were purchased from commercial sources and used without further purification. PAL-CM resin and HMBA-CM resin were from Matrix Innovation, Canada. Fmoc-amino acids were obtained

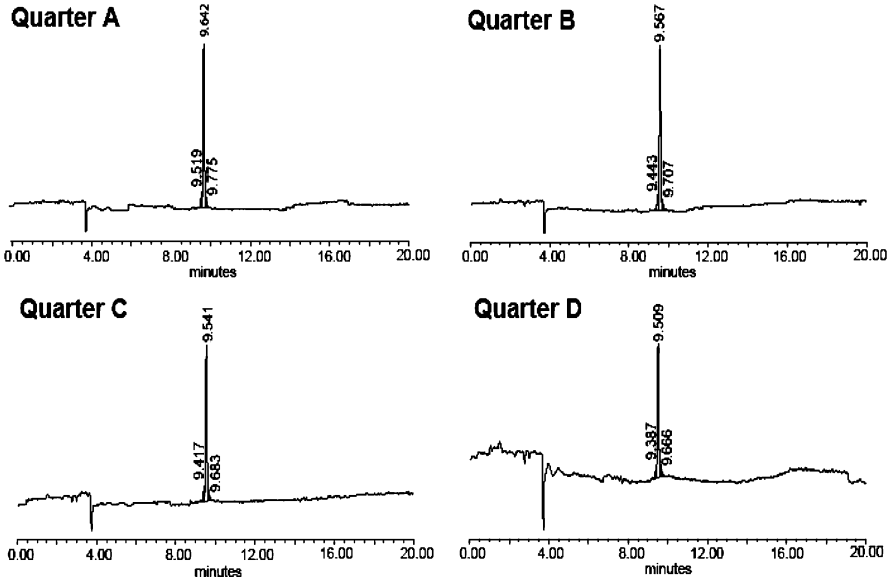

Figure 2. Analytical HPLC chromatograms of DABS-YGGFLGGGGG-NH_2 on four quarters of a bead recorded at 425 nm. HPLC using a reverse-phase C_{18} column (4.6 × 150 mm, 5 μm) and a linear gradient from 100% A to 100% B in 15 min, where A = H_2O (0.045% TFA) and B = ACN (0.036% TFA), at a flow rate of 1.0 mL/min.

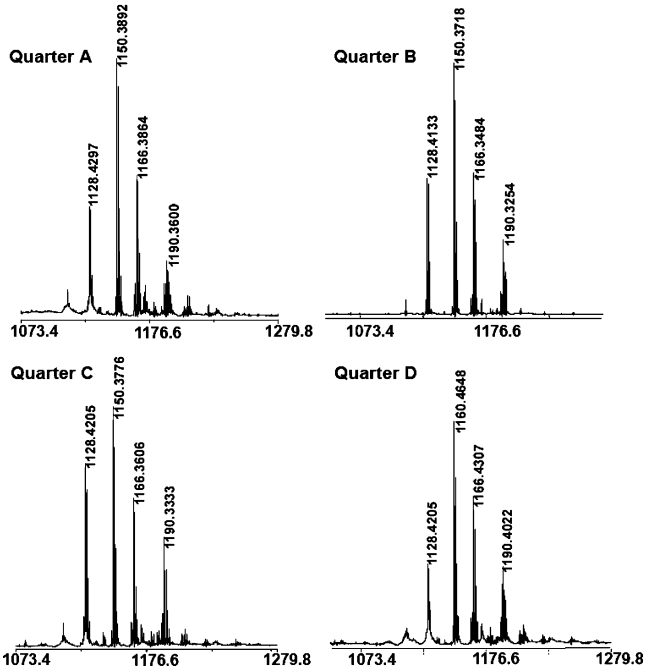

Figure 3. MALDI-TOF of DABS-YGGFLGGGGG-NH_2 of the four quarter beads: *m/z* calcd for DABS-YGGFLGGGGG-NH_2, 1126.4654; found $[M + H]^+$, $[M + Na]^+$, $[M + K]^+$, $[M + Na + K]^+$.

from IRIS Biotech. HPLC was performed on a reversed-phase C_{18} column (4.6 × 150 mm, 5 μm). Linear gradients of 0.045% TFA and 0.036% TFA in ACN were run at a flow rate of 1.0 mL/min. HPLC-MS was performed on a reversed-phase C_{18} column (3.9 × 150 mm, 5 μm) using aqueous 0.1% formic acid and 0.07% formic acid in ACN as eluents. MALDI-TOF mass spectra were recorded in a Voyager DE-RP (Applied Biosystems, Foster City, CA) instrument.

Peptide Synthesis. Manual solid-phase peptide elongation and other solid-phase manipulations were carried out in polypropylene syringes fitted with a polyethylene porous disk. Solvents and soluble reagents were removed by suction. Fmoc removal was carried out with piperidine/DMF (1:4, 1 × 1, 2 × 10 min). Washings between deprotection, coupling, and subsequent deprotection steps were carried out with DMF (5 × 30 s) and CH_2Cl_2 (5 × 30 s) using 10 mL of solvent/g of resin each time.

Previous Washings of CM Resin. HMBA-CM and PAL-CM resins were washed before use as follows: CH_2Cl_2 (3 × 1 min), DMF (2 × 1 min), and swelled in CH_2Cl_2 for 15 min.

Synthesis of YGGFLGGGGG-NH_2 Model Peptide. 1. Peptides on HMBA-CM Resin. The first amino acid was coupled as follows: Fmoc-AA-OH (3 equiv) and DIPCDI (4 equiv) in the presence or DMAP (0.1 equiv) in DMF were added to the resin, and the mixture was allowed to react for 60 min. Then the mixture was filtered, and fresh reactive mixture was allowed to react for another 60 min. Next, an acetylation step with acetic anhydride (6 equiv) and DMAP (0.1 equiv) was carried out. The rest of the synthesis was performed using Fmoc-AA-OH (3 equiv), TBTU (3 equiv) as the coupling reagent, and DIEA (6 equiv) as base. The mixture was allowed to react for 30 min. The coupling was checked with the ninhydrin reaction. After the last Fmoc removal with piperidine/DMF (1:4), the sample was taken and treated with TFA/TIS/H_2O (95:2.5:2.5) for removal of the protecting group.

2. Peptides on PAL-CM Resin. The synthesis was identical to that described above. After deblocking of Fmoc, the resin was washed [CH_2Cl_2 (3 × 1 min), ethanol (2 × 1 min), diethyl ether (1 × 1 min), CH_2Cl_2 (1 × 15 min, 2 × 1 min), and DMF (2 × 1 min)], and the dabsyl chloride compound was coupled (see chromophoric reagent coupling).

Chromophoric Reagent Coupling. Dabsyl chloride (5 equiv) was dissolved in DMF, and triethylamine (20 equiv) was added to complete dissolution. The mixture was added to the YGGFLGGGGG-PAL-CM resin and allowed to react for 5 h. After that, the resin was washed [DMF (2 × 1 min),

CH_2Cl_2 (3 × 1 min), ethanol (2 × 1 min), and diethyl ether (1 × 1 min)].

Scission of the Beads. The beads were manipulated with two needles. Under a microscope, a single bead was picked up with the needles, and then the bead was sliced into four pieces. The pieces were used for HPLC and MALDI-TOF analysis.

Time-of-Flight (TOF) Mass Spectrometry. Peptide on HMBA-CM Resin. The bead quarters were placed on the MALDI plate. Before matrix addition, the peptide was cleaved from the piece of bead using ammonia/THF vapour. Then, 1 μL of ACN/H_2O/TFA (49.75:49.75:0.5) was added to each spot. After evaporation, each spot was treated with 1 μL of AcOH/H_2O/ACN (3:4:3), and the solvent was evaporated again. Finally, a solution of ACN/H_2O/TFA/α-cyano-4-hydroxycinnamic acid (2 mg/mL) (49.5:49.5:0.5:0.5) was deposited onto each spot, and the plate was analysed by MALDI-TOF.

Cleavage and Analytical HPLC of the Peptide from PAL-CM Resin. The bead quarters were deposited into a plastic microcentrifuge tube. The peptide was deblocked and cleaved from the bead quarter with TFA/H_2O (9:1) for 90 min. The solution was filtered and evaporated. Ether was used to wash the peptide. The sample was redissolved in ACN/H_2O (1:1), and injected into the analytical HPLC.

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References and Notes

(1) The following abbreviations are used: ACN, acetonitrile; AcOH, acetic acid; CHCA, α-cyano-4-hydroxycinnamic acid; CM, ChemMatrix; DABS, dabsyl; DMF, *N*,*N*-dimethylformamide; DIPCDI, *N*,*N*′-diisopropylcarbodiimide; DMAP, *N*,*N*-dimethylaminopyridine; DIEA, *N*,*N*′ diisopropylethylamine; Fmoc, 9-fluorenylmethoxycarbonyl; HMBA, 4-hydroxymethylbenzoic acid; HPLC, high performance liquid chromatography; MALDI-TOF, matrix-assisted desorption ionization time of flight; MS, mass spectrometry; OBOC, one-bead-one-compound; OBOR, one-bead-one-reactor; PAL, peptide amide linker; PEG, polyethylene glycol; TBTU, 1-[bis(dimethylamino)methylene]-1*H*-benzotriazolium tetrafluoroborate 3-oxide; TFA, trifluoroacetic acid; THF, tetrahydrofuran.

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