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An Optimized Sensor Array Identifies All Natural Amino Acids

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Supporting Information

ABSTRACT: Wet-chemical discrimination of amino acids is still a challenge due to their structural similarity. Here, an optimized self-assembled eight-member sensor array is reported. The optimized sensor array stems from the combination of elements of different tongues, containing poly(*para*-phenyleneethynylene)s (PPE) and a supercharged green fluorescent protein (GFP) variant. The responsivity of the sensor dyes (PPEs and GFP) is enhanced in elements that contain adjuvants, such as metal salts but also cucurbit[7]uril (CB[7]) and acridine orange; a suitable and robust eight element array discriminates all of the 20 natural amino acids in water at 25 mM concentration with 100% accuracy. The results group well to the amino acid type, i.e., hydrophobic, polar, and aromatic ones.

KEYWORDS: sensor array, amino acid, poly(*para*-phenyleneethynylene)s, cucurbiturils, acridine orange, green fluorescent protein

Amino acids are the building blocks of proteins and peptides.^{1–3} Their identification is attractive even though analytical procedures for amino acid determination based on chromatographic,⁴ spectroscopic,⁵ or electrochemical⁶ methods exist. Current techniques for amino acid discrimination require sample pretreatment and significant instrumentation. Attractive colorimetric and fluorimetric chemosensors have been reported for specific amino acids with distinctive functional groups, such as cysteine, histidine, and aspartic acid.^{7,8} Yet, robust fluorescence-based sensors for all 20 natural amino acids are still surprisingly challenging.

In contrast to the specific sensors for each analyte, sensor arrays (chemical tongues) discriminate analytes based upon patterns formed by collection of signals of differentially selective members of an array.^{9–13} Anzenbacher et al. constructed an attractive indicator-displacement based amino acid sensing array, employing cucurbit[n]uril, where an attached naphthalene's fluorescence is quenched by Eu³⁺ ions.¹⁴ Addition of certain amino acids leads to fluorescence turn-on by displacement of the europium ion; specific amino acids and their amines were identified. Luminol functionalized silver nanoparticles¹⁵ and a multisensor array, employing transition metal ion complexes of 2-imino-phenol-appended calix[4]arene conjugates,¹⁶ also identify specific amino acids, but not all 20 naturally occurring ones. A simple, rapid, and sensitive sensor array that

classifies and identifies all of the 20 natural amino acids is therefore still a challenge and could be attractive in a lab-on-a-chip type approach to ultrafast, chemical protein sequencing. Sensor arrays composed of fluorescent conjugated polymers alone and with simple adjuvants (other polymers, detergents, peptides, etc.) successfully discriminate and identify wide variety of analytes including bacteria, proteins, explosives, carboxylic acids, sugars, wines, teas, whiskies, and honeys.^{17–27} Here, we expand our sensor array for amino acids and construct an improved simple array, discriminating 20 natural amino acids with 100% accuracy at 25 mM concentration. We combine poly(*para*-phenyleneethynylene)s (PPE) and green fluorescent protein (GFP) based arrays tuned up by a pruning process.

RESULTS AND DISCUSSION

Figure 1 shows the structures of poly(*p*-phenyleneethynylene)s P1–P7, the macrocyclic host cucurbiturils (CB[n], *n* = 7 or 8), the dye acridine orange (AO), and the GFP variant GFP-K72 with a high positive net charge induced by recombinant fusion of an unfolded, supercharged polypeptide chain.^{19,27} Figure 2 displays the four starting arrays. Array 1 consists of a positively

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Figure 1. Chemical structures of poly(*p*-phenyleneethynylene)s **P1–P7**, cucurbiturils **CB[7]** and **CB[8]**, tricyclic dye acridine orange **AO**, and green fluorescent fusion protein **GFP-K72**.

Figure 2. Fabrication of a multiple sensor array for the discrimination of amino acids.

charged **GFP-K72** in the presence of different metal cations at pH 7. Array 2 employs the positively charged **P1** also in the presence of the metal cations, while arrays 3 and 4 are supramolecular arrays in which cucurbituril[8] and PPEs or PPEs in the presence of acridine orange and cucurbituril[7] form complex fluorescence-responsive arrays.¹⁹ We note that the fluorescence of **GFP-K72** or **P1** was quenched by metal ions. In arrays 3 and 4, cucurbit[n]urils ($n = 7$ or 8) are used for detection and recognition of amino acids as these interact with the CB host cavity.^{28–30} Array 3 and its function have been discussed.²⁷

Array 3 using the larger **CB[8]** (vide infra) is not efficient for the discrimination of amino acids, and it will not be discussed in detail. In addition to our published array 2, arrays 1 and 4 impart additional selectivity to the array. Attempts to discriminate amino acids just with the cationic GFP were not very successful, but analogously to our published array 1, addition of metal salts unlocked the sensitivity of the GFP toward amino acid analytes. Here we also assume that the GFP forms a nonfluorescent complex with the metal salt, which is reversed by the addition of the analytes. While the used GFP is overall positively charged at pH 7, there will be still a significant number of negatively charged residues that coordinate to the metal salts.

Table 1. Results of Unknown Detection Using a LDA Algorithm

amino acids	number of samples	6 selected elements tongue (metal-based array)		8 selected elements tongue (optimized array)	
		correctly identified	accuracy (%)	correctly identified	accuracy (%)
Ala	4	3	75	4	100
Cys	4	4	100	4	100
Asp	4	3	75	4	100
Glu	4	4	100	4	100
Phe	4	4	100	4	100
Gly	4	4	100	4	100
His	4	4	100	4	100
Ile	4	3	75	4	100
Lys	4	2	50	4	100
Leu	4	2	50	4	100
Met	4	4	100	4	100
Asn	4	4	100	4	100
Pro	4	4	100	4	100
Gln	4	4	100	4	100
Arg	4	4	100	4	100
Ser	4	4	100	4	100
Thr	4	3	75	4	100
Val	4	4	100	4	100
Trp	4	4	100	4	100
Tyr	4	4	100	4	100
Total	80	72	90	80	100

The most remarkable array is the ternary one, composed of acridine orange (**AO**), **P1**, and **CB[7]**. Control experiments show that **CB[7]** enhances FRET between the **AO** and **P1**. Interestingly, both species bind to the cavity of **CB[7]**, as shown by NMR titration experiments (Figures S4 and S5); in the case of **AO**, **CB[7]** forms a 1:1 complex with the dye (Figure S6). **AO** exhibits an emission peak at 530 nm; upon addition of **CB[7]**, a blue shift to 510 nm occurs (Figures 3 and S6).^{31,32} Sensor elements were constructed through in situ assembly of the PPEs **P1–P7**, **CB[7]**, and **AO** at a molar ratio of 1:2:1 (based on a per

Figure 3. Fluorescence emission spectra of (a) P1, AO, the mixture of AO and CB[7] (1:1), P1 and AO (1:1), and P1, CB[7], and AO (1:2:1); (b) P1-CB[7] in the presence of different concentrations of AO (from 0 to 20 μM), [P1] = 2 μM , [CB]7 = 4 μM ; changes in fluorescence intensity of P1-CB[7]-AO (2.0 μM /4.0 μM /2.0 μM) upon gradual addition of (c) Trp and (d) His (from 0 to 30 mM). All spectra were performed in water upon excitation at 410 nm.

repeat unit of the PPEs). Amino acids form hydrogen bonds with CB[7] and dyes due to the amine and carboxylate groups. Tryptophan (Trp) or histidine (His) displace AO or P1 from the cavity of CB[7], shutting down FRET and the emission of AO at 510 nm (Figure 3c and d) decreases. Figure 4 shows the

Figure 4. Proposed schematic illustration of the PPE-CB[7]-AO tongue working with amino acids.

proposed schematic illustration of PPE-CB[7]-AO tongue working with amino acids. Fundamentally, CB[7] acts as a FRET enhancer, but its exact mechanism is not known.

As for the experimental details, the poly(*para*-phenyleneethynylene)s or green fluorescent protein derivative were dissolved in buffers with desired pH values into stock solutions. Then, according to the array, solutions of divalent metal ions or cucurbit[*n*]urils, with or without acridine orange, were added to

the stock solutions. Each complex solution (150 μL) was loaded into a well on a 96-well plate, respectively. Subsequently, 150 μL of a solution of the amino acids was added to each well and mixed. The different fluorescence intensities at λ_{max} were recorded on a microplate reader. The fluorescence intensity change $((I - I_0)/I_0)$ was calculated and used for linear discriminant analysis, where I_0 and I are the fluorescence intensity of the solution in the absence and presence of the amino acids, respectively. Similar procedures were employed to the lower concentration of amino acids (for details, see the Supporting Information).

As a control, we first treated P1–P2 and P5–P7 (Figure 1) with the 20 naturally occurring amino acids (25 mM). The results (Figure S7) indicate that the simple PPE tongue alone is useful for the discrimination of Tyr and Trp but does not discriminate the other amino acids with polar and hydrophobic residues. However, the PPE-CB[7]-AO assembly induces better sensitivity for these analytes (Figure S8). According to the two-dimensional linear discriminant analysis (2D-LDA; Figure 5d, Tables S3 and S4), the PPE-CB[7]-AO tongue discriminates all 20 amino acids. A more simple tongue omitting CB[7] (Figure S9) shows less discriminatory power.

In the next experiment, we tested all four tongues against the 20 amino acids. Figure 5 shows the 2D-LDA plots for the first two factors obtained by the individual sensor arrays 1–4, i.e. the GFP-metal salt tongue, the PPE-metal salt tongue, the PPE-CB[8] tongue and the PPE-CB[7]-AO tongue. The discrimination is fairly poor in the PPE-CB[8] sensor array, while the other arrays work quite well.

Figure S10 shows a two-dimensional canonical score plot obtained by 28 sensor elements; all of the 20 amino acids are reliably discerned. We then performed a screening process,

Figure 5. Two-dimensional canonical score plots for the first two factors obtained by (a) array 1, GFP-metal salts tongue, (b) array 2, PPE-metal salts tongue, (c) array 3, PPE-CB[8] tongue, and (d) array 4, PPE-CB[7]-AO tongue treated with 20 amino acids ($c = 25$ mM) with 95% confidence ellipses. Each point represents the response pattern for a single amino acid to the array. Structures of amino acids are shown in the bottom panel.

employing principal component analysis (Figure 6): This loading plot finds the elements most useful for discrimination and allows to remove weakly performing ones.³³ Excellent discrimination results with a pruned eight-element tongue (all the elements that are marked with red in Figure 6) that identifies all 20 amino acids after LDA (for more details, see the Supporting Information, Figures S2, S3 and Tables S2, S5). None of the high performing elements came from the PPE-CB[8] tongue; control experiments show that the addition of AO does not improve signal of these array-elements (Figure S11).

The fluorescence modulation data of the pruned final tongue were recorded. LDA was performed and converted the training matrix (8 factors $\times$ 20 amino acids $\times$ 6 replicates) into canonical scores. The canonical scores are clustered into 20 different groups. The jackknifed classification matrix with cross-validation reveals a 100% accuracy (Figure S14 and Tables S6, S7). According to the amino acid residue, hydrophobic, polar and aromatic amino acids all grouped very well (Figure 7a). By zooming into a specific part, the discrimination of hydrophobic and polar amino acids becomes quite clear (Figures 7b and 7c). The testing was performed at 25 mM concentration of the amino acid.

To see if we could lower the concentration we also investigated 10 mM solutions. We still discriminate the amino acids, but amino acids with hydrophobic and polar residues do not group well; especially Gln, Ser, and Thr are quite close to the hydrophobic amino acids (Figure 8 and Table S8). Solutions of 5 mM were also investigated; however, the discrimination is not satisfactory (Figure S12).

To validate the efficiency of the optimized sensing system, we performed tests with 80 randomly chosen amino acids. The new cases are classified into groups, generated through the training matrix, based on their shortest Mahalanobis distance to the respective group.^{34,35} We have used our old six-element metal salts based sensor array as comparison and 8 of 80 unknown samples of amino acids were misclassified, representing an accuracy of 90% (Table 1 and Table S9). In stark contrast, the identification of unknowns is improved to 100% when employing the final 8-element tongue (Table 1 and Table S10).

CONCLUSIONS

In conclusion, we have dramatically improved our PPE-based amino acid array by addition of further sensor elements. We have

Figure 6. Loading plot of the principal component analysis plot by the four arrays, identifying the contribution of each element to an axis. The selected eight elements are labeled in red.

Figure 7. (a) Two-dimensional canonical score plot for the first two factors obtained by eight optimized sensor elements treated with 20 amino acids ($c = 25$ mM) with 95% confidence ellipses. Each point represents the response pattern for a single amino acid to the optimized array. Amino acids with hydrophobic, polar, and aromatic residues are given in blue, green, and pink, respectively. (b,c) Detailed view of the amino acids with polar and hydrophobic residues, respectively.

Figure 8. (a) Two-dimensional canonical score plot for the first two factors obtained by eight optimized sensor elements treated with 20 amino acids ($c = 10$ mM) with 95% confidence ellipses. Each point represents the response pattern for a single amino acid to the optimized array. Amino acids with hydrophobic, polar, and aromatic residues are given in blue, green, and pink, respectively. (b,c) Detailed view of the amino acids with polar and hydrophobic residues, respectively.

investigated four different arrays, plucked the best elements from three of the arrays, and created a new, much more powerful array, containing six elements of our old array and two additional elements gleaned from other tongues. We are currently aiming to lower the concentration of detectable amino acids and will investigate microfluidic-type approaches to identify and discriminate amino acids with our hypothesis free arrays. Overall, this is an encouraging development, which shows that simple tongues and sensor arrays discriminate tightly related analytes.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssensors.8b00371.

General information, synthetic details and analytical data for P1–P7, detailed method for obtaining the fluorescence response pattern, experimental setup, additional screening, and linear discriminant analysis data (PDF)

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Author Contributions

The paper was written through contributions of all authors. All authors have given approval to the final version of the paper.

Notes

The authors declare no competing financial interest.

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