Development of a Large Peptoid–DOTA Combinatorial Library

Jaspal Singh,[1*] Daniel Lopes,[3*] D. Gomika Udugamasooriya[1,2,3,4]

[1]*Department of Pharmacological and Pharmaceutical Sciences, University of Houston, Houston, TX 77204*

[2]*Department of Cancer Systems Imaging, MD Anderson Cancer Center, 1881 East Road, Houston, TX 77030-4009*

[3]*Advanced Imaging Research Center, UT-Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75390*

[4]*Department of Biochemistry, UT-Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75390*

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ABSTRACT:

Conventional one-bead one-compound (OBOC) library synthesis is typically used to identify molecules with therapeutic value. The design and synthesis of OBOC libraries that contain molecules with imaging or even potentially therapeutic and diagnostic capacities (e.g. theranostic agents) has been overlooked. The development of a therapeutically active molecule with a built-in imaging component for a certain target is a daunting task, and structure-based rational design might not be the best approach. We hypothesize to develop a combinatorial library with potentially therapeutic and imaging components fused together in each molecule. Such molecules in the library can be used to screen, identify, and validate as direct theranostic candidates against targets of interest. As the first step in achieving that aim, we developed an on-bead library of 153,600 Peptoid–DOTA compounds in which the peptoids are the target-recognizing and potentially therapeutic components and the DOTA is the imaging component. We attached the DOTA scaffold to TentaGel beads using one of the four arms of DOTA, and we built a diversified 6-mer peptoid library on the remaining three arms. We evaluated both the synthesis and the mass spectrometric sequencing capacities of the test compounds and of the final library. The compounds displayed unique ionization patterns including direct breakages of the DOTA scaffold into two units, allowing clear decoding of the sequences. Our approach provides a facile synthesis method for the complete on-bead development of large peptidomimetic–DOTA libraries for screening against biological targets for the identification of potential theranostic agents in the future. © *2016 The Authors. Biopolymers Published by Wiley Periodicals, Inc. Biopolymers (Pept Sci) 106: 673–684, 2016.*

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Additional Supporting Information may be found in the online version of this article.

Correspondence to: D. G. Udugamasooriya; Department of Pharmacological and Pharmaceutical Sciences, University of Houston, Houston, TX 77204. E-mail: gomika@uh.edu

*Both authors contributed equally.

INTRODUCTION

One-bead one-compound (OBOC) combinatorial libraries have been developed and applied for more than two decades, providing a powerful tool for drug-development research.[1–4] Large OBOC libraries of peptides,[5–10] peptoids (oligo-*N*-substituted glycines),[11–17] and other peptidomimetics[18,19] have been reported and used to identify potentially therapeutic

compounds. OBOC libraries have also been used to develop various protein-based[12,14,17,20] and cell-based[5,9,10,13,15,19,21–23] screening platforms to identify potential binding and active molecules. The main focus so far has been to screen the OBOC libraries and identify ligands that have targeting and/or therapeutic value. That focus has limited the design and synthesis of conceptually advanced OBOC libraries that could directly identify molecules with different capabilities, such as imaging, or even provide both therapeutic and diagnostic functions simultaneously. Here, we report the synthesis of a large OBOC library of peptoid–DOTA (1,4,7,10-tetraazacyclododecan-*N*,*N*′,*N*″,*N*‴-tetraacetic acid) compounds in which each molecule has peptoids as the target-recognizing or therapeutic component and DOTA as the imaging component. Both components are built in to every molecule of the library.

When dealing with complex diseases such as cancer, it is of the utmost importance to determine whether a drug reaches its target and is therapeutically effective over the time of treatment. Compounds with both therapeutic and imaging characteristics are extremely important in such applications and are referred to as 'theranostic' agents.[24] In recent years, tremendous effort has been made to develop theranostic agents by bringing together the therapeutic and imaging components to synthesize a single molecule as well as by constructing nanoparticles and macromolecules loaded with those two components.[25,26] The development of multimodal systems using various platforms such as fluorescent-based drug delivery, drug–polymer conjugates,[27–31] polymeric/magnetic/metal nanoparticles,[32–43] dendrimers,[44,45] liposomes,[46–50] micelles,[51,52] and carbon nanomaterials[53,54] are some examples of those efforts. We and others have reported the modification of known therapeutics with common imaging agents such as DOTA.[31,55,56] Historical observations of theranostic development suggest, however, that bringing together significantly different therapeutic and imaging components in the last step of the development process is neither easy nor very useful and eventually creates many problems.[57] First of all, the chemical modification of existing drugs can be a major synthesis challenge. Furthermore, such modifications typically have a negative impact on the activity and pharmacokinetic properties of the original drug. In addition, therapeutics are best if they have a longer circulation time, providing for maximal drug exposure, while imaging agents are expected to be rapidly cleared from non-target regions to reduce the background signal. Combining these two completely opposite characteristics into a single system using existing therapeutics and imaging agents is a huge challenge.

We hypothesized that the development of a simpler and more economical way to synthesize theranostic systems with the potentially therapeutic components and imaging components built together as a single molecule from the beginning of the development process and validated against the target as a single unit could provide solutions to many of those historical complications (Figure 1A). The development of a therapeutically active molecule with built-in imaging components is a daunting task, and structure-based rational design might not be the best approach. Therefore, we propose to develop a large OBOC combinatorial library in which each molecule contains: (I) an imaging moiety, (II) a diversified region that can recognize a biological target, and potentially act as the therapeutic unit, and (III) a variable spacer in the middle that can link the first two components (Figure 1A). The expectation is that when a 'hit' compound is identified from the library, the target-binding moiety could act as a therapeutic unit, as most of the reported 'hit' compounds identified in combinatorial screens display intrinsic activity,[9–15,20,21,58] while it already has a built-in imaging component. In that way, molecules consisting of a targeted/therapeutic unit, a proper spacer, and an imaging component can be "directly" isolated. Here, in order to validate that approach, we report the first step: the development of a unique OBOC library using DOTA as the imaging moiety, amino-ethyloxy-ethyloxy-acetyl (AEEAc) as the spacer, and peptoids as the target recognizing/potentially therapeutic unit, all of which are integrated into each compound in the library (Figure 1B). Our design rationale is to attach the DOTA scaffold to TentaGel resin beads via one of the four arms of DOTA through a linker, leaving three arms of the DOTA free. These free arms can then be used to build the diversified peptoid library (Figure 1B).

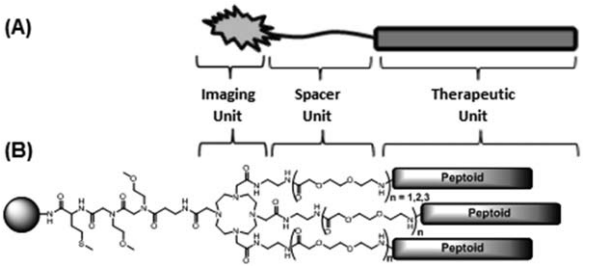
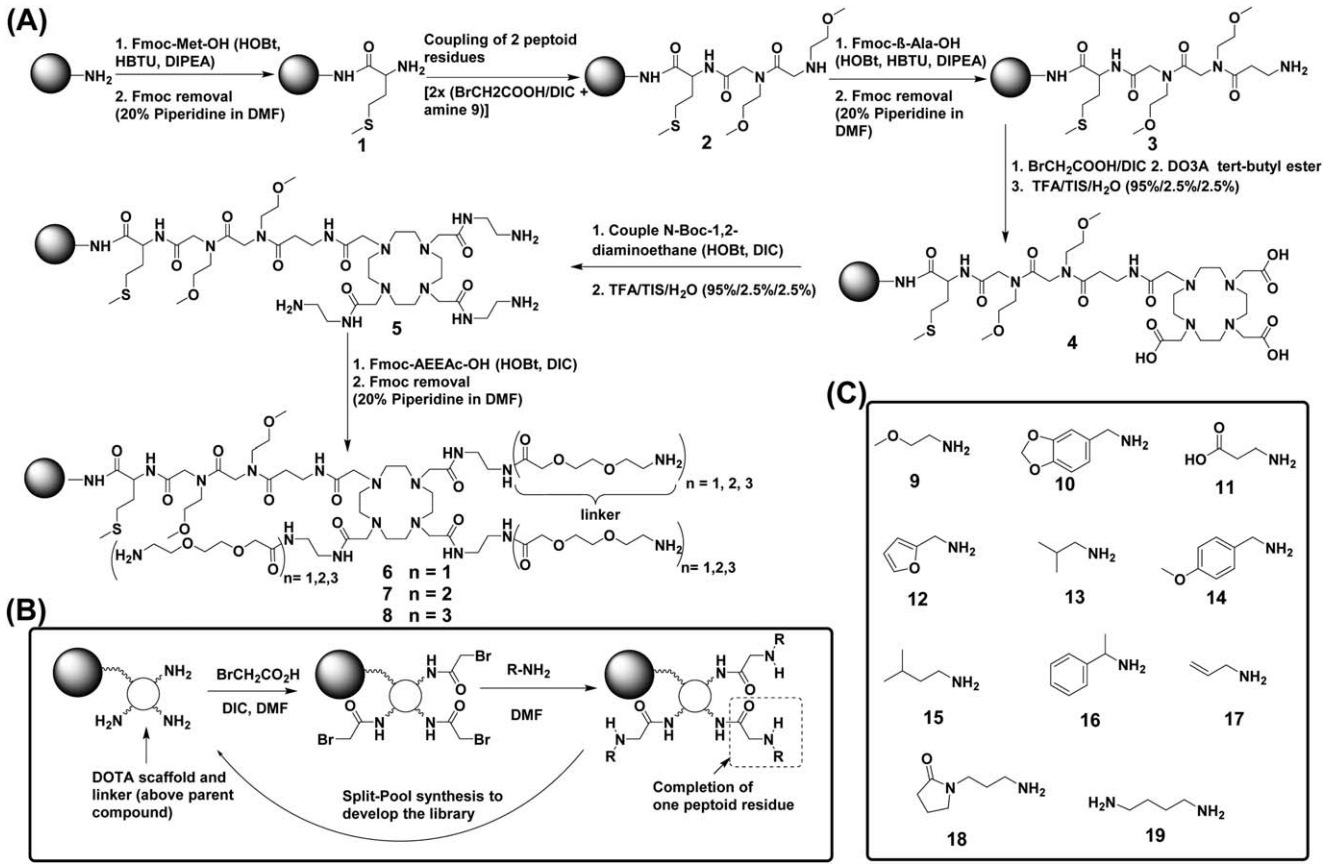


FIGURE 1 (A) Schematic representation of a theranostic unit. (B) On-bead structure of tri-peptoid–DOTA molecule with initial linker, DOTA scaffold, spacer, and diversified peptoid region.

Peptoids are highly biologically amenable and are easy to synthesize compounds. They are also known to be rich sources of protein-binding ligands that exhibit antagonist effects on receptors and intracellular proteins.[11–14,17,20,59–63] Peptoids closely resemble peptides except that the side chains extend from the main-chain nitrogen rather than from the α-carbon.[59,64,65] Peptoid oligomers are protease resistant, non-immunogenic, and achiral, and they adopt conformations different from those of peptides.[66,67] Peptoid synthesis is



SCHEME 1 General synthesis strategy for peptoid–DOTA library. (A) Assembly of linker on resin following DO3A insertion, spacer addition on 3 arms of DO3A. (B) General oligomerization strategy for library construction, (C) list if amines used for the library synthesis.

straightforward. In order to add one residue (equivalent to an amino acid of a peptide), only two chemical steps are required, each of which can be completed by two 15 s microwave pulses (Scheme 1B).[68,69] Bromoacetic acid is coupled onto an amine functional group of the resin, contributing two carbon units, and the bromine is replaced by primary alkylamine groups ($R{-}CH_2{-}NH_2$), completing one peptoid residue. This dramatically expands the repertoire of chemical space, as the 'R' group can be any organic moiety. Large combinatorial libraries of peptoids (containing millions of compounds) can be synthesized easily, inexpensively, and rapidly.[11–15,17,59,70] We chose DOTA as the imaging unit because of its wide applicability in imaging techniques such as MRI, SPECT, and PET.[71–77] Another important advantage of DOTA is that it contains four arms that can be used to obtain the multivalent ligand effect when acting as the drug molecule. Multimeric ligands can interact with multiple copies of receptors on the cell surface via the avidity effect, enhancing the strength of the interaction.[78–81] We take advantage of that multivalent effect, particularly when developing synthetic ligands for cell–surface receptors. We hypothesized that a library of trimer–peptoid ligands on the DOTA imaging chelator, which presents three arms to build the peptoid chain, could directly identify the highest affinity ligands targeting biomarker(s) presented on the surface of cancer cells during a suitable cell screening. The same ability is applicable to conventional protein screens as well to target multiple 'hot spots', which enables the use of such libraries in a wide variety of screening platforms.

EXPERIMENTAL

General

Tentagel macrobeads were purchased from Rapp Polymere (Germany). All the Fmoc protected amino acids, 2-(1H-benzotriazol-1-yl)−1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), and HOBt were purchased from EMD millipore (Billerica, MA). All primary amines, bromoacetic acid, diisopropylcarbodiimide (DIC), *N,N*-diisopropylethylamine (DIPEA), piperidine, trifluoroacetic acid (TFA), and dimethyl formamide (DMF) were purchased from Sigma-Aldrich (Milwaukee, WI); Dichloromethane (DCM) and

acetonitrile were obtained from Honeywell Burdick & Jackson (Morristown, NJ, USA). H-β-Ala-O*t*Bu.HCl was purchased from EMD Biosciences (Gibbstown, NJ, USA). All of the chemical reagents and solvents from commercial sources were used without further purification. 5 mL disposable reaction columns (Intavis AG) were used as reaction vessels for solid-phase synthesis.

MALDI-TOF mass spectra were acquired on an Applied Biosystems Voyager-6115 and Voyager De-Pro using α-Cyano-4-hydroxycinnamic acid as matrix, while MS/MS (MALDI-TOF) for sequencing were performed on a 4700 Proteomics Analyzer (Applied Biosystems).

On-Bead Synthesis of Parent Compound 5

100–200 mg of TentaGel macrobeads resin (Rapp polymer, with a 0.25 mmol/g loading, 300 μm diameters) in a reaction columns were swelled in dimethylformamide (DMF) for 1 h. A combination of established protocols for Fmoc solid phase peptide and peptoid synthesis were then used for the reactions. First of all, the resin was treated with five times (5 × resin loading capacity) of Fmoc—Met—OH, HBTU, HOBt (5 × 0.05 mmol) and DIPEA (10 × 0.05 mmol) in DMF (2 mL) at room temperature overnight. After washing with DMF (10 × 2 ml), Fmoc protecting group was removed by 20% piperidine solution in DMF [2 × (2 ml × 10 min)]. For peptoid coupling, bromoacetic acid (2*M* in DMF) and diisopropylcarbodiimide (DIC) (3.2*M* in DMF) were used for the acylation step and amine solutions (2*M* in DMF) were used for amination step. Thus, the beads were treated with 2*M* bromoacetic acid (1 ml) and 3.2*M* diisopropylcarbodiimide (DIC) (1 ml) and facilitated by microwave oven set to deliver 10% power (2 × 15 s) with a gentle shaking in between for 30 s. After washing with DMF, beads were treated with 2*M* methoxyethylamine (Nmea) (2 ml) following the microwave oven procedure as described above. The procedure was repeated to attach the second peptoid residue with DMF washing at every step. Fmoc—β-Ala—OH was then coupled using the same peptide coupling condition described for the methionine coupling above. After Fmoc removal with 20% piperidine, beads were treated again with bromoacetic acid/DIC mixture under microwave condition. The linker terminal was then treated with 1*M* DO3A-*tris*(*t*-But ester) solution (2 ml) in the microwave oven at 10% power (3 × 15 s), followed by *t*-butyl groups removal using 95% TFA, 2.5% triisopropylsilane, and 2.5% water mixture (3 ml) for 4 h at room temperature. At last the DOTA arms were coupled to *N*-Boc-1,2-diaminoethane, using HOBt/DIC at room temperature for 1 day (the solution was changed after 8 h) followed by Boc removal at the terminals of compound 5.

Synthesis of Test Compounds (20, 21, and 22)

The beads with compound 5, were divided into three reaction vessels and 1, 2, and 3 units of Fmoc-amino-ethyloxy-ethyloxy-acetyl (Fmoc—AEEAc—OH) were coupled to three vessels respectively, using HOBt and DIC coupling protocol to give 6 ($n = 1$), 7 ($n = 2$) and 8 ($n = 3$). After coupling of spacers, desired number of peptoid residues were added using same microwave protocol as discussed above for linker region synthesis.

Library Synthesis

Tentagel beads (2.0 g, 300 μm, 0.25 mmol/g, Rapp-Polymere GmbH, Germany) were swelled in DMF for 2 h before use. Beads were washed thoroughly with DMF. The parent compound 5 was synthesized as discussed above. Beads were then divided equally into three groups named as A, B, and C.

(A) Beads in the group A were coupled with Fmoc—AEEAc—OH in the presence of HOBt/DIC. The Fmoc protecting group was removed with 20% piperidine in DMF. The beads were then treated with bromoacetic acid and DIC mixture for acylation followed by amination with Boc-Nlys (19, Scheme 1C) to couple the 1st peptoid residue. The beads were then divided into 10 reaction vessels and added 10 different amines (9–18, Scheme 1C) after the common bromo-acylation step, for coupling the 2nd peptoid residue, beads from all of the 10 vessels were then pooled together, mixed thoroughly and split again into 10 vessels. Same procedure described above was followed to add 10 different amines as the 3rd peptoid residue.

(B) Beads in the group B were reacted with two consecutive Fmoc—AEEAc—OH addition steps using HOBt/DIC protocol described above. The terminal Fmoc protection group was removed and beads were split into 10 reaction vessels and each vessel was treated with 10 different amines (9–18, Scheme 1C) after the common bromo-acylation step for coupling the 1st peptoid residue. All the beads were pooled and Boc-Nlys was introduced as the 2nd peptoid residue, after the common bromo-acylation step. Beads were again divided into 10 reaction vessels and the same procedure described above was used to add 10 different amines as the 3rd peptoid residue.

(C) Beads in the group C were reacted with three consecutive Fmoc—AEEAc—OH addition steps using HOBt/DIC protocol described above. The terminal Fmoc protection group was removed and beads were subjected to two rounds of split–pool synthesis protocol described above adding 1st and 2nd residues. Then, all the beads were pooled and Boc-Nlys was introduced as the 3rd peptoid residue after the common bromo-acylation step.

At the end of the coupling of first three peptoid residues, each of the A, B, and C groups mentioned above with 1, 2, and 3 AEEAc spacer moieties now carry Nlys on 1st, 2nd, and 3rd positions respectively. At this point, all of those three groups were mixed together and remaining three peptoid residues were added using eight different amines (9, 11–13, 15, 17–19, Scheme 1C) in each step using the split–pool protocol described above.

RESULTS AND DISCUSSION

We recently reported a small on-bead peptoid–DOTA[Eu(III)] library of CEST-MR imaging agents developed using a unique parallel synthesis approach.[82] Here, we used a similar strategy to first attach a DOTA scaffold onto resin beads through a short linker (Figure 1B). Then, we used the remaining three arms of the DOTA to build a peptoid library, with the peptoids separated from the DOTA by a variable AEEAc-based spacer. This variable spacer enabled us to vary the distance between the peptoid chains and the DOTA scaffold. That is important because the true distance between the receptors on the cell surface or between multiple hot spots within the same receptor is usually unknown. The variable spacer regions facilitate compound optimization during subsequent screens, as depicted in Figure 1B. Furthermore, we used AEEAc moieties that typically increase water solubility. One, two, or three AEEAc units were used to provide three different spacer lengths. Finally, we developed a 6-mer peptoid library with five diversified residues (1 position was fixed) using split–pool synthesis at the terminus of each spacer, as shown in Figure 1B and Scheme 1.

Prior to the synthesis of the library, we tested two very important aspects: (I) the synthesis potential of the novel Peptoid-DOTA compounds and (II) the ability to sequence the compounds using mass spectrometry. It is very important to validate every step of the synthesis procedure, as the coupling capabilities of different amines (for the peptoid synthesis) on to the scaffold might vary due to variable molecular weight, charge, polarity, hydrophobicity, steric hindrance, and other properties. Each amine has a random chance to occupy any position of the 6-mer sequence during the split–pool combinatorial synthesis. We standardized the reaction conditions on TentaGel macrobeads (300 μ*M* diameter), which have excellent stability and swelling properties. These beads provide a nonsticky surface that reduces the likelihood of nonspecific binding in subsequent screening experiments. Three test compounds were synthesized with three different spacer lengths (one, two, and three AEEAc units), respectively, and a variety of amines to produce various peptoid regions. The proposed synthetic scheme for the test compounds and for the final library development is shown in Scheme 1. The synthesis procedure for steps 1–6 was modified from the protocol established in our previous study.[82] In brief, we first coupled an initial methionine to the bead to ensure that the growing compounds could be cleaved off the bead (CNBr cleavage) for mass spectrometric analysis. Next, we synthesized a three-residue common linker consisting of two additional peptoid moieties coupled to a β-alanine residue before the DOTA was coupled [Scheme 1A (1–3)]. Next, we added a 1,4,7,10-tetraazacyclododecane-1,4,7-*tris*-tert-butyl acetate (DO3A-tri-Boc-protected DOTA) scaffold to the linker using bromo-substitution through a secondary amine attack, and we removed all of the Boc groups using TFA/TIS/H_2O (95%/2.5%/2.5%) [Scheme 1A (3–4)]. We then coupled the three exposed carboxylic acid groups of the DOTA to *N*-Boc-1,2-diaminoethane to convert the carboxyl functionality into an amine functionality, upon which the spacer region and peptoid chains would be grown [Scheme 1A (4–5)]. Finally, we divided the beads into three portions and coupled one or two, or three units of Fmoc—AEEAc—OH as the spacer units, resulting in precursors 6 ($n = 1$), 7 ($n = 2$), and 8 ($n = 3$), respectively [Scheme 1(A)]. Because the MS/MS sequencing can easily deduce up to 7–8 residues, the actual number of AEEAc moieties (1–3) within the spacers could be readily deduced. After adding the spacer units, we elongated the peptoid chains using a variety of amines on all three precursors [6, 7, and 8; Scheme 1(B)]. The choice of the amines for each test compound was arbitrary (Supporting Information Figure S1), but we used several bulkier amines, which are typically difficult to couple (compounds 21 and 22; Figure 2A) mainly to test their coupling efficiency. We also included aromatic and aliphatic hydrophobic side chains, because native recognition domains are generally rich in such residues. We confirmed the synthesis of the test compounds by MALDI mass analysis. The representative mass and analytical HPLC spectra of compounds 20, 21, and 22 are shown in Figures 2B–2G. Each of those test compounds was synthesized with good yield (20: 83%, 21: 85%, 22: 82%), confirming the facile synthesis of the compounds on the beads.

The next crucial step was to validate the MS/MS sequencing potential of the diversified peptoid regions, which will be unknown when a potential 'hit' compound is identified in a screen. Taking into account that the OBOC library is composed of individual beads, each containing a unique peptoid sequence that needs to be decoded when the 'hits' are found, we characterized and analyzed individual beads from the synthesized test compounds. We subjected all three test compounds (20, 21, and 22) to MALDI-TOF MS/MS sequencing using AB 4700 proteomics analyzer. We were able to assign all the peptoid residues to correct fragments as they were synthesized for test compounds 20 and 22, with one and three AEEAc

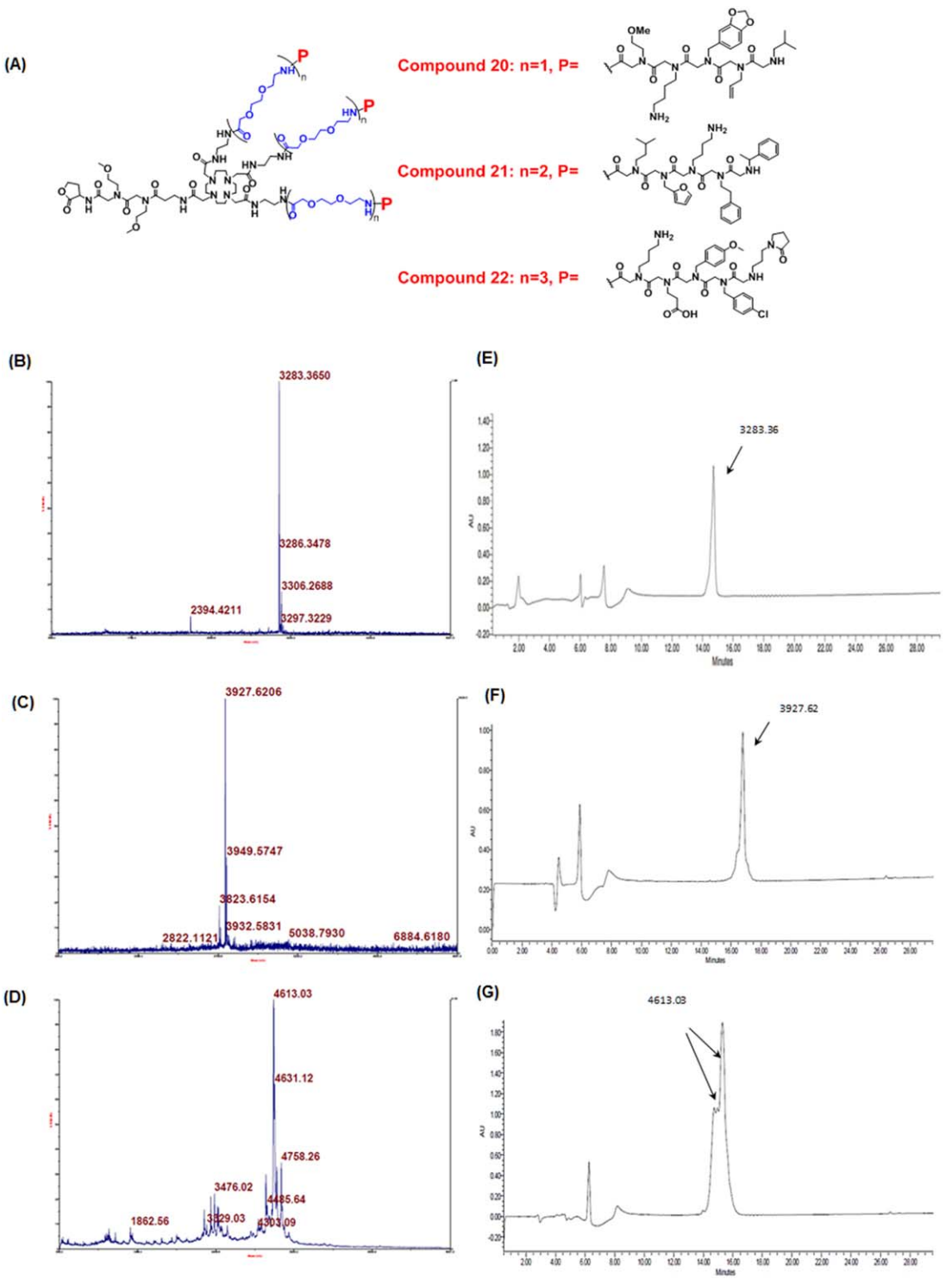


FIGURE 2 (A) Structures of test compounds 20, 21, and 22 with $n = 1$, 2, and 3 AEEAc spacers respectively. (B), (C), and (D) MALDI mass spectra acquired by voyager De-Pro for compounds 20 (expected MS 3282.85 *m*/*z*, observed MS: 3283.36 *m*/*z*), 21 (expected MS 3928.24 *m*/*z*, observed MS: 3927.62 *m*/*z*), and 22 (expected MS 4612.29 *m*/*z*, observed MS: 4613.03 *m*/*z*). (E–G) are analytical HPLC for compounds 20, 21, and 22 respectively.

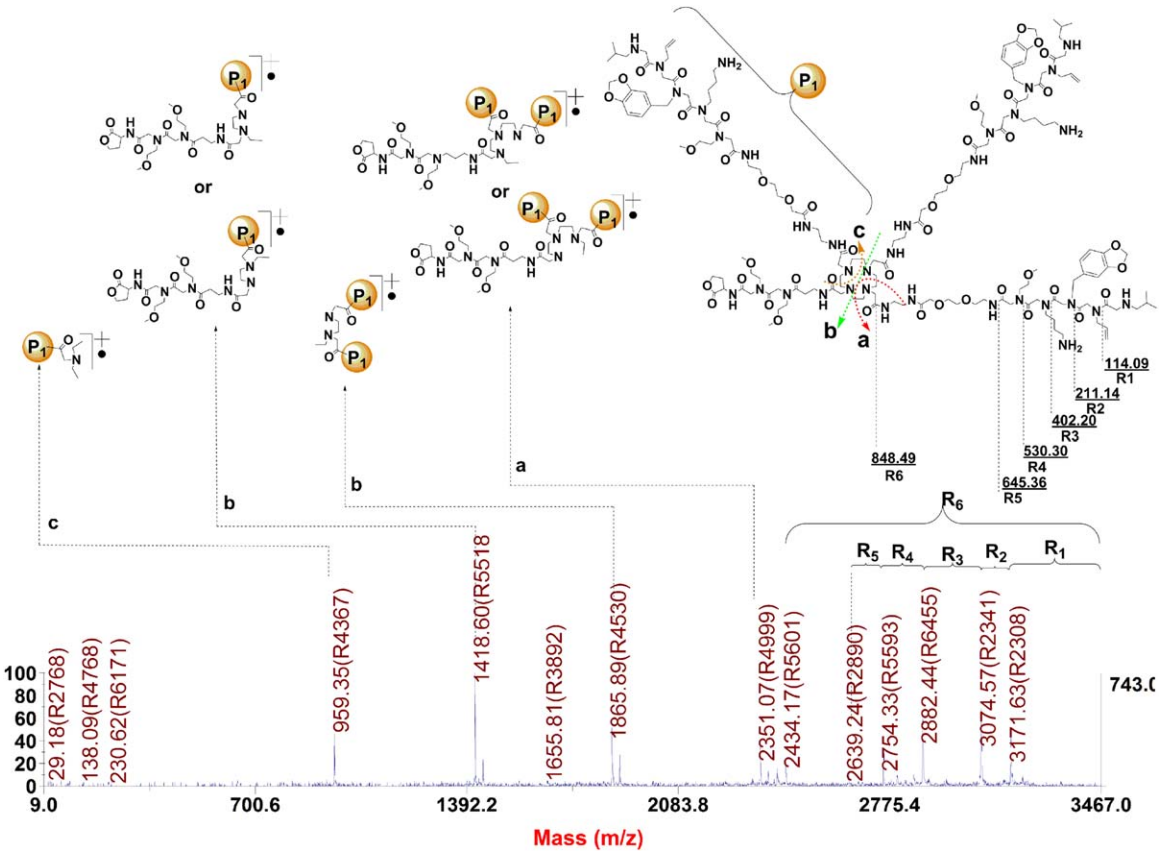


FIGURE 3 MS/MS sequencing of the test compound 20 from crude material cleaved from a single bead.

spacers, respectively. These are shown in Figures 3 and 4. We have chosen a spectrum that did not display all the peptoid fragments for test compound 21, which has two AEEAc spacers, to display the decoding capacity of these compounds under difficult conditions. This is described in Supporting Information Figure S3.

When we looked at the MALDI MS/MS analysis for compound **20** (Figure 3), the ionization patterns of all five terminal peptoid residues (Figure 3, R1–5), the AEEAc spacer, and the 1,2-diaminoethane were clearly apparent within one fragment at *m*/*z* 2434.17 (Figure 3, R6). All three fractions appeared to have broken from a single arm of the three-arm DOTA scaffold, leaving the links on the other two arms intact. Moreover, we observed some unique peaks that were identified as fragments of the DOTA scaffold. Those characteristic peaks corresponded to C—N bond breakages at different sites within the DOTA scaffold, which to the best of our knowledge has not been reported previously for a DOTA scaffold. Typically, C—N fragmentations in peptides and peptoids predominantly occur at CO—NH (amide) bonds. In addition, various mass analysis techniques have demonstrated C—N bond dissociations for larger molecules such as poly(propylene imine) dendrimers,[83–85] but not for a very stable cyclic scaffold like DOTA. Further analysis of compound 20 revealed that the peak at *m*/*z* 2351.07 corresponded to bond dissociation at two sites in such a way that the major fragment consisted of three DOTA nitrogens along with two elongated arms (the bead-linker arm and two peptoid arms; Figure 3, bond cleavage a). There were two terminal nitrogens of the DOTA ring fragment, either of which could have retained an ethyl (N—CH2—CH2⁺) group, resulting in two possible fragments with the same mass (Figure 3, bond cleavage a). The other peaks at *m*/*z* 1865.89 and *m*/*z* 1418.60 represented the results of two C—N bond dissociations in the DOTA, which broke the DOTA scaffold into two halves (Figure 3, bond cleavage b). That cleavage would have produced one peak if the four arms of DOTA were symmetrically modified; but in our synthesis, we always had a shorter arm that was for the bead attachment. Therefore, the two peaks were different. Nonetheless, in both situations, small shoulder peaks resulting from the retention of both ethyl groups on terminal nitrogens were also detected. The fourth major peak in the series with the lowest mass (at *m*/*z* 959.35) likely represented one DOTA nitrogen with a peptoid arm (Figure 3, bond cleavage c). The bond

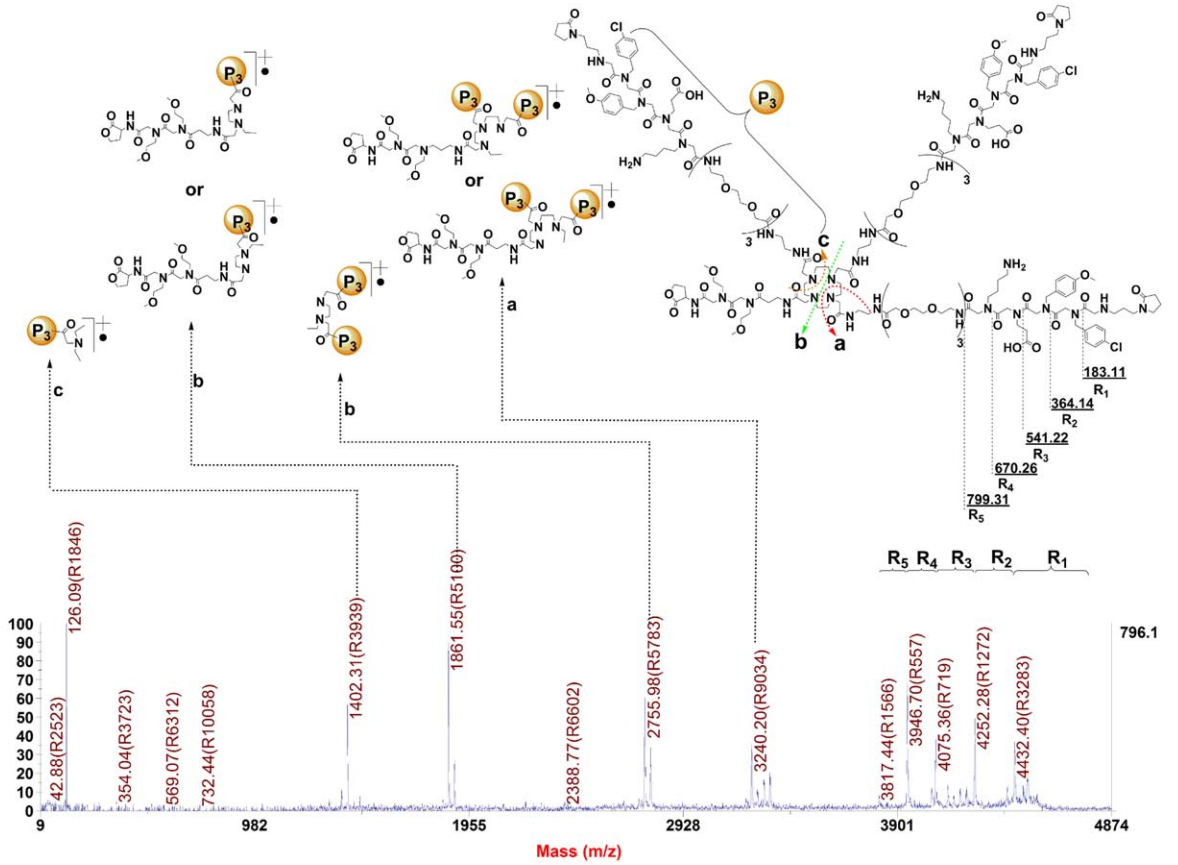
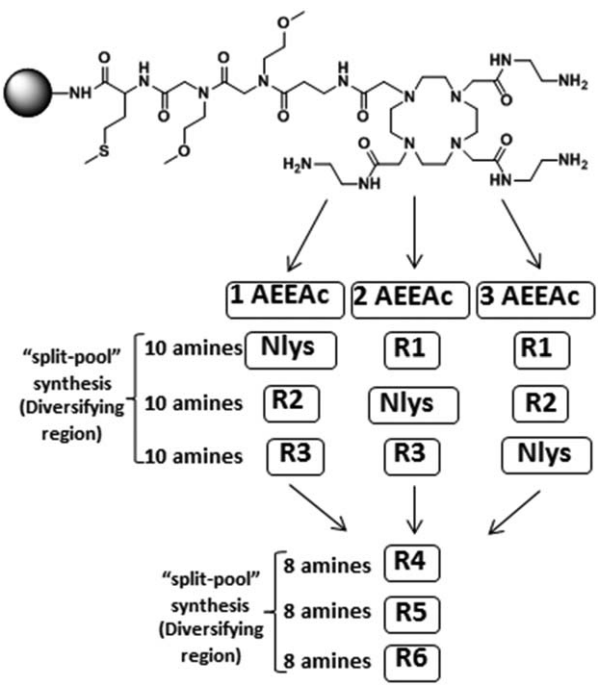


FIGURE 4 MS/MS sequencing of the test compound 22 from crude material cleaved from a single bead.

dissociations within DOTA appeared to have occurred at the ends farthest from the nitrogen, resulting in the retention of both ethyl arms on the nitrogen atom. As a summary, there are four major fragmentation patterns of DOTA scaffold can be observed as follows: (1) Two peptoids arms with the linker that contains three nitrogens, (2) Two peptoids arms that contains two nitrogens, (3) One peptoid arm with the linker that contains two nitrogens, and (4) One peptoid arm that contains one nitrogen.

The very same pattern of peaks for C-N bond breakages within the DOTA scaffold was observed for compounds 21 (Figure S3, Supporting Information) and 22 (Figure 4). Those observations were very unique and provide extremely valuable information for decoding the diversified peptoid regions in library screens. For example, in the single bead MALDI MS/MS spectrum we chose for compound 21, not all of the peptoid residues were cleanly fragmented to give five peaks corresponding to the five terminal peptoid residues R1–5 (Figure S3, Supporting Information). Instead, we observed only two clear peaks that corresponded to R1–2 and R3. Nevertheless, all five peptoid residues (R1–5) were identified and the



SCHEME 2 Peptoid–DOTA OBOC combinatorial synthesis strategy.

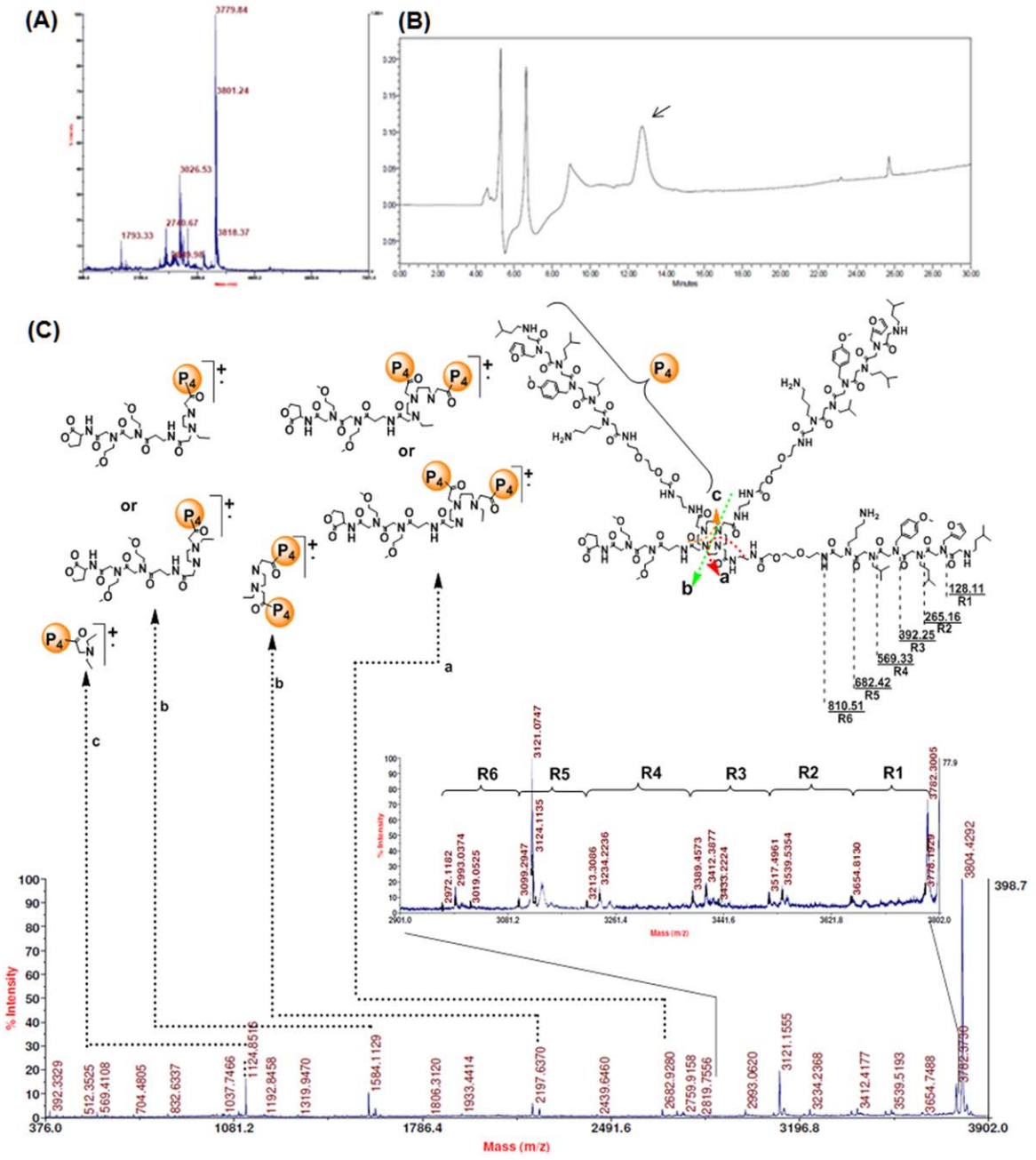


FIGURE 5 Analysis of one of the representative compounds from crude material cleaved off from a single bead randomly chosen from the library. (A) Total mass (MS) spectrum. Expected MS 3778.30 *m*/*z* and observed MS 3779.84 *m*/*z*. (B) Analytical HPLC. Compound peak is marked with an arrow. (C) MS/MS sequencing spectrum and the corresponding analysis. The R1–6 peptoid region peaks considered here are derived from $M + H$ master ion. At the same time, each of those peaks are flanked by corresponding $M + Na$ derived peaks as well (multiple peaks found on the expanded portion of the spectrum).

sequence was narrowed down up to four possible structures with the help of these unique C–N bond ionization peaks. This is possible because we know the exact masses of DOTA scaffold, the AEEAc units and the 1,2-diaminoethane, as described in Figure S3 in the Supporting Information. Thus, the unique C–N bond breakages provide a powerful tool to help reliably decode the diversified peptoid regions of the library.

Confident with the overall synthesis procedure as well as our ability to reliably deduce the peptoid sequences for each arm, we proceeded to synthesize the on-bead peptoid–DOTA

combinatorial library. The design and synthesis strategies are shown in Figure 1 and Schemes 1 and 2. Following the same approach used for the test compounds, we started by attaching methionine to the bead, followed by a three-residue linker, DOTA, and 1,2-diaminoethane coupling to get the main central compound 5 shown in Scheme 1. We then divided that common precursor into three equal parts and coupled it with one or two, or three AEEAc groups to create the variable spacers. Finally, we developed the 6-mer diversified peptoid library region on the spacers using the well-established split–pool synthesis protocol.[1] We decided to introduce a fixed Nlys (19, Scheme 1C) residue at different positions in order to provide additional information to facilitate MS/MS sequencing analysis as well as to improve the solubility of the compounds. We then synthesized the library using Nlys and 10 different amines (9–18, Scheme 1C) as shown in Scheme 2. The first set of pools with one AEEAc spacer was coupled with Nlys as the first residue and then distributed equally into 10 reaction vessels. We treated each vessel with one of the 10 primary amines after the common bromoacetic acid-coupling step in the peptoid synthesis. The beads were washed, pooled, randomized, and redistributed equally into 10 reaction vessels, and the procedure was repeated to add two residues after the Nlys. Similarly, the other two sets with two and three AEEAc spacers were developed with Nlys as a fixed residue at the second and third position, respectively, of the peptoid chain (Scheme 2). The split–pool synthesis was conducted using 10 different amines for all the steps except for the fixed Nlys residue addition. At this point, each bead had DOTA, 1–3 AEEAc spacers, and 3-mer peptoids built on to the 3 arms of DOTA. Then, we mixed the resin beads from all three sets (1–3 spacers, respectively) together and continued to build the library by applying split–pool synthesis with eight different amines (9, 11–13, 15, 17–19, Scheme 1C) until each of the peptoid sequences were six residues in length. At the end, the theoretical diversity of the library was 153,600 compounds; each 6-mer peptoid was built on each of the 3 arms of a central DOTA scaffold attached to beads (same sequence per DOTA or a bead). Finally, we treated the beads with 95% TFA, 2.5% triisopropylsilane, and 2.5% water for 2 h to remove side chain-protection groups and then neutralized the beads with 10% diisopropylethylamine in DMF.

To evaluate the quality of the library, we randomly chose 15 beads, cleaved the compounds off the resin, and analyzed those by MALDI-TOF mass spectrometry. We then subjected the parental $M + H$ ion detected in the initial experiment to tandem MS/MS to generate fragments for sequencing. Eleven out of the 15 resulting compounds were sequenced and at least half of the residues were identified on two more compounds. The total MS, analytical HPLC and MS/MS of one representative compound is shown in Figure 5, and the rest are provided in the Supporting Information (we were unable to obtain analytical HPLCs for all compounds due to technical limitations, as the material cleaved off from a single bead was limited and we gave the priority for MALDI-MS and MS/MS analysis). In the example shown in Figure 5, the ionization patterns of all six terminal peptoid residues (R1–6) were clearly visible (Figure 5, R6). All of the four unique C—N bond cleavage patterns for the DOTA scaffold that were observed for the test compounds (compounds 20, 21, and 22) were exactly reproduced in this library compound as well (Figure 5, bond cleavages a, b, and c). Those unique DOTA cleavage patterns are critically important for deducing the sequences when the breakage patterns of the 6-mer peptoids are not very clear, providing an additional cushion to confidently decode the library. We found that all 10 amines employed in the synthesis were represented in at least one of the 6-mer sequences. With all of those observations, we concluded that the library was of suitable quality to perform screening experiments to isolate novel peptoid–DOTA compounds that can directly be applied as theranostic agents targeting various biological targets.

CONCLUSIONS

We developed a facile on-bead combinatorial synthesis and MS/MS sequencing procedure to establish a unique tripeptoid–DOTA OBOC library. We demonstrated the successful loading of the DOTA imaging scaffold on to the resin using one of the four arms of DOTA. We used the remaining three arms of DOTA to build 6-mer peptoid units that have five diversified residues in each unit using split–pool synthesis. Each peptoid region can act as a recognition and therapeutic unit in the novel molecules, while the DOTA scaffold can be chelated with a variety of metals for PET (^{64}Cu, ^{68}Ga) and MR imaging [Eu(III)]. Three test compounds with different spacer lengths between the DOTA and the peptoids were used to optimize the synthesis procedure. Careful MS/MS sequencing analysis revealed a unique bond cleavage pattern for DOTA that includes C—N bond breakages. After producing the test compounds, we employed the same strategy to develop a library of 153,600 compounds using 10 amines and the split–pool synthesis approach. Randomly chosen beads were analyzed and revealed the same fragmentation patterns displayed by the test compounds, indicating the successful development of the library.

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