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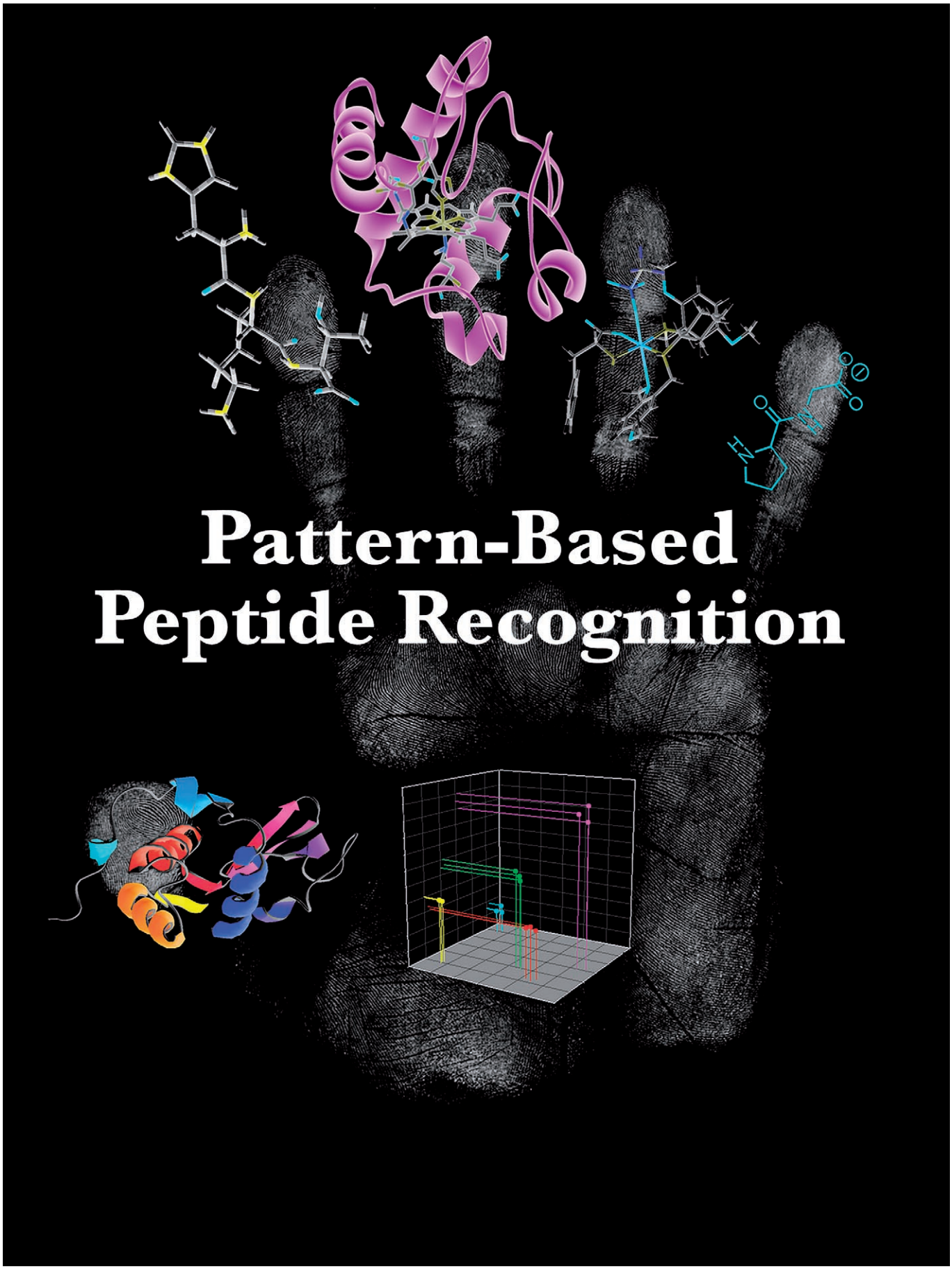


Pattern-Based Peptide Recognition

Byron E. Collins and Eric V. Anslyn*[a]

Abstract: Nature's use of sensor arrays in the mammalian olfactory and gustatory systems has encouraged supramolecular chemists to take a new approach to molecular recognition. Pattern-based recognition involves the use of sensor arrays to create fingerprints for analytes. The use of sensing arrays has paved the way for systems capable of identifying biological analytes that would have been difficult targets using the traditional "lock-and-key" approach to sensor design.

Keywords: molecular recognition · pattern recognition · proteins · sensors

Introduction

The human olfactory and gustatory systems use cross-reactive receptor proteins to create differential response patterns that can be recognized as a specific smell or taste.[1] Recent advances in combinatorial chemistry, as well as chemometric analysis, have provided supramolecular chemists with new opportunities to synthesize and implement systems that closely mimic these biological entities. These pattern-based recognition arrays rely not on the selectivity of any one receptor, but on the composite response of all members of the array.[2] The data obtained from these arrays is used to develop a "fingerprint" representative of the entire array response for an analyte. This type of system offers a significant advantage because one array can be used for the analysis of several different analytes or mixtures, while no one receptor in the array need be absolutely selective for any of those analytes. This approach is particularly intriguing for chemists studying biological systems, because it is often prohibitive to design receptors for large biomolecules such as proteins and enzymes.

With some arrays it is possible to analyze array patterns using the naked eye. However, as the size of the data set increases, this becomes impossible. In these instances, chemometric analytical methods such as principle component analysis (PCA) or linear discriminant analysis (LDA) are typically used to simplify the data set. In the context of this article, PCA is used as an unguided method of analysis to separate data points. It decomposes data into relevant eigenvalues and eigenvectors that may or may not display clustering and separation when plotted graphically. LDA uses discriminant functions to maximize separation between analyte classes. In this sense, LDA is a guided method of separation, because the analyte classes are specified during the calculations.[3] LDA is often used to test the classification ability of an array.

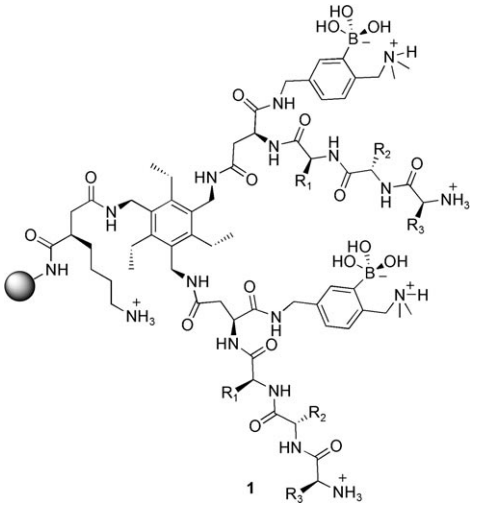
We believe that the combination of differential sensor arrays with pattern recognition protocols will become part of the systems biology revolution. System biology is a field that attempts to correlate genomic, proteomics, and metabolomics with biological function and dysfunction.[4] Typically, biological research takes a "one at a time" approach, called a reductionist approach, to studying biochemical interactions, in which each gene, protein, and/or metabolite is examined independently. In a systems biology approach, the combination and interrelationship of the analytes is examined. This is an application that calls for "fingerprinting" complex mixtures of nucleotides, proteins, and metabolites. In this Concept Article, we examine the earliest and most fledgling advances toward the fingerprinting of proteins, peptides, and amino acids.[5] It is clear that we are witnessing the beginning of a large and expansive field of chemistry that requires the creation of differential probe molecules—an inherently chemical problem.

[a] B. E. Collins, Prof. Dr. E. V. Anslyn
Department of Chemistry and Biochemistry
The University of Texas at Austin, Austin, TX 78712 (USA)
Fax: (+1) 512-471-8696
E-mail: anslyn@ccwf.cc.utexas.edu

Micromachined Arrays

Researchers at the University of Texas at Austin have developed an "electronic tongue" capable of detecting analytes in

aqueous solution.[6] The device consists of a Si/SiN micromachined wafer containing pyramidal wells with small (100 μm) openings in the bottoms. Solid-phase resin-bound receptors are positioned in the array, one bead per well, and the chip is fitted into a flow cell that allows solvent to flow over the top of the wafer and out the holes in the bottom of the wells. This is fitted with a stereoscope and a charge coupled device (CCD) for data acquisition. Using this technology, our group has synthesized a library of resin-bound receptors for the analysis of proteins in solution. The library scaffold consists of a hexa-substituted benzene core with two amino acid functionalized arms (**1**).[7] The "pinwheel" core



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allows for pre-organization of the binding site, and the incorporated boronic acids give the library preference for diols, catechols, and α-hydroxy carboxylic acids.[8,9] The two tripeptide arms are diversified by using split-and-pool chemistry with 19 amino acids (cysteine excluded) creating a library consisting of 19^3 resin bound members, 29 of which were randomly selected for incorporation into the array.

Our group commonly exploits the use of indicator displacement assays (IDAs) to give a colorimetric read-out for quantification of binding events.[10] In an indicator displacement assay, a receptor is first introduced to an indicator that binds in the receptor binding site. Next, introduction of the analyte displaces the indicator. In this case, however, it was found that greater sensitivity was achieved by using an indicator-uptake assay. An indicator-uptake experiment consists of passing an analyte through the array for a known amount of time at a set concentration followed by introduction of an indicator. The rate at which the indicator is adsorbed by the receptors is inversely proportional to the analyte's affinity for a particular receptor. The detection limit for this method (355 μM) was nearly 60 times lower than previous work using IDAs.[11] This was a necessity for the analysis of proteins at biological concentrations. When a 2D PCA scatter plot was created, differentiation was observed between proteins and glycoproteins. However, it was possible to identify three relevant principle axes, and when these were used all of the analytes were separated (Figure 1).

In PCA, those sensors found to contribute most to the formation of a principal component axis will have high loading values. Sensors that contribute very little to the formation of

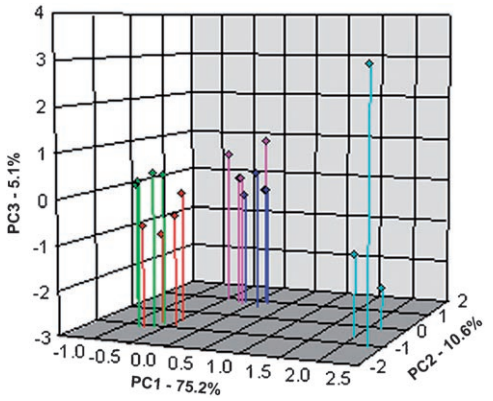


Figure 1. PCA score plot for each protein trial (red: lysozyme; green: elastin; dark blue: ovalbumin; pink: fetuin; light blue: BSA). The additional dimension further separates the proteins.

a PC axis will have low loading values. Because the receptors used were chosen at random, none were likely to be exceptionally selective for any of the proteins analyzed. Five of the beads in this array with the highest loading values on the first PC axis, and two with low loading values, were sequenced by Edman degradation, and there were no similarities among any of these receptors (Table 1). This exemplifies

Table 1. Factor loadings and sequencing results for the four receptors out of library **1** with the highest loading values for principal component 1 (PC1) and the two receptors with the lowest loading values.

Tripeptide sequence	Factor loading (PC1)	Bead number
Ala-Ser-Asp	0.984	12
Ser-Lys-Gly	0.963	9
Arg-Lys-Lys	0.951	15
Gly-Asp-Ser	0.932	2
Asp-Leu-Val	0.928	22
Lys-Arg-Met	0.774	23
Gly-Gln-Gln	0.722	6

the hypothesis that when using PCA analysis, while some rational design is necessary, variety is also important for success.

One of the major complications involved in analysis of biological samples is the presence of multiple potential analytes. The design and synthesis of a receptor selective for each component in a very complex mixture would be greatly prohibitive. To surmount this, we sought to expand our micromachined-array-based system to the analysis of mixtures.

The new receptor design involved a Cu^{II} center with a polyaza tricyclic ligand that created a "horseshoe"-like binding site known to selectively bind tripeptides.[12,13] The core

was proximally functionalized with peptide arms. The resin bound arm consisted of an Asp-Gly-Lys group bound to the core through a succinic acid linker. The other arm was derivatized by using split-and-pool chemistry to create library **2** consisting of 6859 members. The Cu^{II} center made the library selective for tripeptides with copper-coordinating residues at the N termini, while the variable peptide arms provide the differential response. Again, thirty beads were selected at random and analyzed in our micromachined array.

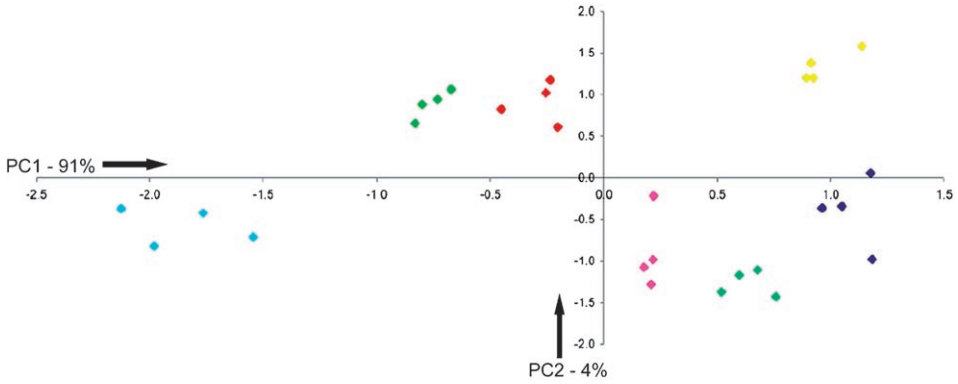
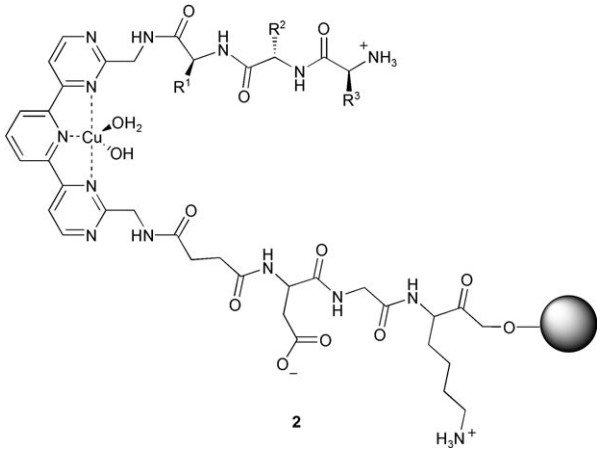


Figure 2. Two-dimensional PCA plot that describes 95% of variance from the original data set. Clustering of the analytes illustrates the ability of our differential array to discriminate various tripeptides and mixtures of tripeptides (dark blue His-Glu-Thr **3**; pink His-Lys-Thr **4**; yellow: Gly-His-Thr **5**; light blue His-Gly-Thr **6**; dark green: **3** and **4**; red **5** and **6**; light green: **4** and **6**).



The tripeptides chosen for analysis (**3**–**6**)) were intended to probe the selectivity of the library by varying the sequence only at the N-terminal or middle residue. The mixture analysis involved 50:50 mixtures of two peptides. The individual tripeptides and the mixtures are all clearly discernable after PCA analysis as shown in Figure 2. In all cases, the PCA plot scores for the mixtures lie between those of the individual tripeptides used indicating that both tripeptides contributed significantly to response of the array.

Again, some of the receptors with the highest loading values along with two receptors with low loading values were fully characterized, and no conclusive structural similarities were observed (Table 2). This indicates that the response from the entire array, not just a few key receptors, is important in the development of the fingerprint for each analyte.

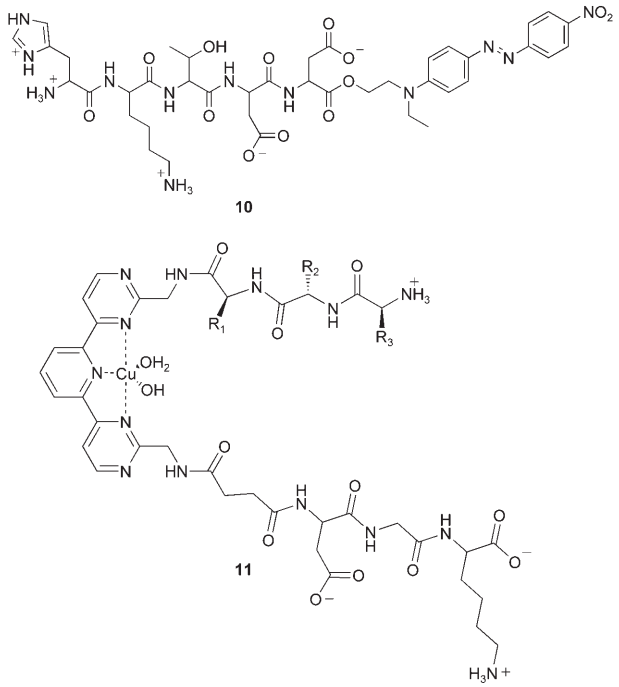
As encouraging as these results were, it was somewhat disturbing that over ninety percent of the total variance of the system was described by one principal component axis. This, along with the rather high loading values for all the receptors used in the array (Table 2) suggested that all of the

Table 2. Sequencing results and factor loading values for PC1 for library **2**.

Tripeptide sequence	Factor loading (PC1)	Bead number
Lys-Ala-Asp	0.989	26
Gln-Val-Gly	0.985	2
Leu-Lys-Ile	0.981	7
His-Ala-Ile	0.954	31
Phe-Pro-Arg	0.901	35
Arg-Gly-Pro	0.844	22

array members responded similarly to each analyte. In our most recent work, we addressed this issue, while also focusing on a valid biological concern. In this work, a solution phase version of library **2** was used for the detection of the tachykinins, α-neurokinin, and substance P, which are neurotransmitter peptides involved in pain transmission in the mammalian brain.[14] Two of the tripeptides analyzed in the previous study (**3** and **4**) along with tetrapeptide His-Lys-Thr-Asp (**7**) were used in this study as α-neurokinin analogues. Also, peptide sequences His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-C(O)-NH_2 (**8**) and Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-C(O)-NH_2 (**9**) were used as α-neurokinin and substance P analogues, respectively. Instead of selecting receptors at random as in previous work, the resin-bound library was screened with a colorimetric variant of a-neurokinin **10**. After screening, only 0.5% of the beads were strongly colored. Six of these beads were selected for array analysis and sequenced by Edman degradation.

The six receptors that displayed the strongest binding were then resynthesized in solution phase for use in a 96-well plate. The array consisted of the six receptors (**11a–f**; Table 3) each with three different metal/counter ion combinations ($Cu^{II}Cl_2$, $Cu^{II}OTf$, or $Cd^{II}OAc$) to make a total of 18 sensors. PCA analysis of the data set indicated four significant PC axes. The three most significant were used for graphical analysis (Figure 3). This demonstrates that in some cases better separation can be achieved through optimization and prescreening of a receptor library.



gands **12–14**, in conjunction with three colorimetric indicators **15–17**, were used to enantioselectively detect several amino acids. Use of a 96-well micro titer plate alleviates the

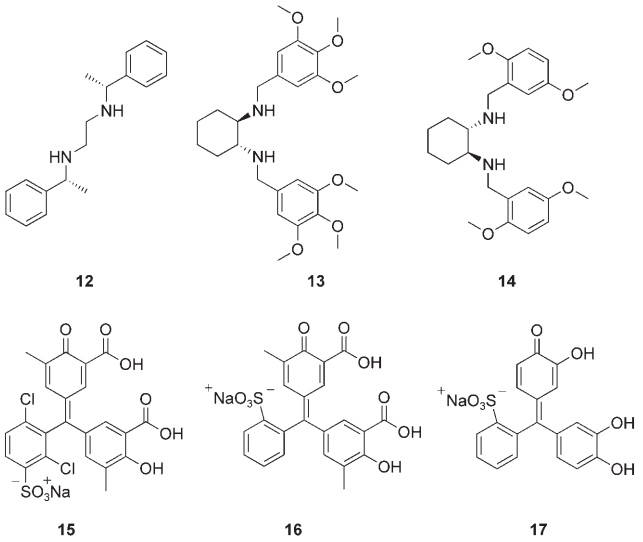


Table 3. Tripeptide sequences for the six receptors chosen from library **11** after pre-screening with chromophore **10**.

Receptor	R^1	R^2	R^3
a	Ala	Asp	Ile
b	Thr	Phe	Thr
c	Thr	Met	Phe
d	Asp	Asp	Ser
e	Pro	Lys	Met
f	Arg	Met	Met

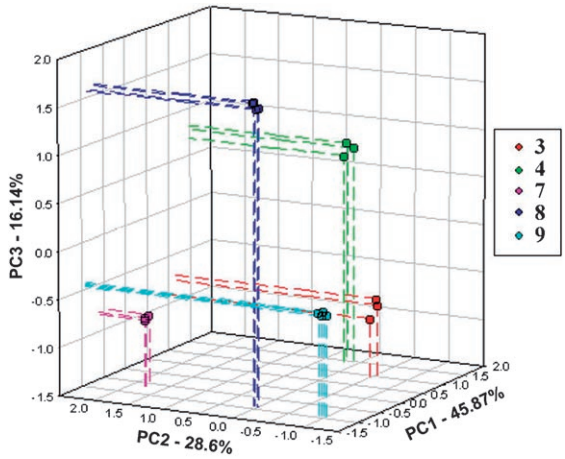


Figure 3. 3D PCA plot showing good clustering and separation of analytes after array analysis with optimized library **11**.

Enantioselective Amino Acid Recognition

We have also focused attention on the use of arrays for enantioselective detection.[15] Here, three bidentate Cu^{II} ligands **12–14**, in conjunction with three colorimetric indicators **15–17**, were used to enantioselectively detect several amino acids. Use of a 96-well micro titer plate alleviates the synthetic requirement of attaching the receptors to a solid support. Ligand **14** has already been shown to quantify the enantiomeric excess of amino acids.[16] In initial studies, receptors **12** and **13** display preference for L-amino acids, while **14** shows preference for D-amino acids. By varying the concentration of ligand, indicator, and $Cu(OTf)_2$, a total of 21 different conditions were used to analyze each amino acid separately.

Interestingly, PCA analysis indicated that, in this instance, binding affinity and chirality were identified as the two most significant components of variance. This can be seen in Figure 4A in which binding affinity increases along the PC1 axis, and all of the L-amino acids have positive PC2 values, while the D-amino acids have negative PC2 values. Omission of the data from receptors of either enantiomeric preference resulted in poor differentiation of the entire data set indicating that, when the data set is split, PCA cannot distinguish these two clear variants (Figure 4B and C). This is analogous to the human gustatory system, which also employs cross-reactive receptors; the D-amino acids are typically classified as sweet and the L-amino acids as bitter.

Multicomponent Sensing Ensembles

It has been demonstrated that multicomponent sensing ensembles can be used to differentiate structurally similar analytes. When a system such as this is shown to be in dynamic equilibrium under thermodynamic control it is known as dynamic combinatorial library (DCL).[17] For example, it has been shown that UV/Vis data from a system consisting of two hexasubstituted benzene receptors and two colorimetric indicators can be analyzed using an artificial neural network

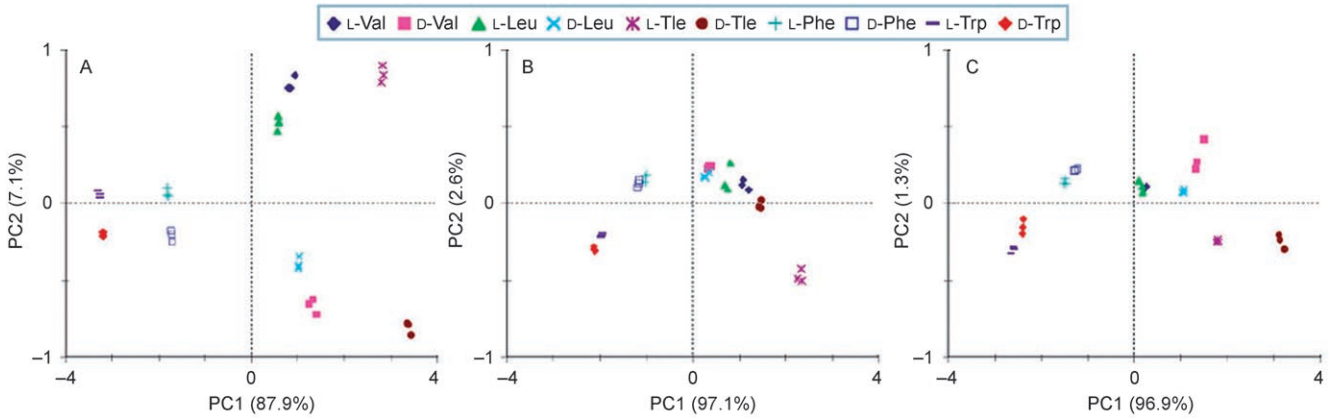


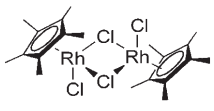
Figure 4. 2D PCA plot for D- and L-amino acids prepared A) from data for all 21 enantioselective IDAs, B) from data for eight IDAs selective for D-amino acids, and C) from data for 13 IDAs selective for L configurations.

(ANN) to distinguish differing concentrations of structurally similar analytes malate and tartrate.[18]

Severin and co-workers have recently demonstrated that DCLs can be implemented by using no synthesis whatsoever.[19] By mixing three commercially available dyes (Arsenazo I, Methylcalcein Blue, and Glycine Cresol Red), which are known to coordinate metal ions, with two metal salts ($NiCl_2$ and $CuCl_2$), a DCL consisting of all possible combinations of metal-dye complexes results. The resultant pool of metal-dye complexes has its own characteristic UV/Vis absorption spectrum.

It was shown that all of the components of this system were indeed in a dynamic equilibrium, and that addition of a dipeptide caused a reproducible shift in this equilibrium as evidenced by the change in the UV/Vis absorption spectrum (Figure 5A). After demonstrating that structurally dissimilar dipeptides afford clearly distinct UV/Vis spectral changes, the scope of the DCL was tested by analyzing several similar dipeptides. Because the absorption changes were all similar, eight relevant wavelengths were chosen as the data set for linear discriminant analysis (LDA). As shown in Figure 5B, all of the dipeptides including stereoisomers L-Phe-Ala and D-Phe-Ala were clearly discernable. It has also been shown that this DCL can be optimized to differentiate sequence isomers by varying the concentration of the Ni and Cu relative to each other.[20] The optimized system was dependent on the analyte in question.

In another study from the same group, IDAs were performed using an organometallic Cp*Rh complex (**18**; Cp = cycopentadienyl) as a receptor to differentiate all twenty natural amino acids.[21] Again, this system was implemented without any synthetic requirements. IDAs were performed for each amino acid using three different indicators at various pH values.



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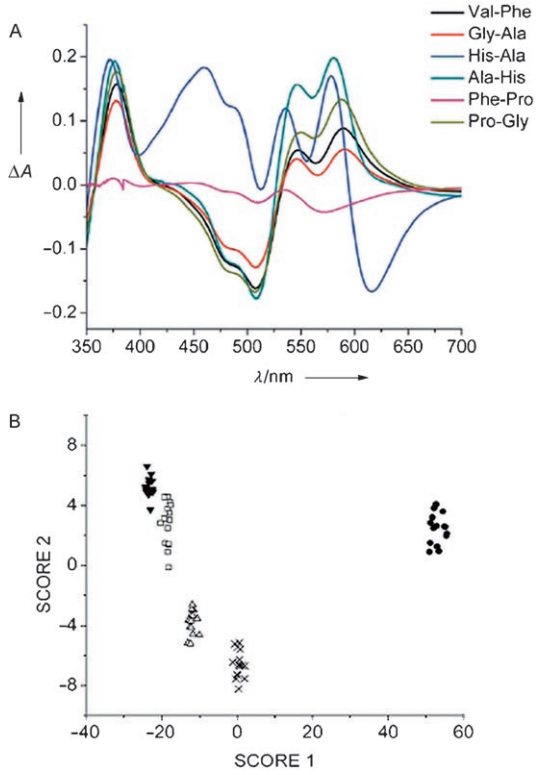


Figure 5. A) Changes in the UV/Vis spectrum upon addition of different dipeptides to an aqueous DCL, B) 2D LDA score plot for the analytes Gly-Ala (●), Val-Phe (□), Ala-Phe (▼), Phe-Ala (△), and D-Phe-Ala (×).

After analysis with **18** and gallocyanine, the amino acids were split into a high affinity (His, Cys, Met, Asp, and Asn) and a low affinity group. These two groups were further analyzed separately. After complete analysis, both groups were analyzed with LDA to determine the predictive power of the system. The high affinity group gave accurate assignments 100% of the time, while the low affinity group was

99.4% accurate. The most common misclassifications involved isoleucine and valine. The low affinity group was further analyzed with PCA in order to visualize the clustering patterns of the amino acids (Figure 6). The only area of

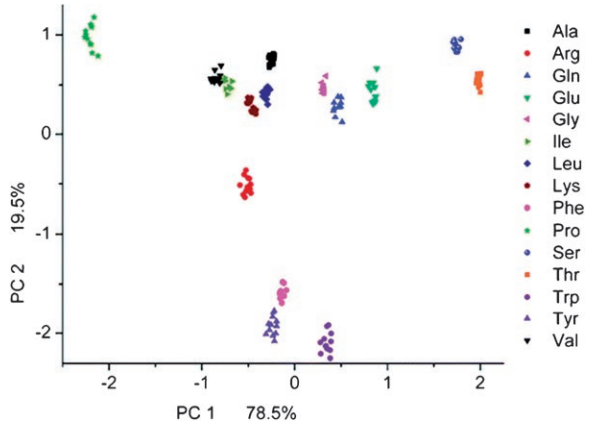


Figure 6. PCA score plot showing discrimination of 15 amino acids.

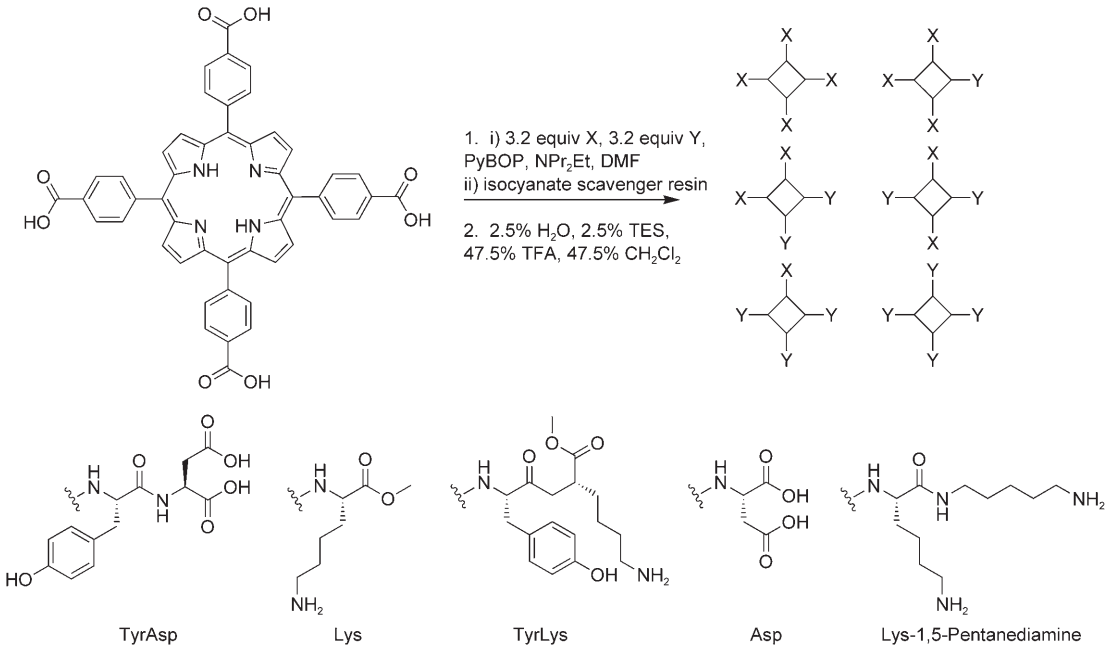
overlap occurs with Ile and Val, and all of the amino acids seem to be at least loosely group based on their side chains.

Porphyrin Arrays

Hamilton et al. have developed a library of functionalized porphyrins capable of discriminating proteins without the necessity of labeling target proteins.[22] To address the necessity for a more robust process applicable to a wide range of biologically important proteins, Hamilton synthesized a library of tetraphenyl porphyrins (TPPs; **19**), the fluorescence intensities of which change upon interaction with a protein (Scheme 1).

The large hydrophobic cores of the TPPs have been shown to provide a good binding surface, while the amino acid based arms contribute diversity and selectivity by altering the overall charge of the sensors.[23] The TPP library was synthesized by using a mixed condensation strategy that involved treating a TPP core with two peptidic arms at once, thus affording six library members from each reaction. From the resulting 35-member library, eight fluorophores were chosen for incorporation into the array based on their charges. Upon incubation of the array with one of four proteins (cytochrome *c*, cytochrome *c*551, ferredoxin, and myoglobin) the resulting fluorescence changes were combined to form a fingerprint for each protein. In the initial proof of concept experiments, the target proteins were characteristically different enough that naked eye classification was possible (Figure 7).

The scope of the TPP library was expanded through incorporation of PCA for data analysis.[24] By developing a 3D Euclidian plot, it was possible to distinguish non-metal-containing proteins as well as protein mixtures (Figure 8). Significantly, a mixture of cytochrom *c* (a strong interacting protein) and lysozyme (a weak interacting protein) gave a signal distinct from either of the two individual components, indicating that a strongly quenching protein does not mask a weakly quenching one. It was also observed that, through expansion of the TPP library from 8 to 16 members, the overall average distance between data points increased from 1.27 to 1.62.



Scheme 1. Mixed condensation synthesis of library **19**.

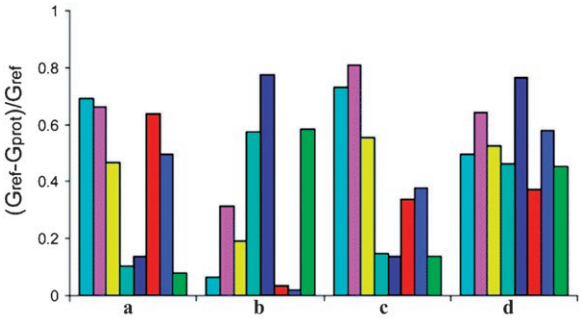
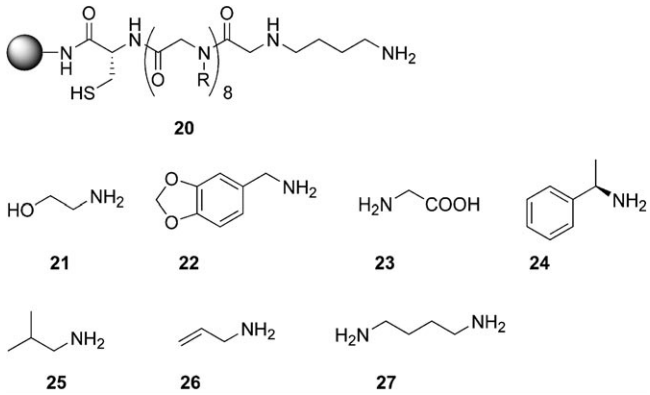


Figure 7. Fingerprints of a) cytrochrome *c*, b) ferredoxin, c) cytrochrome *c*551, d) and myoglobin based on the eight porphyrin array.



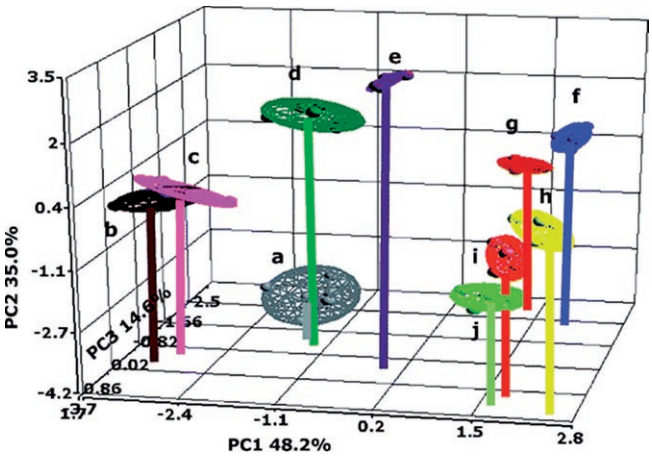


Figure 8. 3D PCA mapping of the 10 samples identified by an eight porphyrin array: protein concentrations 7.5 μM (1.5e) or 15 μM (3e). Abbreviations: cytochrome *c* (Cytc), ferredoxin (FD), lysozyme (Lys), and α-lactalbumin (Lact). Samples: a) Lact 1.5e; b) FD 1.5e; c) FDLact 1.5e; d) CytcFD 1.5e; e) CytcFD 3e; f) Cytc 3e; g) Cytc 1.5e; h) CytcLys 3e; i) CytcLys 1.5e; j) Lys 1.5e.

Small-Molecule Microarrays (SMM Arrays)

Most of the studies discussed thus far have involved either 96-well microtiter plates or micromachined arrays. However, small-molecule microarrays (SMMs) offer the significant advantage of incorporating a much greater number of sensors than either of the previously mentioned platforms, while requiring less space. Kodadek and co-workers have recently demonstrated that SMMs can be used as a platform for the fingerprinting of proteins in biological solutions.[25]

In this example, a combinatorial library of 7680 octameric peptoids **20** was first synthesized on solid resin with amines **21**–**27**. The library members were then separated into 96-well plates and cleaved from the resin. An array spotter was then used to spot each peptoid onto a maleimide functionalized glass slide. The array was then analyzed in two different ways.

In the first analysis, one of three fluorescently labeled proteins, Ubiquitin (Ub), maltose-binding protein (MBP), or anti-glutathione S-transferase (GST) was incubated with the array in the presence of a 100-fold excess of crude *E. coli* extract. After washing, the array was analyzed with a microarray scanner. The fluorescence intensity changes at each spot were converted into a bar graph that was unique for each of the three proteins. The entire bar graphs were too complicated to discern, but focusing on smaller areas of the graphs provided visual distinction (Figure 9). As an alternative method, nonlabeled GST was hybridized to the array, again in the presence of a 100-fold excess of crude *E. coli* extract, and then probed with fluorescently labeled GST antibodies.

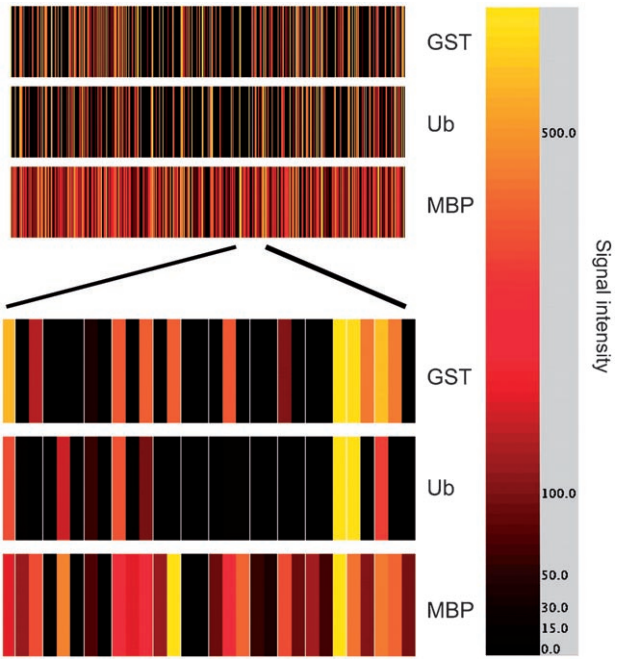


Figure 9. Protein fingerprints made by color-coding the relative response of each sensor in an array. The expanded portions more clearly demonstrate the differences in the fingerprints.

Each experiment was duplicated, and scatter plots were used to examine the reproducibility of the fingerprints. These plots showed good correlation between arrays of the same protein, and much lower correlation for plots compar-

ing different proteins. A scatter plot comparing the fingerprints of native GST and fluorescently labeled GST gave a large number of off-diagonal points as well, indicating that the array can distinguish between tagged and native proteins.

Conclusion

As of the spring of 2007, only a handful of studies using arrays of synthetic receptors for the fingerprinting of proteins, peptides, and amino acids have been reported, all of which were summarized in this Concept Article. Yet, even with the currently limited examples, the power of differential arrays of synthetic receptors is evident for protein recognition. Our group, as well as that of Hamilton, have shown that proteins can be differentiated. In our study, post-translation glycosylation events were distinct from normal proteins. Further, our group, as well as that of Severin, have shown that peptides can be distinctly fingerprinted. In fact, we have found that even complex decapeptides can be differentiated with cross-reactive receptors. Finally, in a more biologically sophisticated approach, Kodadek has used arrays of peptoids followed by antibody visualization, to create bar codes for the identity of proteins. Clearly the ground is set to expand upon the use of synthetic receptors for the type-casting, quantifying, and monitoring of complex mixtures of proteins. This class of analytes is just one of those being studied for a system biology approach to medicine, but the concepts presented herein for proteins are just as valid for nucleotides and metabolites. Therefore, the future of chemical research using synthetic receptors is sure to contain differential receptors for nucleotides, proteins, and metabolites.

Acknowledgements

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