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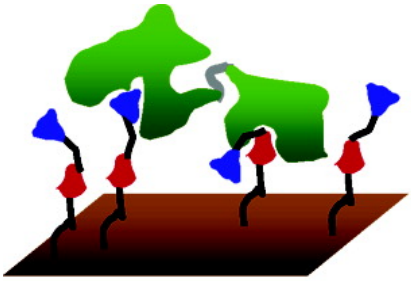

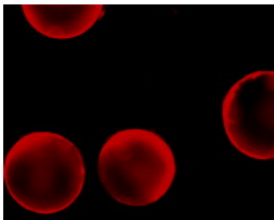

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Mixed-Element Capture Agents: A Simple Strategy for the Construction of Synthetic, High-Affinity Protein Capture Ligands

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The advent of DNA microarray technology has revolutionized many areas of biology by providing a global view of how gene expression patterns change in response to specific mutations or external stimuli.[1] However, since nucleic acid-based techniques are blind to many important regulatory events, such as posttranslational modifications of proteins, there is great interest in the development of similarly parallel analytical technologies at the proteomics level.[2] Currently, most protein profiling experiments rely on the multidimensional separation of proteins (or tryptic peptides) in a given sample followed by mass spectrometric analysis.[2] The development of protein-detecting arrays has lagged far behind, principally due to the dearth of high-quality protein-binding agents that would be immobilized on each element of a protein-detecting array.[3] Many workers in the area assumed that antibodies could be employed for this purpose. However, this approach has been frustrated by the poor quality of most commercially available antibodies as well as issues related to maintaining the activity of a folded macromolecule on the array surface.[4] Thus, there is a need for the development of general and robust techniques to isolate high-affinity protein capture agents, other than antibodies, by using methods that could potentially be rendered high-throughput. We report here a simple approach to the creation of high-affinity capture elements that involves co-immobilization of two synthetic, noncompeting protein ligands to a surface. Model experiments using peptides and an engineered multidomain protein show that such a mixed-element capture agent (MECA) binds its target protein with sufficiently high affinity and specificity to support the development of practically useful protein-detecting microarrays.

The approach that we have used is based on the well-known fact that if two modest-affinity, noncompeting ligands are linked together appropriately, the resulting bidentate ligand will have an affinity that can approach the product of the individual binding constants. While there are several examples of the creation of high-affinity protein ligands using this concept,[5] the effort required to design or discover an optimal linker has limited use in areas such as proteomics that will require high-throughput. We suspected that the issue of linker optimization would be irrelevant for an immobilized capture agent, since a densely functionalized surface would present a wide variety of geometric combinations of the two ligands[6] (Figure 1). Statistically therefore, some fraction of such a surface should represent high-affinity bidentate binding sites for the target protein.

To test this idea, a fusion of two monomeric proteins, the maltose binding protein (MBP) and the Mdm2 protein, was created as model for a monomeric protein containing two domains. The biophysical data shown in the Supporting Information confirm that, as expected, the fusion protein is also monomeric. MBP and Mdm2 were chosen for this model system because peptide ligands, VFFKDKKF and MPRFMDYWEDL, with μM dissociation constants were in hand for each polypeptide.[7]

Figure 1. Schematic representation of the MECA concept. Two noncompeting ligands (red and blue shapes) could be immobilized individually (left) or as a linear fusion (right), allowing for two appropriately positioned molecules to collaborate in binding the target protein tightly.

Table 1. Solution Dissociation Constants for the MBP−Mdm2·Peptide Complexes as Determined by Isothermal Calorimetry

	MBP-binding peptide	Mdm2-binding peptide	fusion peptide
K_d (μM)	30	32	22

We compared the binding affinities of the MBP- and Mdm2-binding peptides by themselves relative to that of a linear fusion of the two peptides separated by a single serine residue. Table 1 shows the results of solution binding experiments monitored by isothermal titration calorimetry (see Supporting Information for primary data). Under the conditions employed, the equilibrium dissociation constants (K_D's) of the complexes formed by the MBP-binding peptide and the Mdm2-binding peptide with the MBP−Mdm2 fusion protein were 30 and 32 μM, respectively, whereas the fusion peptide-MBP−Mdm2 complex was found to have a K_D of 22 μM. The least-squares fit of the raw data provide N values ranging from 1.1 to 1.4, suggesting 1:1 binding stoichiometries. This indicates that the randomly chosen serine linker is far from optimal for supporting bidentate binding in solution, resulting in little increase in the solution affinity of the fusion peptide for the protein.

To evaluate the apparent affinity of the immobilized peptides for MBP−Mdm2, the experiment shown in Figure 2A was conducted. The MBP-binding, Mdm2-binding, and fusion peptides were synthesized on TentaGel resin, which has a long flexible poly(ethylene glycol) linker arm, that ensures peptides synthesized close to the resin are equally accessible for binding. After synthesis the peptides are deprotected without releasing them from the beads. The peptides were then incubated with 1 μM Texas Red-labeled MBP−Mdm2 under stringent conditions (1 M NaCl + 1% Tween-20 and 100-fold excess of nonspecific proteins from an *Escherchia coli* lysate). After thorough washing with the same buffer lacking the labeled protein, the beads were then photographed. Two experiments were conducted. Each contained approximately equal numbers of the beads displaying the MECA and either of the two possible individual peptides. The photomicrograph shows two distinct bead populations, one of which is barely visible, and the other, bright (Figure 2A). Several of each type were picked, and

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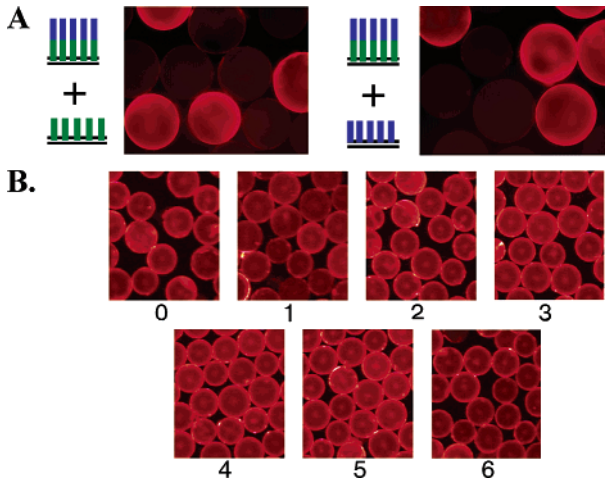

Figure 2. (A) TentaGel beads displaying the MECA (NH_2-VFFKDKKF-S-MPRFMDYWEDL) and beads displaying either (left) NH_2-VFFKDKKF, the MBP-binding peptide or (right) NH_2-MPRFMDYWEDL, the Mdm2-binding peptide were incubated with Texas Red-labeled MBP−Mdm2 fusion protein under stringent conditions. Edman sequencing confirmed the bright beads in each experiment displayed the MECA. (B) Binding of Texas red-labeled MBP−Mdm2 to MECAs in which the MBP- and Mdm2-binding peptides were separated by zero to six amino acids.

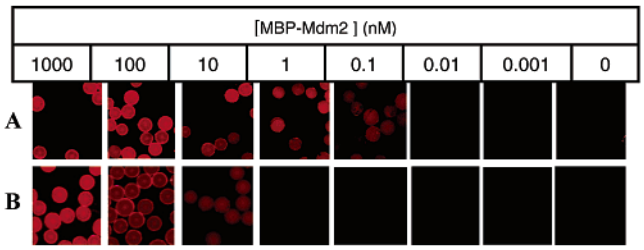

Figure 3. Retention of Texas Red-labeled MBP−Mdm2 by the MECA from solutions with the indicated concentration of target protein. (A) No competitor protein. (B) 100-fold excess of proteins derived from an *E. coli* lysate.

the identity of the peptide was determined by Edman sequencing. These data confirmed that the beads displaying the fusion peptide were indeed the high-affinity population, whereas the dark beads displayed the individual peptide. The striking difference between the relative protein affinities of the fusion and individual peptides in solution and when immobilized argues that high affinity capture of the protein on the bead requires the concerted action of two fusion peptides with one molecule of MBP−Mdm2; i.e., the surface acts as a linker (akin to Figure 1).

In further support of this model, there was no apparent difference in the affinity of fusion peptides for MBP−Mdm2 when the linker was varied from zero to six residues (Figure 2B).

To evaluate the functional affinity of the MECA, the fusion peptide-displaying beads were mixed with increasingly dilute solutions of Texas Red-labeled MBP−Mdm2. As can be seen in Figure 3A, labeled protein could be captured on the beads down to a concentration of 100 pM, showing that even low abundance proteins can be bound by this type of synthetic ligand. Even in the presence of 100-fold excess *E. coli* proteins, the synthetic ligands captured the fusion protein in a solution as dilute as 10 nM (Figure 3B). Other experiments using solutions of equivalent protein concentration, but larger amounts of the target, verified that the loss of signal at 100 pM and 10 nM, in the absence and presence of competitor proteins, respectively, was not due to the sensitivity of detection (data not shown). Indeed these results reflect the limit of the binding affinity.

The data presented above show that two noncompetitive, modest-affinity ligands can be combined to form a high-affinity MECA using a linker of arbitrary length and geometry. This extends our previous observations that peptides which bind homodimeric proteins with modest affinity in solution also act as high-affinity capture agents when immobilized due to the same type of avidity effect.[6] These findings, while limited to a small number of examples thus far, support the idea that it will be generally feasible to create high-affinity protein capture agents from two or more simple peptides, peptidomimetic compounds, or other small molecules. In analogy with the model system discussed here, it seems reasonable to propose that a MECA for any given protein could be created by combining "hits" from library screens against different domains of the same protein. Work to explore this strategy is underway.

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Supporting Information Available: Experimental procedures for cloning, protein purification, peptide synthesis, Texas Red labeling of proteins and on-bead fluorescence assays, biochemical/biophysical data for the fusion protein and its binding to peptides (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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