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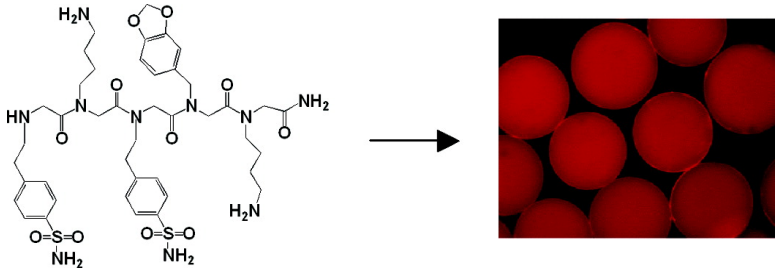
J. Am. Chem. Soc., **2003**, 125 (46), 13995-14004• DOI: 10.1021/ja036417x • Publication Date (Web): 24 October 2003



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Isolation of Protein Ligands from Large Peptoid Libraries

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Abstract: The isolation of ligands for large numbers of proteins is an important goal in proteomics. Whereas peptide libraries are rich sources of protein-binding molecules, native peptides have certain undesirable properties, such as sensitivity to proteases that make them less than ideal for some applications. We report here the construction and characterization of large, chemically diverse combinatorial libraries of peptoids (N-substituted oligoglycines). A protocol for the isolation of specific protein-binding molecules from these libraries is described. These data suggest that peptoid libraries will prove to be inexpensive and convenient sources of protein ligands.

Introduction

There is a great deal of interest in the development of methods for massively parallel protein profiling, i.e., the simultaneous measurement of hundreds or even thousands of proteins in a biological sample. It is anticipated that such technology would be an important tool in medical diagnostics and basic research. Currently, this field is dominated by approaches based on the multidimensional separation of proteins or proteolytic digests followed by mass spectrometric analysis.[1,2] However, another approach that has generated interest is the development of protein-detecting arrays.[3,4] Such devices would be comprised of large numbers of protein-specific binding agents, each addressed to a unique location on a chip or to a specifically encoded bead.

The major barrier to the construction of such devices is lack of large numbers of suitable protein-binding agents. Few commercially available antibodies have sufficient binding affinity or specificity or both for this type of demanding application.[5] Furthermore, antibody production by classical means is an expensive, inefficient, and time-consuming process that is not well suited to proteomic-scale projects. Therefore, there is great interest in the development of rapid and economical methods for the isolation of high-affinity recombinant antibodies,[6−11] protein aptamers,[12] large peptides,[13] and nucleic acid aptamers.[14,15] Despite significant advances in these areas, there remains a case for the development of relatively low molecular mass, synthetic protein ligands for proteomics applications. Such molecules will be easier to mass-produce and purify than biological macromolecules and can be tailored to facilitate linkage to a surface in a defined manner. Furthermore, they do not require a stable tertiary structure, and therefore devices based on these ligands should be much more robust than those displaying biological macromolecules as capture agents. However, while there has been enormous interest in the use of protein-binding small molecules in chemical genetics studies,[16] there has been relatively little effort in the development of immobilized synthetic molecules as capture agents.[17] This is because synthetic molecules isolated from libraries generally exhibit affinities for their target protein at least 1000-fold lower than that of a good antibody. In other words, typical synthetic molecule−protein complexes display equilibrium dissociation constants (K_D's) in the micromolar range, which are insufficient to retain dilute proteins from complex samples. While it is often possible to derive higher affinity ligands from such lead compounds, traditional methods to do so are generally too slow and labor-intensive for proteomics applications.

Our laboratory[18,19] reported a potentially simple solution to this problem recently. We found that when two modest affinity ligands that bind noncompetitively to a protein are co-im-

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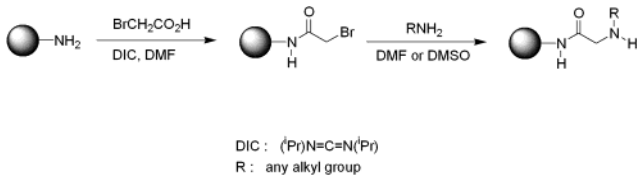
Scheme 1



mobilized at high density on a surface, they can cooperate to capture the target polypeptide with high affinity and specificity. This general strategy has been used many times to derive high-affinity soluble ligands from two or more low-affinity precursors. In solution, however, optimization of the linker is required for high affinity.[20,21] Co-immobilization of two noncompetitive ligands on a solid support eliminates this issue, presumably since the surface presents the two binding elements in many different spacings and geometries. In other words, the surface acts as a "library of linkers", and some fraction of a densely functionalized surface represents a high-affinity bidentate ligand for the target protein. Using peptide ligands as models, we have shown that surfaces modified with these mixed element capture agents (MECAs) can capture subnanomolar levels of target proteins with good specificity.[18,19]

These promising preliminary results support the idea that protein-detecting arrays of real utility could be constructed from simple, readily obtainable compounds. However, native peptides are sensitive to proteases, which could be a significant drawback in the analysis of cell extracts. Thus, we have turned our attention to the development of suitable methods for the generation and screening of large libraries of peptide-like molecules that are immune to proteases. Of the many families of peptide-like compounds that have been reported, we chose to focus on peptoids (oligo-N-substituted glycines)[22−25] since they are almost ideally suited to the preparation of large, chemically diverse combinatorial libraries.[26] This is because peptoids can be made by a "submonomer" route[27] (Scheme 1), in which the side chain is installed by nucleophilic attack of a primary amine on an α-bromo amide moiety. Thus, the "diversity element" in a split-and-pool synthesis is a primary amine,[26] hundreds of which are available commercially.

Although peptoids appear to have such favorable properties for library work, only a few such studies have been reported.[23] Several years ago, Zuckermann and co-workers reported the isolation of two α_1-adrenergic receptor (α_1-AR) ligands with K_i's in the low nanomolar range from a relatively small (ca. 5000 compounds), biased library of two- and three-residue peptoids.[25] More recently, a very small (12-compound) library of peptide−peptoid hybrids was screened to provide an excellent SH3 domain ligand.[28] Similarly, peptoid ligands with analgesic[29] and antimicrobial[30] activities have also been identified from relatively small combinatorial libraries. While these efforts do not represent the screening of large, naïve libraries that would be necessary for a major proteomics ligand discovery effort, they are nonetheless encouraging. Therefore, we decided to carry out pilot studies in which much larger peptoid libraries were synthesized, characterized, and screened against a target protein.

We report here that, as expected, the synthesis of very large peptoid libraries is facile and efficient. In addition, a relatively straightforward protocol was developed that allows the isolation of high-quality protein ligands from these libraries. These data suggest that peptoid libraries will be an excellent source of robust, specific, and inexpensive protein ligands of utility in the construction of MECA-type ligands for the construction of protein-detecting arrays and for other applications.

Results

Synthesis and Characterization of a 78 125-Compound Peptoid Library. The first goal of this project was to construct a peptoid library of many thousands of compounds. As a first step, some new amines were confirmed as good building blocks (2, 5, 8, and 11 in Figure 1). In addition, several other amines have been shown by others to work well in peptoid synthesis, and these are also included in Figure 1.[24−26,31,32] All the amines shown in Figure 1 were found to provide excellent yields in the submonomer protocol (Scheme 1). In each case, this was determined by the synthesis of a test pentameric peptoid in which the amine in question was used in steps two and four, and residues 1, 3, and 5 were derived from the well-behaved benzylamine.[26] We will refer to this test as the "benzylamine sandwich assay". In each case, the desired pentamer was isolated in at least 85% yield.

A major issue proved to be the selection of the solid support. Since the ultimate goal of our efforts is to identify compounds capable of capturing proteins when immobilized on arrays, we plan to screen libraries on resin (vide infra), rather than physically segregating the beads and releasing the compound into solution as is generally done for chemical genetics screens.[33,34] Thus, it was important to identify a resin with: (1) good swelling properties in organic solvents and in water to support both efficient synthesis and to provide ready access of the bound peptoids to proteins in aqueous buffer, (2) a sufficiently high loading capacity that the structure of "hits" from a screen can be determined unambiguously by direct

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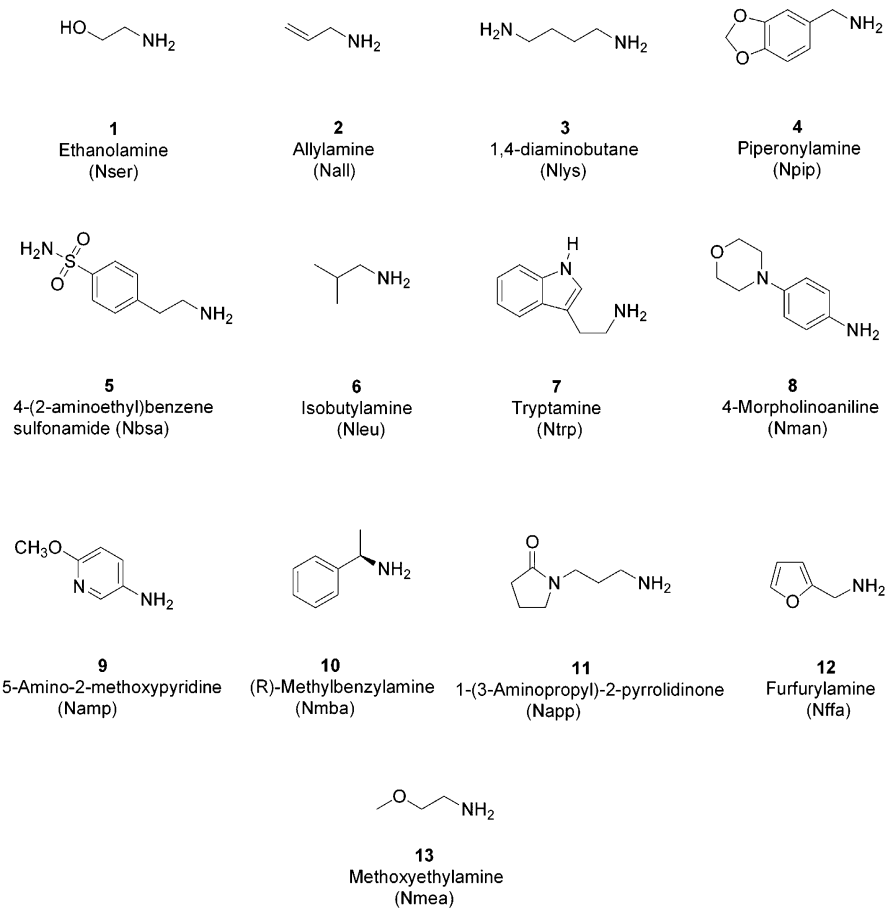

Figure 1. List of amines used for the preparation of the libraries. In brackets we include the corresponding nomenclature of the peptoid units.

Edman or mass spectrometry (MS)-based sequencing, eliminating the need for encoding, and (3) a low fluorescence background, since the screening experiments will most conveniently be carried out with fluorescently labeled protein (vide infra). We found that PEGA, a polyacrylamide-based resin which is employed by several workers in the combinatorial chemistry field, generally satisfied these criteria. However, these beads are extremely fragile mechanically, and this introduced serious problems in the screening experiments due to the large number of broken beads that were present in any library. Therefore, we decided to employ a much more stable polystyrene-based bead. After considerable experimentation, TentaGel Macrobeads (140−170 μm in diameter from Rapp Polymere) were selected as the resin of choice. While it has a hydrophobic core, the TentaGel resin is derivatized with poly(ethylene glycol) chains that not only greatly improve the swelling of the beads in aqueous solution, but also provide a "nonsticky" surface that is ideal for reducing nonspecific protein binding during screening experiments. As will be detailed below, the major drawback of TentaGel beads was their high level of intrinsic fluorescence. This complicated screening experiments, but could be tolerated (vide infra).

The first library constructed employed amines **1**−**5** and had the general formula X_3−Nser−X_4 where X represents any of the monomers derived from amines **1**−**5** (Figure 1). A standard split-and-pool synthesis scheme[35] using 1.5 g of beads was employed to create the combinatorial library, which has a theoretical diversity of 78 125 compounds. The protocol employed to create the first four residues was a slight modification of the published submonomer procedure[24,26] in which the acylation step was carried out with 2 M bromoacetic acid and 3.2 M diisopropylcarbodiimide (DIC) in DMF for 40 min at 37 °C followed by displacement of the bromide with 2 M primary amine for 1 h. For the subsequent residues, the amine addition step was allowed to proceed for 90 min at 37 °C. All the primary amines were dissolved in DMF, except 4-(2-aminoethyl)benzene sulfonamide, which was dissolved in DMSO. The resin was pooled into a 250-mL glass peptide synthesis reaction vessel, mixed by bubbling argon through the suspension for 15 min, and split before each acylation step. At the end of the synthesis, the side-chain protecting groups, if present, were removed by treating with 95% TFA, 2.5% water, and 2.5% anisole for 2 h. The resin was then neutralized with 10% DIEA in DMF, washed with DCM, and dried until further use.

To determine the quality of the library, several tests were conducted. Unfortunately, the amount of compound present on a single bead is too small to allow direct characterization by HPLC or spectroscopic means, but to further address the likely purity of the library members, an 8-mer peptoid was synthesized on Rink amide MHBA resin using amines **1**−**5** (sequence: Nser-Nlys-Nall-Nlys-Nbsa-Npip-Nbsa-Npip-$CONH_2$). The final product was released from the beads using 95% TFA, 2.5% water, and 2.5% anisole, and the material was characterized by

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(1) Npip-Npip-Nbsa-Nser-Nlys-Npip-Nbsa-Nlys
(2) Nbsa-Npip-Nlys-Nser-Nbsa-Nser-Nbsa-Npip
(3) Nbsa-Nlys-Npip-Nser-Nbsa-Nser-Nall-Nlys
(4) Nbsa-Nbsa-Nall-Nser-Nall-Npip-Nall-Npip
(5) Nall-Nall-Nbsa-Nser-Nlys-Nbsa-Nser-Nall
(6) Nlys-Nlys-Nser-Nser-Npip-Nall-Nbsa-Nser
(7) Nlys-Nlys-Nser-Nser-Npip-Nbsa-Nall-Nbsa
(8) Nall-Nlys-Nser-Nser-Nbsa-Nser-Nlys-Nlys
(9) Npip-Nlys-Nall-Nser-Nbsa-Nser-Nbsa-Nall

Figure 2. Text sequences of nine random peptoids picked from the 78 125-compound library. The sequences were determined by automated Edman degradation.

HPLC and mass spectrometry. As shown in the Supporting Information, the data indicated that the major peak in the HPLC corresponded to the expected compound. While this experiment cannot account for potential context-dependent effects in the synthesis of a combinatorial library, it does demonstrate that all of the monomers work well in the synthesis, consistent with the previous tests of each monomer using the benzylamine sandwich assay.

To evaluate diversity, 10 beads from the library were chosen at random, and the displayed peptoids were sequenced by automated Edman degradation.[36] Liskamp reported that peptoids can be sequenced by Edman chemistry using several beads as the input.[37] However, for library screening experiments, it is critical to be able to sequence a single bead. An automated approach would be even more advantageous, as it would eliminate the practical difficulties involved in handling individual beads for long periods of time, over several cycles of chemistry. We therefore adapted a commercial peptide sequencer (ABI 476A) for the sequencing of peptoids. Although larger (400−500 μm) TentaGel macrobeads are available commercially that allow spectroscopic analysis of the compounds derived from a single bead, such beads introduce practical limitations on the size of the libraries that can be constructed and, hence, were not employed in this study.[38] The typical HPLC protocol used for sequencing peptides was modified slightly to allow the gradient to run longer (see Experimental Section for details). As shown in Figure 2, when 10 beads were picked from the library and subjected to Edman degradation, the derived sequence of each peptoid was different, as expected for a large, diverse library. The chromatographic traces from these sequencing runs (see Supporting Information) also showed that each of the peptoids was full-length. At each step of the Edman process, we generally observed only one major peak, with the exception of a small amount of signal resulting from the previous and subsequent monomers in the peptoid, which is commonly observed in peptide sequencing using this chemistry.

Isolation of Mdm2-Binding Peptoids From the Library. With a high-quality peptoid library in hand, we next turned to developing appropriate conditions for on-bead screening. To facilitate future efforts to automate screening using a fluorescence-activated bead sorter, we had hoped to employ fluorescently

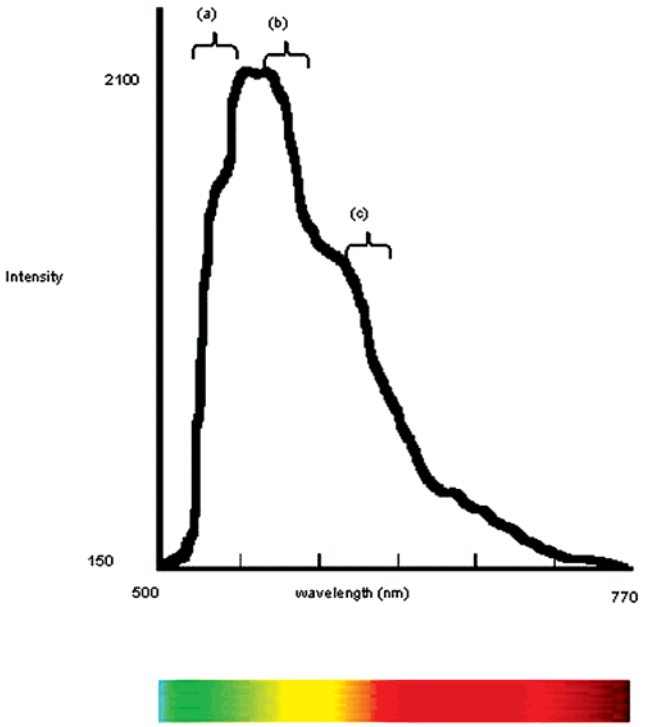

Figure 3. Fluorescence emission spectrum of a TentaGel bead. Excitation: 460−490 nm. Emission of some fluorescent dyes: (a) fluorescein, (b) tetramethylrhodamine, (c) Texas Red.

labeled proteins in screening experiments. However, as mentioned above, the TentaGel resin employed had a surprisingly high level of intrinsic fluorescence, particularly in the green region of the spectrum (Figure 3). This "background fluorescence" rendered the use of many organic dyes, such as fluorescein, impractical for screening experiments (data not shown). However, the intensity of the bead fluorescence dropped off significantly in the red region of the spectrum. Thus, we decided to evaluate Texas Red-labeled proteins as potential targets in the screening process.

The human Mdm2 protein is a negative regulator of p53 function and a potential anti-cancer drug target. We employed a fragment of Mdm2 (residues 1−188) fused to maltose-binding protein (MBP) as our initial target, since this fusion protein was expressed at higher levels than the Mdm2 fragment alone. This region of Mdm2 includes the region of the native protein that binds the p53 activation domain,[39] and there have been several previous reports of the isolation of peptide or small molecule ligands for this region of Mdm2.[40−42] Thus, we suspected that this protein would represent a reasonable target for the initial peptoid library screening experiments.

Detailed screening conditions are given in the Materials and Methods section. In general, we found that it was critical to employ challenging conditions to eliminate low-affinity or low-specificity hits (data not shown). For example, we have settled on the use of a high salt- and detergent-containing buffer (1 M

(36) A single bead was placed into the chamber of the Edman sequencer, and the peptoid was sequenced on resin.
(37) Boeijen, A.; Liskamp, R. M. J. *Tetrahedron Lett.* **1998**, *39*, 3589−3592.
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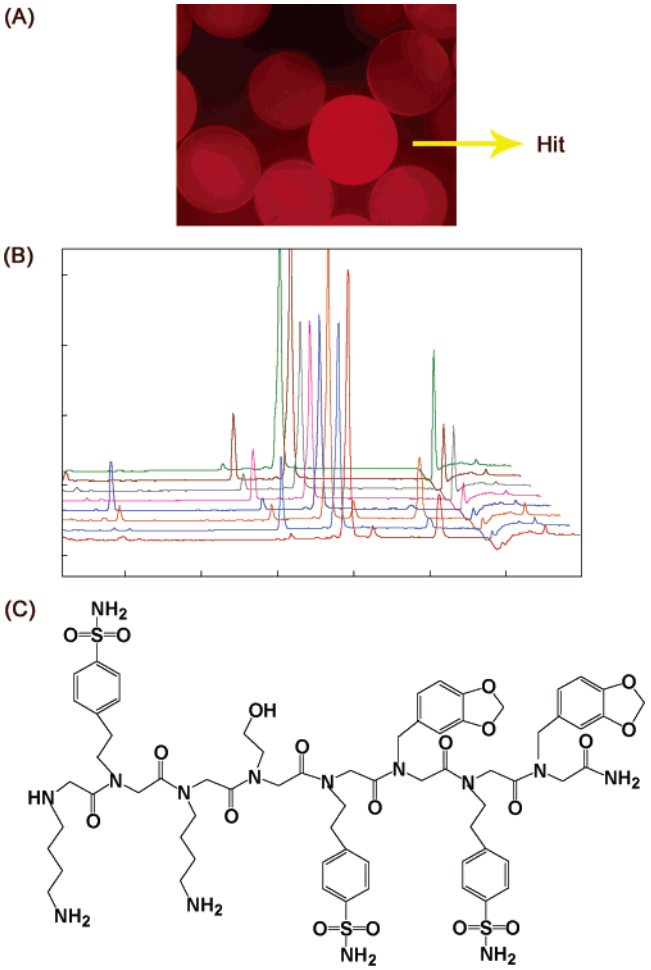

Figure 4. Isolation of a putative peptoid ligand for MBP−Mdm2. (A) A photomicrograph showing a field of beads that contains the one picked as a putative "hit". (B) An Edman sequencing trace of the bright bead shown in part A. (C) Sequence of the isolated peptoid deduced from automated Edman degradation from the single bead.

NaCl and 1% Tween-20). The concentration of the Texas Red-labeled protein was only 50 nM since experiments conducted at higher protein concentrations indicated that a larger fraction of the library registered as "hits", presumably representing weaker ligands. Finally and perhaps most importantly, we employed a 1000-fold excess (based on mass) of cleared *Escherichia coli* lysate to demand high specificity. As will be detailed elsewhere (L. Troitskaya and T. Kodadek, in preparation), screening experiments that employed only a single competitor protein such as bovine serum albumin (BSA) provided poorer results.

After incubating the labeled maltose-binding protein (MBP)−Mdm2 fusion protein with the bead library for 1 h under these conditions and then washing the beads six times with the same buffer, beads that exhibited above background fluorescence were identified visually using a fluorescence microscope. Figure 4A shows a photomicrograph of a field containing a bead that we scored as a hit in this experiment. As can be seen, this bead (marked with the arrow in the figure) is clearly brighter than the surrounding beads, but all of the others are far from dark. This is, in part, a reflection of the intrinsic fluorescence of the beads (Figure 3) as well as the low-level, nonspecific binding of some of the labeled protein to many beads in the library, despite the presence of high levels of competitor. Fortunately, while this background is annoying and reduces the speed at which libraries can be screened visually, it is tolerable. Eleven hits (~0.014% of the input beads) with a fluorescence well above were identified in this experiment.

To identify the sequence of each peptoid hit, the bright beads were picked manually using a pipet. The individual beads were then heated to 95 °C in 1% SDS[43] and placed in the chamber of an automated Edman sequencer. Some consensus was observed among the hits at positions 1, 2, and 8. The Edman sequencing trace of the brightest bead among the 11 hits is shown in Figure 4B, which clearly identified its sequence (Figure 4C).

Validation of the Putative Mdm2 Ligands. A critical issue in any library screening experiment is to validate the resynthesized ligands. Bead library-derived ligands often fail in typical solution binding assays for many reasons. For example, avidity or context effects unique to the solid surface on which the library was constructed could allow it to work well on resin but behave poorly in solution binding assays. Even more problematic is the possibility that the true ligand might have been a minor component on the bead due to some sort of side reaction during the synthesis and is not the compound expected from the sequencing data.

The putative hit (see Figure 4C) was resynthesized on Rink resin, cleaved, and purified to apparent homogeneity by HPLC. Binding of the synthetic peptoid to MBP−Mdm2 was then analyzed by isothermal titration calorimetry (ITC).[44] The data (see Figure 5A) indicated an equilibrium dissociation constant of 37 μM. When the titration experiment was repeated with MBP, little or no binding was observed (Figure 5B). This observation suggests that the peptoid ligand isolated is specific and that it recognizes the Mdm2-derived domain of the MBP−Mdm2 fusion protein against which it had been selected.

Given our long-term goal of constructing protein-detecting microarrays based on peptoids or other synthetic compounds, it was of even greater interest to us to determine the binding properties of the resynthesized compound when affixed to a solid surface. To this end the experiments shown in Figure 6 were carried out. First, the hit was resynthesized on TentaGel and the protecting groups removed without removing the peptoid from the bead. It was then incubated with either Texas Red-labeled MBP−Mdm2 or Texas Red-labeled MBP at the protein concentration indicated in the figure in the presence of 2% BSA as competitor. As can be seen in Figure 6A, the beads captured the MBP−Mdm2 protein efficiently, while little MBP binding was observed. This corroborates the ITC data. To eliminate the possibility that the Texas Red label contributes significantly to binding of the protein to the immobilized peptoid, we also conducted an experiment using native MBP−Mdm2. Unlabeled protein was incubated with the peptoid hit on TentaGel beads in the presence of a 1000-fold excess of *E. coli* proteins. The beads were then pelleted and washed. As shown in Figure 6B, lane 3, SDS-PAGE/Western blot analysis revealed that the immobilized peptoid had retained about 10% of the MBP−

(43) We have observed that the beads that have been exposed to lysates during screenings did not sequence well. Presumably this was as a result of the beads becoming coated with the lysate components that interfered with Edman degradation. Consistent with this view, peptoid-displaying beads that had not been exposed to lysates sequenced quite well. By washing the beads with 1% SDS for 20 min at 95 °C, followed by rinsing with distilled water, we alleviated this problem.

(44) Leavitt, S.; Freire, E. *Curr. Opin. Struct. Biol.* **2001**, *11*, 560−566.

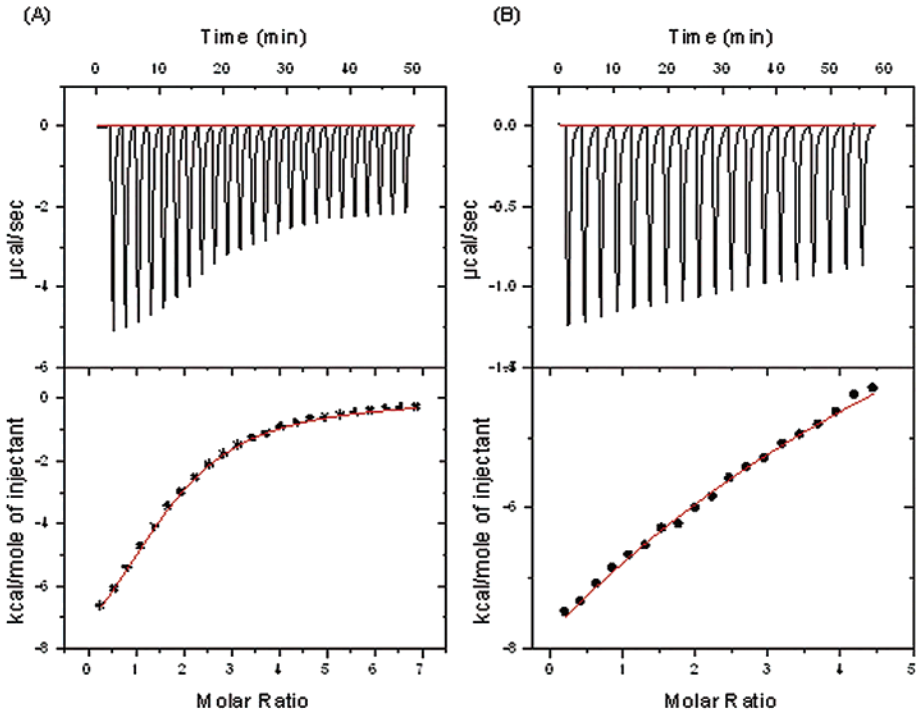

Figure 5. Characterization of the peptoid−protein complex by ITC. ITC traces for binding of Nlys-Nbsa-Nlys-Nser-Nbsa-Npip-Nbsa-Npip to (A) MBP−Mdm2 and (B) MBP. The top panel shows the raw data, whereas the bottom panel shows the integrated curve of the experimental points (solid circles) and the best fit (solid red line) of the curve. The K_D values derived from these data were 37 μM for the MBP−Mdm2·peptoid complex and greater than 1 mM (i.e., little or no binding) for MBP, indicating that the peptoid targets the Mdm2-derived polypeptide.

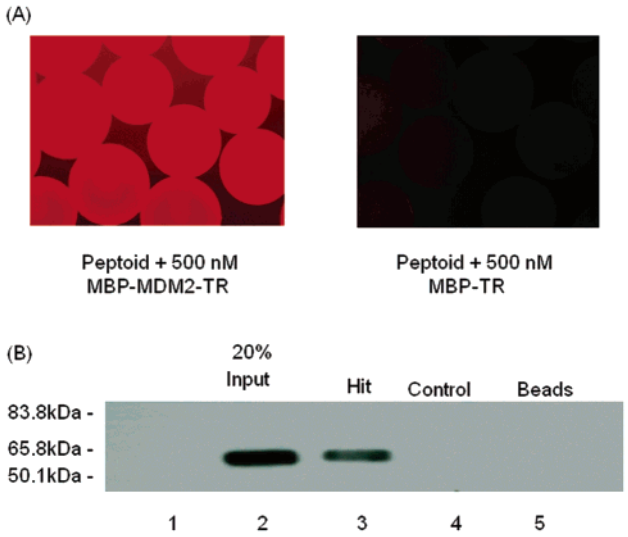

Figure 6. Characterization of the on-resin binding properties of the Mdm2-binding peptoid. (A) TentaGel beads displaying Nlys-Nbsa-Nlys-Nser-Nbsa-Npip-Nbsa-Npip were incubated with 500 nM of Texas Red-labeled MBP−Mdm2 (left panel) or 500 nM Texas Red-labeled MBP (right panel). (B) Capture of native protein. TentaGel beads displaying the peptoid indicated were incubated with 1 μM native protein (indicated below) and a 1000-fold excess of *E. coli* extract. The protein retained was analyzed by SDS-PAGE. A Western blot using anti-Mdm2 antibody is shown. Lane 1: molecular mass standards. Lane 2: 20% of the input. Lane 3: protein retained by TentaGel beads displaying the hit Nlys-Nbsa-Nlys-Nser-Nbsa-Npip-Nbsa-Npip. Lane 4: protein retained by the control peptoid Nmba-Nbsa-Nleu-Nlys-Npip-Nmba-Nleu-Nleu. Lane 5: protein retained by TentaGel beads lacking a displayed peptoid (bead only control).

Mdm2 protein present (note that the protein was present in molar excess over the peptoid, and thus complete retention of the input was not possible). No detectable MBP−Mdm2 protein was retained when this experiment was repeated with a random peptoid (lane 4) or TentaGel beads lacking a displayed peptoid (lane 5).

Table 1. Description of the Libraries Constructed in This Study[a]

formula	amines employed	no. different compounds (theoretical)
X_3−Nser−X_4	1, 2, 3, 4, 5	78 125
X_5	3, 4, 5, 6, 8, 9, 10, 11, 12, 13	100 000
X_8	3, 5, 6, 7, 10	390 625
X_6	3, 4, 5, 6, 7, 10, 11, 12, 13	531 441

[a] Numbers refer to the compounds shown in Figure 1. X represents any of the possible monomers derived from the amines shown.

We conclude from these experiments that the peptoid selected in the library screening experiment is a bona fide Mdm2-binding compound capable of capturing the protein from complex mixtures such as model cell extracts.

Larger, More Chemically Diverse Peptoid Libraries. With the above results in hand, we turned to the construction of larger or more chemically diverse libraries or both to support future larger-scale screening experiments against many different protein targets. As before, standard split-and-pool synthesis[35] on TentaGel was employed to create the library as detailed in the Experimental Section. The first library utilized amines **3**−**6** and **8**−**13** and consisted of five residue peptoids (Table 1), providing a theoretical diversity of 100 000 compounds. While not much larger than the 78 125-member library discussed above, this library is far more diverse chemically, since 10 different amines were employed in its construction, with each position randomized. The second library employed only five monomers, but was longer, consisting of randomized octamers, providing a theoretical diversity of 390 625 compounds (Table 1). Finally, an extremely large library of randomized hexamers was made using nine different amines, providing a theoretical diversity of 531 441 compounds (Table 1).

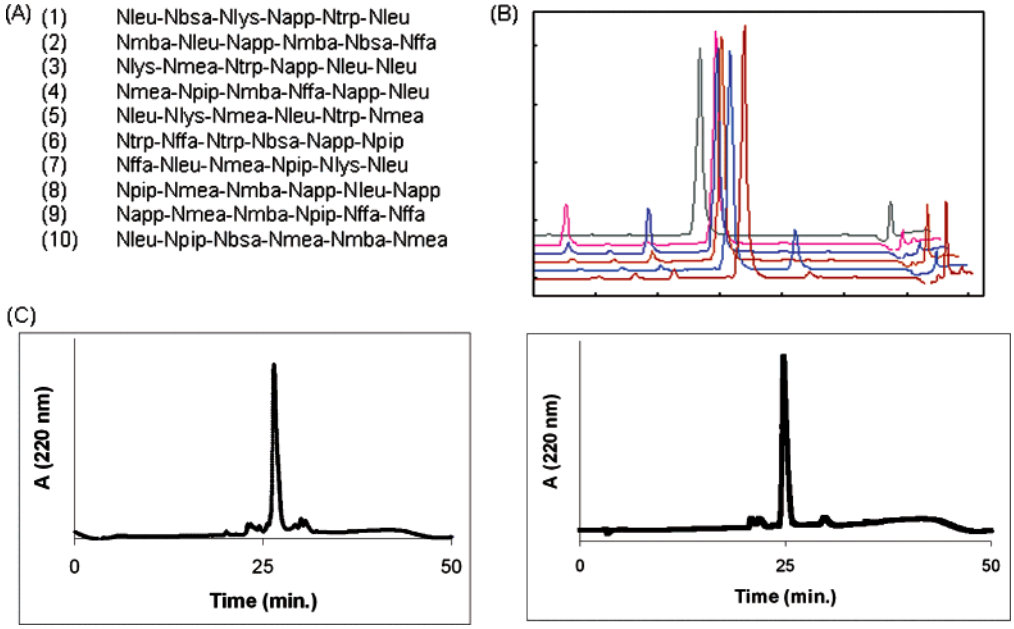

Figure 7. Characterization of a large peptoid library containing more than half a million compounds. (A) Sequences of the peptoids obtained from 10 beads picked randomly from the library. (B) Representative Edman traces obtained from one of these beads. (C) HPLC traces of two hexamers (Ntrp-Nmea-Npip-Nlys-Nffa-Nmba and Nbsa-Nleu-Napp-Napp-Nffa-Nmea-Npip) that, between them, contain each of the amines employed in the construction of the library.

All of these libraries were characterized for quality in the same way as described above for the 78 125-compound library. Some of the data for the largest of the libraries are presented below. Figure 7A shows the results of sequencing 10 beads chosen at random from the library. As expected, all were different. The Edman traces again suggested that full-length peptoids were obtained in each case (Figure 7B). Two mixed sequence hexamers, Ntrp-Nmea-Npip-Nlys-Nffa-Nmba-$CONH_2$ and Nbsa-Nleu-Napp-Nffa-Nmea-Npip-$CONH_2$, were synthesized and shown by HPLC to be >85% pure. Between them, these hexamers contain all of the monomers that were subsequently employed in the library construction. The results again suggest that in the absence of unexpected context effects, all of the coupling steps proceed in high yield.

To determine if these libraries would be facile sources of protein ligands as well, part of the 100 000-compound library was screened against Texas Red-labeled glutathione-*S*-transferase (GST) using conditions similar to those described above except that lower salt and detergent concentrations were employed. Of the approximately 50 000 beads used in this screen, 0.5% of the total population displayed red fluorescence well above the background. One of the brightest beads was picked and the peptoid sequence determined by Edman degradation to be Nbsa-Nlys-Nbsa-Npip-Nlys-$CONH_2$ (Figure 8). Since we are primarily interested in evaluating the ligands isolated from these screens for their ability to retain proteins from biological samples when attached to a surface, we conducted a number of on-bead assays with Nbsa-Nlys-Nbsa-Npip-Nlys-$CONH_2$ (Figure 9). As shown in Figure 9A, the resynthesized compound retained Texas Red-labeled GST, but not a control protein (MBP) when immobilized on TentaGel. Furthermore, unlabeled GST was retained by the TentaGel−peptoid beads in the presence of a 1000-fold excess of *E. coli* extract (Figure 9B). As shown in Figure 9C, this was the case using GST concentrations of 1 μM to 100 nM. When the protein concentration was 10 nM, little or no fluorescence above background was observed (not shown). Finally, solution binding studies were performed employing ITC, resulting in an equilibrium dissociation constant of 62 μM for the peptoid−protein complex (see Supporting Information).

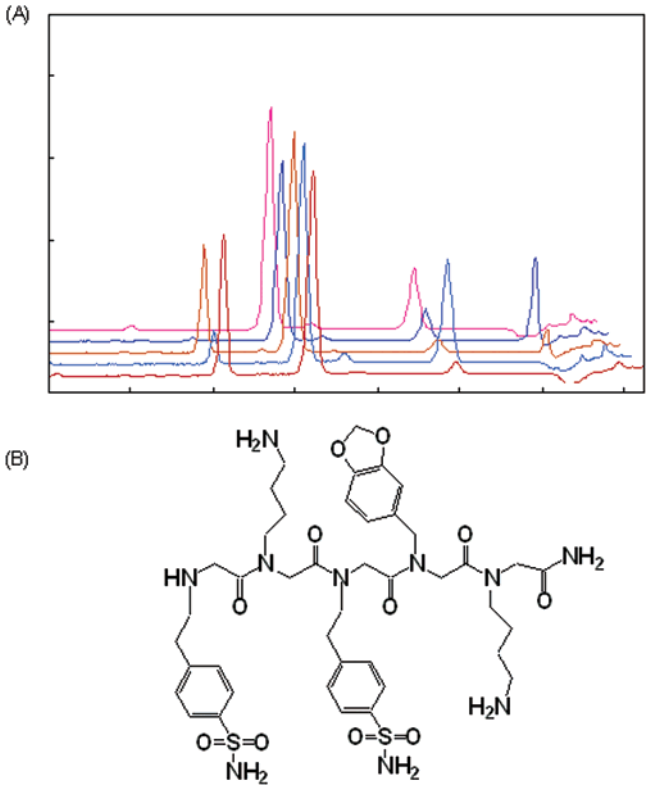

Figure 8. Identification of a GST-binding peptoid from a library of 100 000 pentamers. (A) Edman traces of the hit picked from the screening experiment. (B) Sequence of the peptoid derived from the Edman traces.

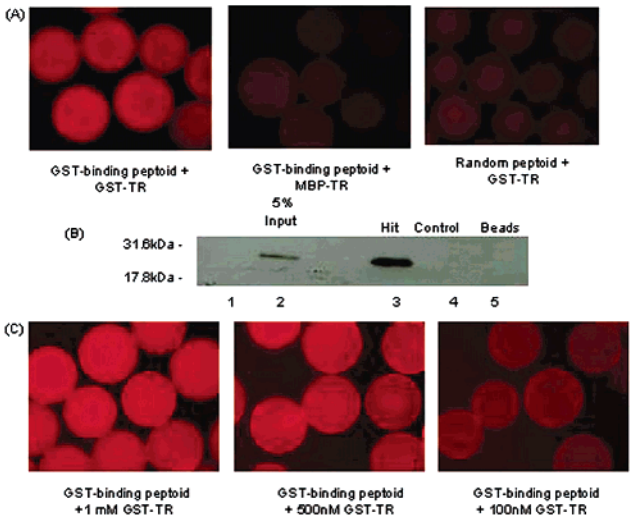

Figure 9. Characterization of the on-bead binding properties of the peptoid obtained in the screen against GST. (A) Photomicrographs obtained after incubation of TentaGel beads displaying the putative GST-binding peptoid Nbsa-Nlys-Nbsa-Npip-Nlys (left and middle panels) or a control peptoid Npip-Nser-Nbsa-Nall-Nlys-Npip (right panel) with 500 nM Texas Red-labeled GST or 500 nM Texas Red-labeled MBP. BSA (2%) was included in each solution to reduce nonspecific interactions. (B) Capture of native GST by TentaGel-displayed peptoid. A Western blot obtained using anti-GST antibodies is shown. Lane 1: molecular mass standards. Lane 2: 5% of the input (1 μM GST + 1000-fold excess *E. coli* extract). Lane 3: GST retained by TentaGel-Nbsa-Nlys-Nbsa-Npip-Nlys. Lane 4: GST retained by TentaGel-displayed Nmba-Nbsa-Nleu-Nlys-Npip-Nmba-Nleu-Nleu (the control peptoid). Lane 5: GST retained by TentaGel beads without a displayed peptoid (beads only control). (C) Dilution experiment measuring the capture of Texas Red-labeled protein by TentaGel-displayed Nbsa-Nlys-Nbsa-Npip-Nlys at the protein concentrations indicated. All solutions contained a 100-fold excess of *E. coli* extract.

Discussion

Peptoid libraries are a promising source of protease-insensitive protein-binding molecules. The development of the so-called submonomer synthesis of peptoids (Scheme 1) by Zuckermann and colleagues provides a convenient route to libraries by the standard split-and-pool approach using inexpensive, commercially available amines as the source of diversity. We have employed this chemistry to create quite large combinatorial libraries of peptoids (Table 1), the largest exceeding half a million compounds. The data shown in Figures 2 and 7 indicate that these libraries are of good quality.

Since our immediate goal is to create arrays of protein capture molecules immobilized on chips or encoded beads,[3,4] we chose to develop conditions to screen these compounds on resin rather than in solution. This has been accomplished. For the two examples provided in this report, peptoids that recognize Mdm2 and GST, respectively, the binding data suggest that the ligands bind their targets with good specificity. Of particular importance to us is the fact that the immobilized peptoids are able to capture unmodified protein efficiently in the presence of a large excess of bacterial proteins, thus simulating the situation of a moderately abundant native protein in a crude extract (Figures 6B and 9B). Many studies of protein-binding ligands often fail to assess this important aspect of binding specificity and instead focus only on experiments using a single purified protein. The Mdm2·peptoid and the GST·peptoid complexes displayed K_D's of about 37 and 62 μM, respectively, under the conditions employed (Figure 5 and Supporting Information). In the course of characterizing hits from peptoid and peptide library screens against many different proteins (unpublished data), we have found this result to be fairly typical. In general, very high affinity ligands that bind in the nanomolar regime can only be derived from much larger libraries[13] that are not accessible by split-and-pool synthesis. Nonetheless, we are confident that high-affinity capture agents can be constructed by co-immobilization of two or more noncompetitive ligands in a fashion that would support cooperative, bivalent binding to the target protein. We have demonstrated this principle using peptide ligands.[18,19]

Work has begun to screen some of the libraries reported here, particularly the 100 000- and 531 441-compound libraries, against a variety of target proteins with the eventual aim of constructing a protein-detecting microarray from these ligands. These efforts will be reported in due course.

Materials and Methods

Reagents and Instrumentation. All of the reagents and solvents were purchased from commercial suppliers and used without further purification. TentaGel macrobeads (140−170 micron diameter; substitution: 0.51 mmol/g) were obtained from Rapp Polymere. Analytical HPLC was performed on a Biocad Sprint system with a C18 reversed-phase HPLC column (Vydac, 5 μM, 4.6 mm i.d. × 250 mm). A gradient elution of 10−50% B in 20 min followed by 50−80% B in 5 min was used at a flow rate of 1 mL/min (solvent A: H_2O/0.1% TFA; B: CH_3-CN/0.1% TFA). MALDI-TOF MS was performed on a Voyager-DE PRO biospectrometry workstation (Applied Biosystems) using α-hydroxy cinnamic acid as the matrix. A New Brunswick Scientific Innova 4400 incubator shaker was used to perform the peptoid syntheses at 37 °C. Microwave-assisted peptoid syntheses were performed on a 1000W Whirlpool microwave oven (model MT1130SG) set to deliver 10% power. Edman sequencing of peptoids was performed on an ABI 476A Protein Sequencer (Applied Biosystems). The fluorescence spectra of the beads were recorded with a hyperspectral microscope constructed in the laboratory of Prof. Harold Garner (UT-Southwestern).[45] The on-bead fluorescence assays were visualized with a Nikon Eclipse TE300 fluorescence microscope equipped with a Chroma 61002 triple band filter set and a CCD camera. MetaMorph software was used to acquire and process the photomicrographs. ITC experiments were performed on a MicroCal VP−ITC instrument.

Syntheses of Peptoid Libraries at 37 °C. The syntheses of the 8-mer libraries were performed in standard 25-mL glass peptide synthesis reaction vessels (Chemglass) in an incubator shaker at 37 °C. TentaGel macrobeads (1.5 g; 140-170 μm; substitution: 0.51 mmol/g) were distributed equally into five peptide synthesis reaction vessels, 5 mL of DMF was added, and the beads were allowed to swell at room temperature for 60 min. The DMF was drained, and to each vessel was added 1.5 mL of 2 M bromoacetic acid and 1.5 mL of 3.2 M diisopropylcarbodiimide (DIC). The reaction vessels were placed on an incubator shaker set at 37 °C and 225 rpm for 40 min. The vessels were drained, and the beads were thoroughly washed with DMF (8 × 3 mL). The beads in each of the vessels were treated with one of the five primary amines (see Table 1) at 2 M concentration and allowed to react in the shaker at 37 °C for 60 min. All the amines were dissolved in DMF, except 4-(2-aminoethyl)benzene sulfonamide which was dissolved in DMSO. The vessels were drained and washed thoroughly with DMF (8 × 3 mL). The beads in each of the reaction vessels were pooled into a large 250-mL peptide synthesis vessel, drained, suspended in 50 mL of dichloromethane/DMF (2:1), and randomized by bubbling argon for 15 min. The beads were distributed equally into five 25-mL peptide synthesis vessels, and the procedure was repeated. The protocol was slightly modified for the final four residues of the library, where the displacement of the bromide by the primary amine was carried out

(45) Schultz, R. A.; Nielsen, T.; Zavaleta, J. R.; Ruch, R.; Wyatt, R.; Garner, H. R. *Cytometry* **2001**, *43*, 239−247.

for 90 min, instead of 60 min. In the case of the 78 125-compound library, the fourth residue from the amino terminus was fixed and thus, all the reaction vessels were treated with ethanolamine for the bromide displacement step.

Microwave-Assisted Peptoid Library Syntheses. The syntheses of the 5-mer and the 6-mer libraries were performed employing a microwave-assisted protocol[46] on 1 and 2 g of beads, respectively. In this protocol, both the acylation and bromide displacement by the primary amine were performed twice for 15 s in a 1000W microwave oven set to deliver 10% power. The beads were shaken manually for 30 s between microwave pulses to ensure proper mixing. All the other steps were identical to the 37 °C procedure.

Protection and Deprotection of Additional Functional Groups in Primary Amines. The functional groups in amines **1** (hydroxy), **3** (primary amino), and **7** (secondary amino) were protected following previously reported literature procedures.[47,48] The following procedure was used to cleave the protecting groups at the end of the library synthesis.

The beads were washed thoroughly with DMF (8 × 3 mL) and then with dichloromethane (3 × 3 mL), drained, and treated with 6 mL of 95% TFA, 2.5% water, and 2.5% anisole for 2 h. The cleavage cocktail was drained, and the beads were washed thoroughly with dichloromethane (8 × 3 mL). The beads were neutralized by treating with 10% diisopropylethylamine in DMF for 5 min, washed with dichloromethane (5 × 3 mL), and dried until further use.

For resynthesis and characterization of peptoids by HPLC and MALDI-TOF, syntheses were performed on 50 mg of Fmoc-Rink amide MHBA resin (substitution: 0.73 mmol/g; Nova Biochem). The beads were swollen in DMF for 30 min, drained, treated twice with 20% piperidine in DMF for 10 min (2 × 2 mL), and washed with DMF (8 × 3 mL). The peptoid sequence was constructed by the microwave-assisted protocol[42] and washed thoroughly with DMF (8 × 3 mL) and dichloromethane (3 × 3 mL). The peptoid was released from the resin with concomitant removal of protecting groups by treating the beads with 6 mL of 95% TFA, 2.5% water, and 2.5% anisole for 2 h. The suspension was filtered and the filtrate concentrated by blowing nitrogen over the solution. The concentrated filtrate was dissolved in 2 mL of 1:1 acetonitrile/water and lyophilized. The resultant solid was subjected to HPLC and MALDI-TOF analysis.

Sequencing Peptoids by Edman Degradation. The sequencing of peptoids was performed on an ABI 476A protein sequencer, using the FSTNML program and a standard gradient (Gradient 1). The FSTNML program was slightly modified by adding a 60 s "wait" step at the end of the cycle to enable the gradient to run slightly longer than normal.

Protein Purification. GST was expressed in *E. coli* BL21-RIL from the commercially available plasmid pGEX-2T (Amersham Biosciences). The cells were grown until an OD_{600} of 0.8 was reached, at which time 1 mM IPTG was added to the medium to induce protein expression. After further growth at 37 °C for 3 h, the cells were harvested, sonicated, and centrifuged at 22 000 rpm. The cleared lysate was then incubated with glutathione−agarose beads equilibrated with phosphate-buffered saline (PBS) at 4 °C for 1 h. The beads were washed with 10−12 volumes of PBS, packed into a column, and further rinsed with PBS. GST bound to the beads was eluted with 10 mM reduced glutathione/PBS, and fractions were collected and analyzed on a 12% denaturing polyacrylamide gel. The fractions containing highly purified GST were pooled and dialyzed against PBS + 10% glycerol. The protein concentration was estimated using Coomassie Plus Protein Assay Reagent Kit using BSA as a standard. MBP−Mdm2 (residues 1−188) was overexpressed from pMAL−Mdm2 in BL21-RIL cells.[18] Herein, the conditions were slightly modified; cells were grown in the presence of 0.2% glucose and induced at $OD_{600\ nm} = 0.5$ with 0.3 mM IPTG and grown for an additional 3 h. Tris-HCl (20 mM) + NaCl (200 mM) + EDTA (1 mM), pH 7.4, was used as the buffer. The protein was bound to amylose resin and after thorough washing, eluted with 10 mM maltose.

Protein Labeling with Texas Red. The protein solution (preferably 2 mg/mL) was adjusted to pH 8.3 with 0.2 M $NaHCO_3$ buffer. To this 5 μL of 50 mg/mL Texas Red solution in DMF was added with mild vortexing to mix the sample. This solution was incubated with tumbling at room temperature for 1 h, after which the reaction was quenched with 1.5 M hydroxylamine. Dye-conjugated protein was separated from excess dye using a desalting column. Measurement of the absorbance of the sample at 280 and 595 nm indicated that, on average, these conditions resulted in each protein molecule acquiring one molecule of Texas Red.

Preparation of *E. coli* Lysate for Screening Experiments. The *E. coli* (BL21-RIL strain) cells were grown overnight at 37 °C in Luria broth. The cells were harvested by low-speed centrifugation, washed, and resuspended in sonication buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 0.1% Tween20 + protease inhibitor). The cells were then sonicated and centrifuged at 22 000 rpm to remove cell debris and provide the cleared cell lysate. The concentration of the lysate was estimated using the Bradford assay with BSA as a standard.

Library Screening and Identification of Hits. TentaGel beads (150 mg; approximately 78 000 beads) harboring the combinatorial library X_3−Nser−X_4 were swollen in TBST (50 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Tween20) for 1 h, after which they were blocked with *E. coli* lysate at room temperature for 1 h. The lysate was removed, and the beads were incubated with 50 nM Texas Red-conjugated MBP−Mdm2 in TBST containing 1 M NaCl + 1% Tween20, in the presence of a 1000-fold excess of *E. coli* lysate (assuming the average molecular mass of the proteins in the lysate to be the same as of the target protein), for 1 h at room temperature. The beads were washed with TBST (6 × 1 mL) and visualized under a fluorescence microscope fitted with a Texas Red filter. The brightest beads were isolated manually with a pipet tip.

In another experiment, 100 mg of library X_5 was screened for GST binding peptoid ligands. The beads were blocked with 5% milk/TBST and then incubated with 1 μM Texas Red-labeled GST in the presence of 1000-fold excess of *E. coli* lysate

After picking the putative "hits", each bead was heated in a 1% SDS solution for 20 min, followed by three washes with 1× PBS. They were then sequenced by Edman degradation.

Isothermal Titration Calorimetry. ITC experiments were conducted on a MicroCal VP−ITC instrument. For the titration, 70 μM MBP−hMdm2 or 30 μM GST in PBS + 10% DMSO was taken in the sample cell. To this, 15 μL aliquots of the peptoid solution in the same buffer were added from a computer-controlled 250 μL rotating syringe. The syringe was set at 400 rpm with intervals of 3 min between injections to attain baseline stabilization. The heat absorbed or released accompanying the titration was recorded as differential power (DP) by the instrument software. Experiments were carried out with *C* values between 1 and 400. The total heat recorded was then fitted via a nonlinear least-squares minimization method. Titration of the ligand solution with the buffer alone gave the heats of dilution. Titration with MBP alone was recorded under identical conditions.

Protein Capture Assays Using TentaGel-Displayed Peptoids. TentaGel beads (5 mg; displaying the respective hit sequences, Nlys-Nbsa-Nlys-Nser-Nbsa-Npip-Nbsa-Npip-$CONH_2$ and Nbsa-Nlys-Nbsa-Npip-Nlys-$CONH_2$ or a random sequence Npip-Nser-Nbsa-Nall-Nlys-Npip-$CONH_2$) were equilibrated in PBS for 60 min. The buffer was removed, and the beads were blocked with 2% BSA for 60 min to saturate any nonspecific binding sites. The beads were then washed with PBS (×3 times) and incubated with 500 nM (unless indicated otherwise) of a Texas Red-labeled protein (MBP−Mdm2 or GST) in 2% BSA (in 1× TBST buffer) in a 300 μL volume for 60 min. The

(46) Olivos, H. J.; Alluri, P. G.; Reddy, M. M.; Salony, D.; Kodadek, T. *Org. Lett.* **2002**, *4*, 4057−4059.
(47) Uno, T.; Beausoleil, E.; Glodsmith, R. A.; Levine, B. H.; Zuckermann, R. N. *Terahedron Lett.* **1999**, *40*, 1475−1478.
(48) Pons, J.-F.; Fauchére, J.-L.; Lamaty, F.; Molla, A.; Lazaro, R. *Eur. J. Org. Chem.* **1998**, *853*, 3−859.

beads were washed with TBST six times to remove any unbound protein and photographed under a fluorescence microscope.

Experiments that employed native (unlabeled) proteins were performed as follows. Beads (10 mg) displaying the peptoid were exposed to 1 μM protein in the presence of 1000-fold excess *E. coli* lysate, 0.2% Tween20, and 0.2 M NaCl in a total volume of 2 mL at RT for 2 h. The beads were washed thrice with TBST (20 mM Tris-buffered saline + 0.1% Tween20). SDS-PAGE loading dye (10 μL of 2×) was then added directly to these beads, and they were boiled for 10 min. The entire supernatant was loaded onto a 12% denaturing polyacrylamide gel and analyzed by Western blot using anti-Mdm2 antibodies for MBP−Mdm2 and anti-GST antibodies for GST.

Dilution Experiment. TentaGel beads (15 mg) displaying Nbsa-Nlys-Nbsa-Npip-Nlys-$CONH_2$ were equilibrated in PBS for 60 min. The buffer was removed, and the beads were incubated with *E. coli* lysate for 60 min to block any nonspecific binding sites. The beads were washed with PBS three times and split into three Eppendorf tubes. The beads were incubated with 1 μM, 500 nM, or 100 nM, respectively, of Texas Red-labeled GST in the presence of a 100-fold excess of *E. coli* lysate in a 300 μL volume for 60 min. The beads were washed with TBST six times to remove any unbound protein and visualized under a fluorescence microscope. The experiment was also done at 10 nM protein, but little or no fluorescence above background was observed (not shown).

Acknowledgment. This work was supported by a contract from the National Heart Lung and Blood Institute Proteomics Center Program (NO1-HV-28185) and grants from the National Cancer Institute (R21CA093287-01) and the Welch Foundation (I-1299). We thank Prof. Harold Garner for assistance using the hyperspectral imaging microscope and Prof. Lee Bulla for use of the fluorescence microscope with which all of the photographs were taken.

Supporting Information Available: Edman traces of 10 random beads picked from the 78 125- and 531 441-compound libraries; HPLC and MALDI-TOF of a representative mixed sequence 8-mer, NSer-Nlys-Nall-Nlys-Nbsa-Npip-Nbsa-$CONH_2$; MALDI-TOF spectra of mixed sequence 6-mer, Ntrp-Nmea-Npip-Nlys-Nffa-Nmba-$CONH_2$, and NH-Nbsa-Niba-Napp-Nffa-Nmea-Npip-$CONH_2$; solution binding studies of peptoid−GST complex by ITC; and table of retention times of hydantoins derived from Edman sequencing (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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