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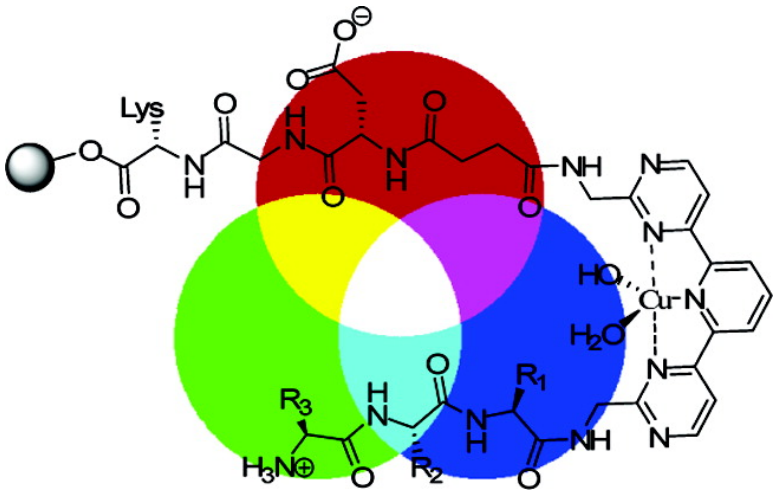

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A Differential Array of Metalated Synthetic Receptors for the Analysis of Tripeptide Mixtures

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Abstract: A novel library of resin-bound receptors within a cross-reactive differential array for the identification and discrimination of tripeptides and tripeptide mixtures is reported. Pattern recognition using principal component analysis showed complete discrimination of four similar tripeptides and three tripeptide mixtures. The library is comprised of a Cu(II)-centered core with two proximally appended tripeptide arms emanating outward. One tripeptide arm was prepared using combinatorial chemistry to generate the differential nature of the library. Thirty resin-bound receptors were randomly selected from the library and placed within a silicon microchip array that included integrated microfluidics elements, and an indicator-uptake assay was used for colorimetric signaling. The indicator Orange G yielded an accurate measure of the degree of association between receptors and analytes as determined by kinetic analysis of the indicator-uptake assays. Within this paper we detail the method used for differential sensing using a novel receptor library. This work further demonstrates the power and utility of a differential array of synthetic receptors for identification and discrimination of complex bioanalytes.

Introduction

Selective synthetic receptors have been used for binding many challenging targets including bioanalytes such as heparin, phosphate, and small peptides.[1−15] Receptors for more structurally or conformationally complex analytes have been obtained by screening libraries of receptors.[16,17] The need to screen receptor libraries results from the inherent difficulty of designing specific receptors for large analytes, such as proteins and peptides. This is attributed to the difficulty in preorganizing multiple binding moieties on a receptor to complement a target protein or peptide. Another factor that complicates protein and peptide recognition is conformational dynamics, which is particularly true for a short flexible peptide.

In addition to size and dynamic complications in the analysis of biomolecules, most real-world applications involve the analysis of multicomponent fluids containing multiple analytes of potential interest. It is, thus, extremely difficult and synthetically prohibitive to design selective receptors for each of the vast number of bioanalytes making up a mixture. However, extraordinary levels of discrimination exist for complex mixtures of analytes within the mammalian sensing system. For example, the mammalian senses of smell and taste employ multiple recognition elements to provide unique aroma or flavor responses.[18] The sense of taste is mediated by several variable receptors located within papillae on the tongue. Upon ingestion of an analyte, there is a cross-reactive (differential) response from the receptors resulting in a complex composite signal that mammals recognize as a unique taste perception. Sensing assays that mimic this cross-reactive system have the potential to elicit unique composite signals for complex mixtures of analytes without requiring specific receptors designed for each analyte.

Recently, arrays of sensors have been employed to create diagnostic signals (patterns) with high accuracy.[19−22] Furthermore, a number of groups have designed synthetic receptors within cross-reactive arrays to obtain differential responses to analytes.[23−27] Others have even evaluated the analysis of urine

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doped with steroids, ions and mixtures of ions, and patterns for various sodas.[28−31]

In our design strategy, the core of the receptor is derivatized with one or more functional groups to establish and enhance affinity for a particular analyte class. Functional groups could include basic or acidic moieties for ion-pairing, heteroatoms for metal coordination, and many others. Proximally appended from the core are differential binding moieties, such as peptide chains, that can be readily varied between the receptors. In this type of system, receptors are designed to target particular classes of analytes but not necessarily to be selective for single analytes. The array's selectivity is manifested from the collective response of the differential receptor ensemble to an analyte. Introduction of an analyte to the array of differential receptors produces a unique "pattern" based upon the composite cross-reactive response. Chemometric methods can make pattern identification more facile by reduction of the response data.[20] In combination with chemometric tools, a single differential receptor array can potentially discriminate multiple analytes without requiring the design of specific receptors.

Several potential applications exist for solution-based analysis using differential synthetic receptor arrays. These include environmental testing for pollutants, on-line process monitoring, and medical diagnostics for identification of disease states and risk markers. Additionally, differential arrays may be useful in the fields of proteomics and metabolomics, which are essentially mixture analyses. Prior to tackling these challenging applications, the progression must be made from fundamental analyses of simple analytes, to complex bioanalytes, and ultimately to complicated mixtures in natural media. However, only a limited number of reports involving arrays for solution-based analysis exist, and there are far fewer involving bioanalytes. To date, lipopolysaccharides have been targeted by polymeric acetylene receptors,[32] amino acids by ruthenium-coordinated receptors,[23] proteins by metalated tetraphenylporphyrin receptors,[26] proteins and glycoproteins by *o*-aminomethyl boronic acid receptors,[33] and nucleotide phosphates by bisguanidinium receptors.[34] Vast biochemical space remains for analysis: peptides, hormones, specific enzyme classes, and complex mixtures akin to biological solutions.

The challenge of developing an array for diagnostic sample analysis lies within identifying an analyte in a complex clinical or field sample. A first step is to determine how a differential receptor array responds to both single analytes and mixtures. Ideally, unique patterns would be obtained for both the mixtures and single analytes. It is expected that patterns for mixtures should be composites of the individual component responses. Herein, we report the use of a combination of an integrated microfluidics system with a novel synthetic combinatorial library of receptors that accomplishes the analysis and recognition of tripeptides and tripeptide mixtures using a differential receptor array.

Results and Discussion

Prior to our current work, a combined research effort was undertaken to develop an "electronic taste chip". This effort produced a chip-based microsphere array system for the digital analysis of analytes in solution.[35,36] These arrays have been shown to mimic several cross-reactive features exhibited by the human sense of taste.[37,38] The instrumentation for the array assays included a charge-coupled device (CCD) attached to a stereoscope for collection of colorimetric signal modulations, a fluid delivery system, and a flow-cell for housing the silicon wafer that holds the microspheric resin-bound receptors. The instrumentation permits automated collection of optical red, green, and blue transmissions recorded from each receptor within the array. Each receptor effectively operates as a microreactor and microanalyzer. Repeated use of the receptor array for detecting multiple analytes and generating reproducible results is readily executable.

We have recently completed the design and array application of two libraries of synthetic receptors for the analysis and discrimination of nucleotide phosphates and proteins and glycoproteins.[33,34] The separation and identification of analyte and mixture patterns was determined with principal component analysis (PCA), a chemometric method.[20,39] As stated earlier, our approach to differential sensing is to create libraries of synthetic receptors that are biased toward particular analyte classes. In this study, tripeptides and tripeptide mixtures were targeted because they are inclusive of the dynamic nature of many complex bioanalytes. We developed a new combinatorial library of receptors on tentagel resin using standard split-and-pool combinatorial chemistry.[40] Thousands of unique receptors are readily generated with this protocol. In the resulting library each bead bears a unique receptor biased in part by the metallocore toward ligating analytes, and differentially enhanced by the receptors' appended tripeptide arms.

To probe binding events occurring at the receptor sites, a signal-modulating event is incorporated into the array. Previously, an indicator-displacement array was employed.[34,41] However, we recently determined that a staining, or indicator-uptake, signaling assay increases the sensitivity of the analysis in bead-based arrays.[33] Further refinements of the indicator-uptake protocol in the current study resulted in even greater sensitivity.

Receptor Design Criteria. Recently, a single selective receptor (**1**) was synthesized for the tripeptide His-Lys-Lys (HKK), and an association constant ($K_a = 1.0 \times 10^6\ M^{-1}$) was

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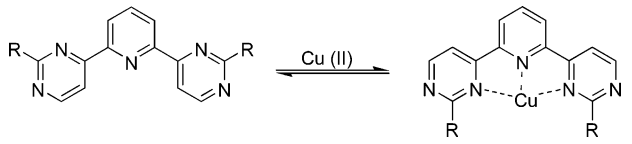
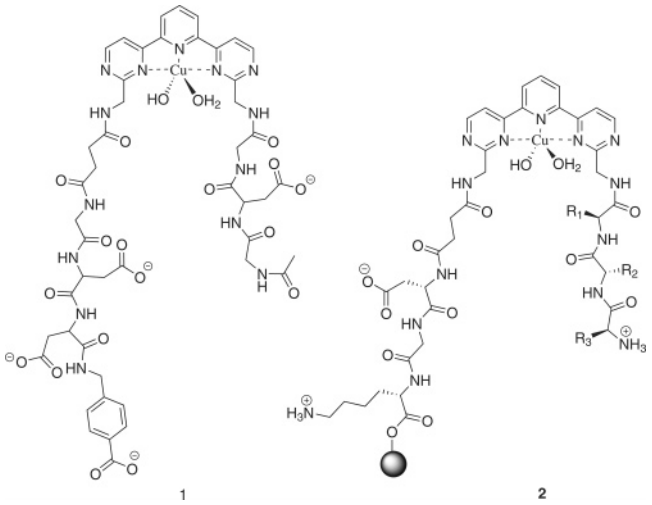
Scheme 1 [a]



[a] The ligand adopts a "horseshoe"-like conformation upon coordination to Cu(II). When the ligand is coordinated to the metal, the peptidyl R groups are held in closer proximity to the metal center.

obtained in a 1:1 water:methanol solution buffered with HEPES at pH 7.4.[10] We reported that tripeptides with Cu(II)-ligating amino acids, coordinated through the N-terminus, bound selectively to our receptor versus tripeptides without ligating amino acids. It was also demonstrated that the overall strength of association between HKK and the peptide binding arms of **1** was enhanced by a factor of 3, and the selectivity of **1** was modified, by manipulating the peptide sequences. Incorporation of several acidic residues into the peptidic components of **1** improved the selectivity of the receptor complex for HKK by increasing ion-pairing interactions.



To differentiate various tripeptides and tripeptide mixtures, library **2** was designed and synthesized with a core tridentate ligand coordinated to a Cu(II) center. Two appended peptide arms were added for differentiation and greater affinity. The ligand core of the receptor is a polyaza tricyclic heterocycle and is a known Cu(II) ligand.[42] The association constant between Cu(II) and the core was previously determined as 7.8×10^4 M^{-1}.[42] Crystallography indicated that the metal−ligand complex adopts a "horseshoe"-like molecular cleft by preorganizing the two arms of the receptor in closer proximity of one another (Scheme 1).[42]

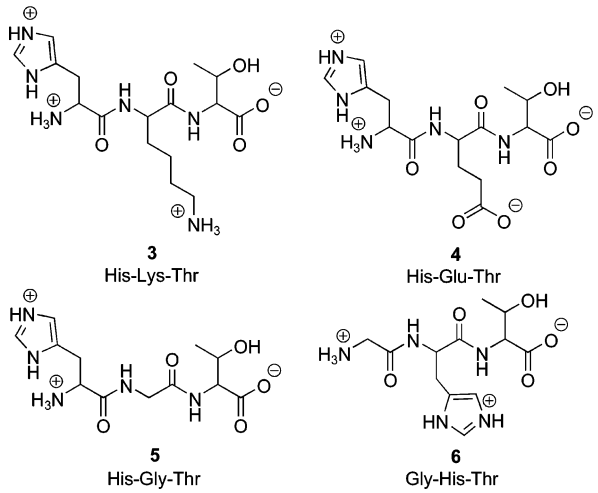
As mentioned earlier, combinatorial chemistry is an excellent source for generating diversity (cross-reactivity) within synthetic receptor arrays.[33,34] By exploiting the wide variety of functional groups in amino acids, thousands of receptors with differential binding activities were generated expeditiously with combinatorial chemistry in the synthesis of library **2**.

Synthesis. The first peptide arm (that which is affixed directly to the resin) of **2** was synthesized as the tripeptide Asp-Gly-Lys. Succinic acid was used as a spacer to attach the first peptide arm to the core. Because a diamino core is incorporated, it is necessary to "turn" the direction of the synthesis using the diacid spacer. In other words, the core is coupled to the acid of the succinic acid spacer on the peptide affixed to the resin, and to the carboxyl group of the first amino acid of the second peptide arm.

The details for the synthesis of the core of library **2** and the synthesis of a peptide arm incorporating the succinic acid linker have been described previously.[10] As with the previous work, solid-phase synthesis with a tentagel amino resin and 1-hydroxybenzotriazole, TBTU, and *N*-methylmorpholine were used for the peptide forming reactions. After the core was attached to the resin-Lys-Gly-Asp-succinic acid arm, library **2** was developed with split-and-pool Fmoc-protecting group synthetic protocols.[40,43]

The variable tripeptide arm of **2** was synthesized by incorporating one of 19 natural amino acids (cysteine was not used to eliminate the potential for disulfide linkages) at each of three sites on the peptide arm. This resulted in a library of 19^3 (6859) unique members. Thirty members of **2** were randomly selected and placed into the array for pattern recognition of tripeptides and mixtures.

Analyte Choice. Tripeptides **3**−**6** were prepared. Three tripeptides terminated at the N-termini with histidine (**3**−**5**), a known Cu(II) ligand, and the fourth tripeptide terminated with glycine at the N-termini (**6**). These tripeptides were prepared to test the ability of our library to recognize four tripeptides (13 μM each) that differ only in the middle or N-terminal residue. The tripeptides included an acidic (**4**), two basic (**3**, **6**), and one nonpolar (**5**) residue at the middle position. These differences were reflected in the differential responses obtained from the receptor array. In all cases, the third residue was threonine. Threonine increased the overall polarity of the tripeptides, subsequently increasing the solubility. Tripeptides **3** and **4**, **4** and **5**, and **5** and **6** (13 μM in water buffered with HEPES at pH = 7.4) were mixed. It was expected that mixing the analytes would result in pattern responses that are composites of the two individual tripeptide pattern responses.



Analysis. A silicon wafer microchip etched with 35 pyramidal pits was used to hold the resin-bound receptors. Thirty members of library **2** were randomly selected and placed in the array.

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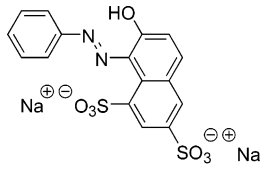
Scheme 2. Indicator-Uptake Signaling Protocol

An advantage of this array design is that each receptor embedded within the array acts as both a reaction vessel and an analysis chamber. The remaining five sites of the array were occupied by *N*-acetylated resin blanks.[36] The receptor-loaded array was placed into the integrated microfluidics flow cell and positioned on the imaging station that permits bottom illumination of the resin-bound receptors. The microfluidics structure delivers solutions to the top of the array. The solutions then pass through the resin beads and exit the array through openings in the bottom of each pyramidal pit. Mounted to the imaging platform is a 12-bit charge-coupled device (CCD) that is interfaced to a computer for data analysis. The CCD quantifies red, green, and blue pixel intensities by capturing images at determined intervals. Introduction of solutions to the array is performed by an external fluid delivery system that permits controlled delivery rates through the microfluidics flow cell. After an analysis, the array can be "reset" by flushing the system with acid and base washes. As configured, a disadvantage of this system is that the CCD captures only red, green, and blue pixel information, so the system can only monitor binding events if a colorimetric signaling event is incorporated. This was executed by the addition of an indicator-uptake signaling protocol in our array assay.

Colorimetric Indicator Analysis. Previous differential array studies within our group have incorporated either an indicator-displacement[34] or an indicator-uptake[33] signaling assay. When using pattern recognition to differentiate nucleotide phosphates with an indicator-displacement assay, the analyte concentration required was 20 mM.[34] This is a relatively high concentration for the analysis of bioanalytes. To increase the sensitivity, a new signaling protocol akin to staining was created, termed "indicator-uptake".[33] In this method an analyte is first delivered to the array at a slow rate (0.5 mL/min) to maximize adsorption of analytes to the receptors. At the low concentrations of analytes employed, it is believed that several binding sites on each bead in the array remain empty following the initial analyte delivery. Following analyte delivery and a brief buffer wash to remove unbound analyte, the indicator is delivered at a fast rate through the flow cell (1.0 mL/min). The indicator occupies binding sites that remain open. If an analyte interacts strongly with a receptor and binds to more sites, less indicator-uptake will occur. On the other hand, if the analyte:receptor interaction is weak, a larger indicator-uptake occurs. Therefore, the colorimetric event correlates indicator-uptake to analyte binding at each receptor within the array. At the end of an analysis, HCl (300 mM) and NaOH (150 mM) are delivered to the array to remove all bound analyte and indicator (Scheme 2).

The indicator used for this study was the acidic dye Orange G (**7**, 60 μM). It is believed that this indicator interacts with the peptide arms via Cu(II) ligation. The analyte delivery flow rate was changed to 0.5 mL/min from 0.25 mL/min as used in a prior study.[33] This change likely modified the extent of binding that occurred between the analytes and receptors. The indicator concentrations were also increased 20-fold from previous studies, from 3.0 μM to 60 μM.[33] These two minor changes resulted in a significant decrease in the analyte concentration required, from 355 μM in our previous study with proteins and glycoproteins to only 13 μM for tripeptides and mixtures.



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During indicator-uptake analysis, 215 12-bit CCD images were obtained for each resin bead in the array. These images were analyzed by drawing an area of interest (AOI) covering a maximum portion of each bead. From within the AOI, blue pixel intensity ($\lambda \approx 420-500$ nm) values were obtained from each CCD image. For analysis, only the blue channel intensities were used because they had the greatest signal-to-noise ratio. The blue channel intensity values were converted to an "effective blue absorbance" value, A_B, using Beer's law ($A_B = -\mathrm{Log}(I_B/I_N)$), where I_N was the average blue pixel intensity of a blank *N*-acetylated bead. This correction factor removes residual background noise due to lamp fluctuations. For each trial, a slope of indicator-uptake was obtained for each receptor by snapping one image every 2 s. These slopes describe the kinetics of indicator-uptake for each receptor.

Figure 1 shows four indicator-uptake slopes taken from four different receptors from the array in response to His-Glu-Thr (**4**). As observed in Figure 1, each receptor responds uniquely to **4**. Multiple trials demonstrated that these kinetic slopes are reproducible. It is also required that each receptor responds differently from one tripeptide or mixture to the next. The kinetic slopes defined for each of the 30 receptors in the array are combined to provide an overall response. This cumulative response is defined as the pattern for an individual tripeptide or mixture. This is similar to multiple receptors on the tongue giving differential responses to an analyte that leads to a single unique flavor response. For a complete analysis of multiple analytes and mixtures, it is required that each receptor also responds differently from one another in response to a single analyte. If each receptor responded identically to a single tripeptide, it would not illustrate a differential response, but rather that the primary mode of association between receptors and analytes was identical across the array regardless of the composition of the peptidic arms. If this had occurred it could have been attributed to the metal-ligating core, because that is the common feature between receptors in library **2**. However, as previously noted, all the receptors in the array responded uniquely.

In the current analysis the resin was initially saturated with Cu(II). The tripeptide or tripeptide mixture was then added through the array, followed by the indicator Orange G. Twelve-bit images were captured during the indicator-uptake, and data

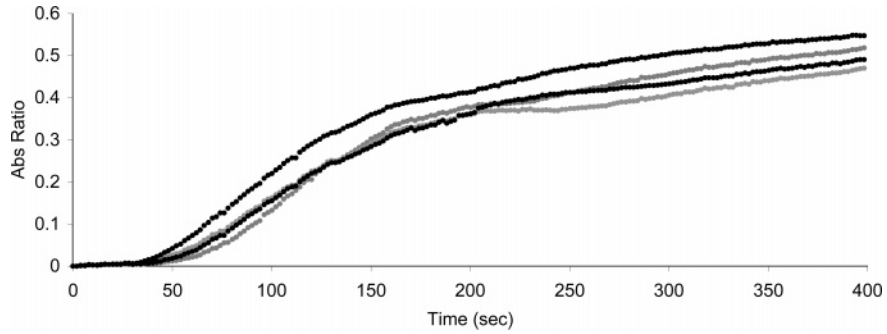

Figure 1. Indicator-uptake slopes using Orange G from four receptors in the differential array in response to a single analyte. All receptors uniquely respond to a common analyte; thus, this represents a cross-reactive or differential array.

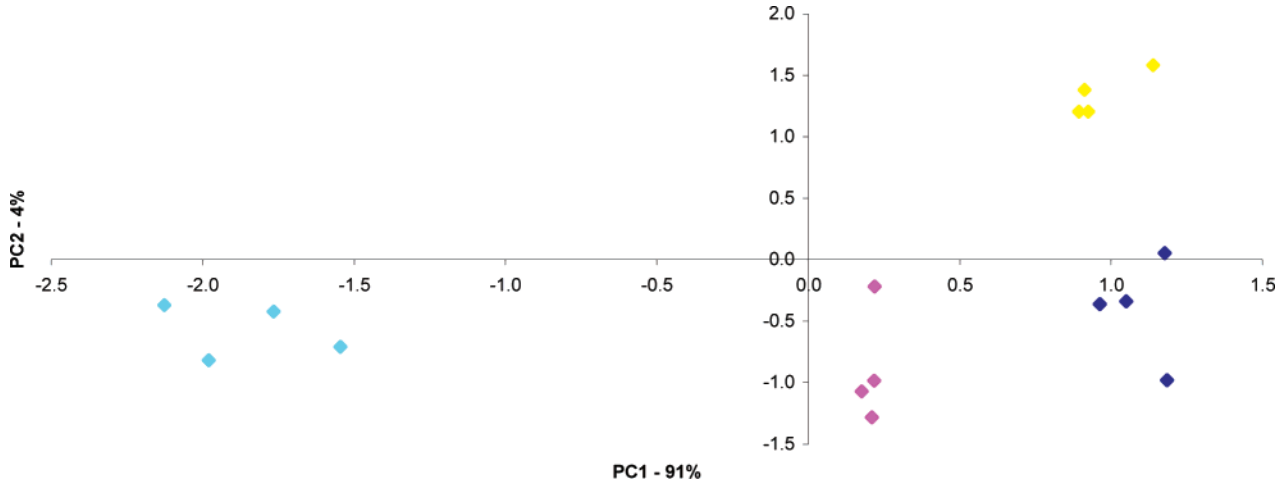

Figure 2. Two-dimensional PCA plot describing 95% of the variance from the original data set. Clustering of the analytes illustrates the ability of our differential array of resin-bound receptors to discriminate various tripeptides: His-Glu-Thr, dark blue; His-Lys-Thr, pink; Gly-His-Thr, yellow; and His-Gly-Thr, light blue.

were extracted to obtain the indicator-uptake slopes for each receptor. The copper, analytes, and indicator were rinsed from the array using acid and base washes. Four trials were completed for each tripeptide or tripeptide mixture, and patterns were determined with PCA.

Principal Component Analysis and Tripeptide Patterns. PCA is a mathematical approach that reduces the dimensionality of a data set to a size that can be accommodated graphically.[20] In the analysis of the differential array, such a large number of indicator-uptake slopes are calculated that it is impractical to visually determine an observable pattern for an analyte without employing a degenerative mathematical application to the data. In PCA, the multiple slopes generated from a single trial of one tripeptide/mixture are reduced to data points known as scores on principal component axes. Therefore, a 30-dimension pattern from the array is reduced to a single score point on a two-dimensional PCA chart. The PCA chart has two or more principal component (PC) axes in which the first principal component (PC1) is calculated to describe the maximum amount of variance in the original data set. Therefore, spatial separations along PC1 are more significant than those of subsequent axes that describe diminishing levels of variance.

The indicator-uptake rates display a complicated behavior involving a combination of fluid mixing, analyte transport, and complex binding dynamics. Thus, the uptake curves deviate from linear behavior (Figure 1). As described, the patterns are obtained from the cumulative slopes from all the receptors within the array. Since the array uptake responses were not linear, it was necessary to determine if the PCA plots were different depending on the time increments from which the slope was obtained. Thus, we split the curves of Figure 1 into three sections: (1) the entire area from 50 to 400 s, (2) the steep slope region from 50 to 175 s, and (3) the gentle slope from 175 to 400 s. A PCA plot was generated for each slope region, and the graphs were consistent in all slope areas, indicating that the differential array responses were consistent throughout the indicator-uptake.

A single receptor taken from the array would be expected to be, at best, weakly discriminating between various tripeptides. However, using a differential array of receptors from library **2** and PCA, we obtained excellent discrimination and separation of tripeptides on a PCA plot. The PCA plot was generated from the full indicator-uptake slope region from 50 to 400 s (see Figure 1). As illustrated in Figure 2, individual tripeptides cluster, and different tripeptides are clearly separated with PCA. The two PC axes describe 95% of the variance in the original data set, with PC1 describing 91%.

The separation of analytes is not due simply to the interaction between the N-terminal amino acid and the copper center of the receptor. If three tripeptides with His in the N-terminus would have clustered together, the graph would not have shown distinct separation. This suggests that the peptide arms are important for discriminating between the individual tripeptides. Therefore, specific interactions occurring between the peptides

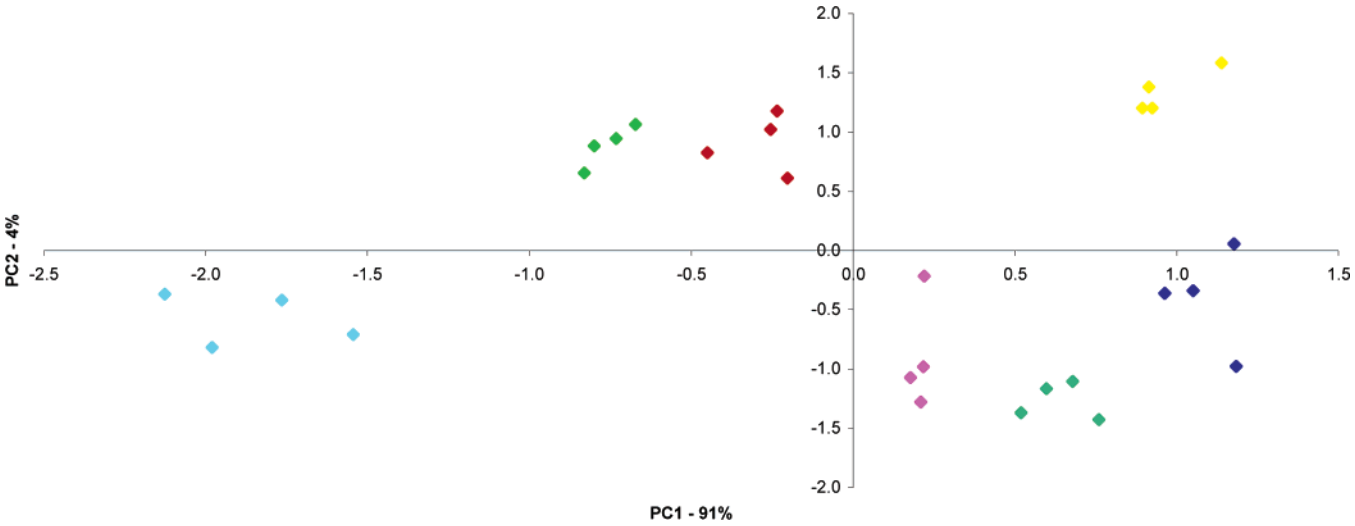

Figure 3. Two-dimensional PCA plot that describes 95% of the variance from the original data set. Clustering of the analytes illustrates the ability of our differential array of resin-bound receptors to discriminate various tripeptides and mixtures of tripeptides: His-Glu-Thr, dark blue; His-Lys-Thr, pink; Gly-His-Thr, yellow; His-Gly-Thr, light blue; His-Lys-Thr & His-Glu-Thr, green; His-Gly-Thr & Gly-His-Thr, orange; and His-Lys-Thr & His-Gly-Thr, green.

of the receptors and the tripeptide analytes give different modes of binding that are reflected in the PCA plot. Evaluating the slope responses, those analytes on the far right side of the PCA had cumulatively higher slope responses than those on the left. As described earlier, higher slope values correlate to weaker binding interactions between receptors and analytes. Therefore, in Figure 2, His-Gly-Thr (**5**) has the strongest interaction because the majority of the receptors have lower indicator-uptake slopes than with the other analytes. Conversely, Gly-His-Thr (**6**) has the weakest interaction, so several of the receptors within the array had large indicator-uptake slopes. The nature of these receptors was still differential, as several receptors did not respond in this fashion.

Mixture Analysis and Patterns. Most real-world samples, whether environmental or biological, are comprised of multiple components. For a full application of our array, a mixture analysis was included. The responsiveness of our receptor array to mixtures was tested by attempting to discriminate single tripeptides from composite mixtures. Three tripeptide mixtures were evaluated at concentrations identical to those of the single tripeptides (13 μM, 6.5 μM for each tripeptide in the mixture). As shown in Figure 3, the mixtures separated from one another, and from the single tripeptides. This initial result demonstrates that our differential array is sensitive to mixtures and is capable of discriminating between mixtures and the individual tripeptides.

All of the mixtures have scores on the PCA plot that lie between the scores of their individual components. This implies that, in all cases, the interactions of the mixtures are governed by both tripeptides in the mixtures.

Characterization of Receptors. The contribution of an original variable (resin-bound receptor) axis to the new principal component axes is determined by measuring the cosine of the angle between the new principal component axis and the original variable axis. If the cosine of that angle is close to −1 or 1, then the original variable is significant to the formation of the new axis. These values are known as factor loading values, and they can be used to identify the receptors within the array that are most significant to the formation of the first PC axis in both Figures 2 and 3.[44] Once the significance of each bead was identified, Edman degradation was used to determine the specific residues on several beads. This provided full characterization of several receptors within the array.

Table 1. Sequencing Results and Factor Loading Values for the First Principal Component (PC1)

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

The analysis of the factor loading values provides interesting insights into the operation and functionality of the array response. Indeed, nearly all of the 30 receptors were found to be important to the formation of PC1, meaning their values were close to −1 or 1. This is intuitively sensible, as PC1 describes such a large portion of the original variance. Therefore, there is likely a common interaction between the tripeptides/mixtures and the Cu(II) center of the receptor for all analytes. However, the strength of association between the variable peptide arms of the receptor and the analytes is the discriminating factor resulting in the separation and clustering of analytes on the new principal component graph.

We chose to fully characterize four receptors from the array with the largest loading values, and two with the smallest (Table 1). However, the receptors with smaller loading values are still relatively significant. The two receptors with smaller loading values both had a proline and an arginine, but in different positions. This may indicate that one or both of these residues caused either a charged or a steric interference with the analytes, thereby limiting association. The receptors with the largest loading values have basic amino acids conserved, lysine and histidine, as well as a number of different aliphatic residues: glycine, leucine, isoleucine, valine, and alanine. However, it would be errant to draw any large conclusions from this information, as the factor loading values across the array are relatively similar.

(44) Beebe, K. R.; Pell, R. J.; Seasholtz, M. B. *Chemometrics: A Practical Guide*; Wiley-Interscience: New York, 1998.

Conclusions

The current approach to differential sensing represents a powerful tool for the analysis of dynamic analytes and complex mixtures. We have shown that the use of library **2** in differential arrays can successfully analyze tripeptides and tripeptide mixtures. By employing an enhanced indicator-uptake assay, 13 μM concentrations of the analytes could be targeted. Furthermore, pattern recognition using principal component analysis showed good discrimination of the analytes and mixtures. We demonstrated that, while the interaction of the metallocenter with the analytes is important to binding, it is the variable peptide arms of the receptors that provide discrimination of the analytes. This is another example of targeting complex bioanalytes with differential (or cross-reactive) arrays that would have required highly labor-intensive design and synthesis to develop multiple selective receptors. We have also delineated in detail our protocol for employing a differential synthetic receptor array.

Currently, we are designing receptors with new binding functionalities for the analysis of other bioanalytes. We are also looking into the effect of mixing multiple libraries to discriminate bioanalytes that may be difficult to discriminate with just a single receptor library. Last, developing a functional tool for medical or environmental diagnostics will necessitate more advanced studies of crude mixtures in complex media.

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Supporting Information Available: Full experimental details and characterization data for tripeptides **3**−**6**. This material is available free of charge via the Internet at http://pubs.acs.org.

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