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# Pattern-Based Detection of Different Proteins Using an Array of Fluorescent Protein Surface Receptors

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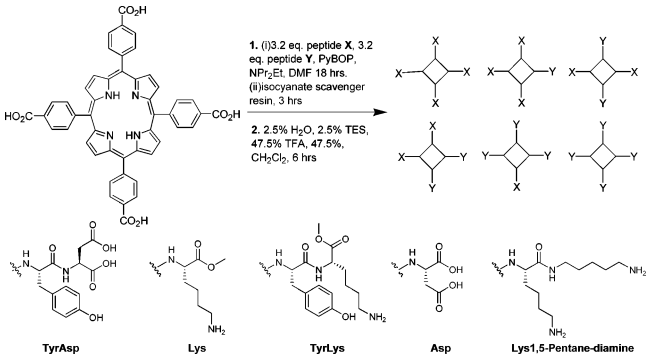
There is currently a pressing need for simple, robust, and high throughput strategies for the detection of specific proteins in different environments.[1] For example, large-scale proteomics projects require the facile detection of signaling proteins as they rise and fall as a result of cellular stimulus.[2] Similarly, in medical diagnostics, it is important to determine the presence or absence of the characteristic protein signature of a disease. Finally, current concerns about bioterrorism have raised an immediate need for field detectors that can identify airborn or dissolved pathogens and their toxic protein byproducts.[3] Any protein detectors must be responsive and/or applicable to a wide range of different targets. As a result, there has been extensive development of multiple protein binding molecules in an array format, with the expectation that different proteins will interact with the array in distinctive ways. Effective protein-detecting arrays have been based on monoclonal antibodies,[4] antibody fragments,[5] or nucleic acid aptamers.[6] However, these strategies suffer from chemical instability of the biopolymer recognition molecule and the need to label the protein target for detection. Kodadek has described two principal hurdles to the development of protein-detecting arrays: (1) the need for large numbers of stable, easily prepared ligands that each bind to a protein target with high affinity and selectivity, and (2) the incorporation of a means for detecting the ligand/protein interaction into the array.[1]

In the present paper, we report a simplified approach to this problem based on the use of solution arrays of fluorescent protein surface receptors. We have previously shown[7] that a synthetic tetraphenylporphyrin (TPP) derivative containing four negatively charged carboxamide substituents can bind to the positively charged surface of cytochrome *c* with low nanomolar affinity. The TPP unit is well-suited for protein surface recognition due to its large hydrophobic surface area (>300 Å$^2$) that can be easily functionalized on its periphery (*m*, *p*-phenyl or $\beta$-pyrrole positions) to match a complementary hydrophobic and charged domain on a protein surface. More importantly, TPP derivatives are highly fluorescent and can show emission intensity changes on binding to a protein target. We therefore reasoned that a large number of TPP derivatives peripherally functionalized with substituents of different charges, size, hydrophobicity, and symmetry might form an array of receptors having different binding characteristics. None of the receptors need be either specific or highly efficient for any single protein analyte, but when arranged in an array format they should respond quite distinctively to proteins with varying surface characteristics, yielding a composite response unique for each analyte.[8−10] Moreover, changes (or lack of them) in the fluorescence of the TPP derivatives would provide information on the surface characteristics of the protein analyte.

A library of TPP derivatives functionalized with different amino acids or amino acid derivatives could be rapidly synthesized using a mixed condensation strategy (Scheme 1, top). The "one-pot" reaction of 1 equiv of meso-tetracarboxyphenylporphine with

***Scheme 1.*** (Top) Mixed Condensation Synthesis of Porphyrin Receptors; (Bottom) The Five Peptidic Components




PyBOP and an excess of two different peptidic derivatives chosen from a pool of five (Scheme 1, bottom) led to a mixture of six different products. Four of them and the mixture of the two geometrical isomers, $X_2Y_2$, were easily separated, due to their different polarities, and subsequently deprotected using 2.5% water, 2.5% triethylsilane, 47.5% trifluoroacetic acid, and 47.5% dichloromethane, a mixture which minimized cleavage of the methyl esters and alkylation of the amines by free cations. Iteration of this porphyrin functionalization procedure starting with every possible combination of the two peptidic components followed by separation and deprotection resulted in the isolation of 35 unique fluorophores comprising every possible charge combination from +8 to −8 and from 4 to 8 hydrophobic groups.

Eight members of the library were then selected to form a preliminary group of receptors having different functionalities (Figure 1, left). Next, 5 $\mu$M solutions of these porphyrins in 5 mM phosphate buffer (pH 7.4) containing 0.05% of Tween 20 and 0.5% of DMSO were arrayed in the first five columns of a 96 well quartz plate, a different porphyrin in each row (**A**−**H**). When observed under UV light (302 nm), each well shows a bright and intense red fluorescence. To test the protein recognition ability of this preliminary array, we selected four proteins having different surface characteristics, ranging from the very acidic ferredoxin (pI 2.75[11]) to the highly basic cytochrome *c* (pI 10.6), and incubated 3 equiv of each protein in columns 2−5 (column 1 as blank). When irradiated with UV light (302 nm), the plate displayed a pattern of fluorescent and nonfluorescent wells (Figure 1, right).

We have previously shown[7] that proteins containing a metal center with unpaired electrons efficiently quench the porphyrin fluorescence upon binding, due to the proximity of the metal to the porphyrin ring in the complex. The wells that show quenching would then correspond to a binding interaction between the synthetic receptor and the protein, while the wells that remain fluorescent should contain species that do not form complexes. In this way, "naked-eye" detection would enable the rapid screening of the collection of fluorophores for the identification of high affinity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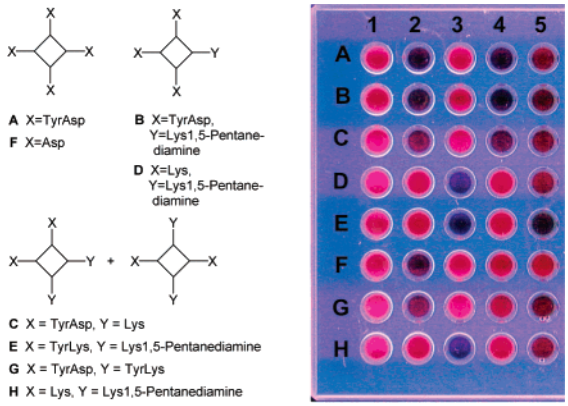


***Figure 1.*** (Left) Schematic structure of porphyrin receptors **A**−**H**. (Right) Picture of part of the 96 well quartz plate irradiated with UV light (302 nm). Each well contains 200 $\mu$L of a 5 $\mu$M solution of porphyrins **A**−**H** (5 mM phosphate buffer, pH 7.4, containing 0.05% of Tween 20 and 0.5% of DMSO); in the wells of columns 2−5 were added 3 equiv (as 500−600 $\mu$M stock solutions in 5 mM phosphate buffer, pH 7.4) of the following proteins: **2**, cytochrome *c* (pI = 10.6, MW ≈ 12 500); **3**, ferredoxin (pI = 2.75,[11] MW ≈ 5500); **4**, cytochrome *c*551 (pI = 4.7,[12] MW ≈ 9000); **5**, myoglobin (pI = 6.8, MW ≈ 18 000).

***Table 1.*** Dissociation Constants[a] and Structural Properties of Selected Synthetic Receptors and Proteins

| porphyrin | charge | aryl groups | protein | pI | $K_d$ ($\mu$M) |
|---|---|---|---|---|---|
| **A** | −8 | 8 | myoglobin | 6.8 | 1.76 ± 0.08 |
| **B** | −4 | 7 | cytochrome *c* | 10.6 | 1.59 ± 0.07 |
| **E** | +6 | 8 | myoglobin | 6.8 | 3.7 ± 0.1 |
| **F** | −8 | 4 | cytochrome *c* | 10.6 | 0.67 ± 0.02 |
| **H** | +6 | 4 | ferredoxin | 2.75 | 0.37 ± 0.05 |

[a] Determined at 5 mM sodium phosphate, 0.05% Tween-20, pH 7.4, 298 K.

ligands for protein surfaces. This hypothesis was confirmed by individual titration experiments between porphyrin−protein pairs that occupy nonfluorescent wells (Table 1); titration of the protein into the porphyrin solution resulted in quenching of the receptor fluorescence due to complex formation. The dissociation constants calculated upon fitting the data to 1:1 binding isotherms are in the low and sub-micromolar range, confirming that appropriately functionalized porphyrins provide receptors suitable to interact with a range of protein surfaces. As expected, titration of ferredoxin into a solution of **A** did not result in a significant fluorescence decrease (Supporting Information). The quenching in the different wells cannot be directly correlated to the relative binding affinities, because the mechanism of quenching may vary according to the location of the porphyrin on the protein surface.

The response of the array reflects the charge complementarity between the porphyrin receptors and the target protein. In fact, cytochrome *c* is bound by the negatively charged fluorophores **A**, **B**, **C**, **F**, and **G**, and ferredoxin interacts with the positively charged porphyrins **D**, **E**, and **H**. Cytochrome *c*551 has a prevalence of acidic groups on its surface, but the hydrophobic area containing the heme edge is surrounded by a few basic residues, thus forming

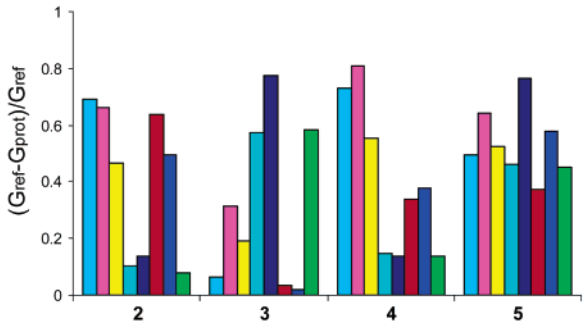


***Figure 2.*** Fingerprints of cytochrome *c* (**2**), ferredoxin (**3**), cytochrome *c*551 (**4**), and myoglobin (**5**) based on the eight porphyrin array. Each bar quantifies the extent of color attenuation measured as the quantity $(G_{ref} - G_{prot})/G_{ref}$, where $G_{ref}$ is the average gray value for the blank wells and $G_{prot}$ is the average gray value for the protein-containing wells.

a region which favorably interacts with the acidic porphyrins **A**, **B**, **C**, and, to a lesser extent, **F** and **G**. Myoglobin, which has a fairly neutral surface, has some affinity for most of the porphyrins except **F**, which has the highest charge and the lowest hydrophobicity.

Thus, each column of the plate corresponds to a unique fingerprint, characteristic of a specific protein. This allows for the unambiguous visual identification of each protein within the four investigated (Figure 2).

In conclusion, we have developed an approach to the generation of an array of porphyrins with different groups on their periphery. When incubated with different proteins, the array responds with a unique pattern of fluorescent/nonfluorescent spots which represents a characteristic fingerprint for the protein.

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**Supporting Information Available:** Synthetic procedures and analytical data, titration data (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

## References

(1) For an insightful discussion of this problem, see: Kodadek, T. *Chem. Biol*. **2001**, *8*, 105−115.
(2) Zhu, H.; Snyder, M. *Curr. Opin. Chem. Biol*. **2001**, *5*, 40−45.
(3) Bronze, M. S.; Greenfield, R. A. *Drug Discovery Today* **2003**, *8*, 740−745.
(4) De Wildt, R. M. T.; Mundy, C. R.; Gorick, B. D.; Tomlinson, I. M. *Nat. Biotechnol.* **2000**, *18*, 989−994.
(5) Boder, E. T.; Midelfort, K. S.; Wittrup, K. D. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 10701−10705.
(6) Hesselberth, J.; Robertson, M. P.; Jhaveri, S.; Ellington, A. D. *Rev. Mol. Biotechnol.* **2000**, *74*, 15−25.
(7) Jain, R. K.; Hamilton, A. D. *Org. Lett.* **2000**, *2*, 1721−1723.
(8) Albert, K. J.; Lewis, N. S.; Schauer, C. L.; Sotzing, G. A.; Stitzel, S. E.; Vaid, T. P.; Walt, D. R. *Chem. Rev.* **2000**, *100*, 2595−2626.
(9) Lavigne, J. J.; Anslyn, E. V. *Angew. Chem., Int. Ed.* **2001**, *40*, 3118−3130.
(10) Rakow, N. A.; Suslick, K. S. *Nature* **2000**, *406*, 710−713.
(11) Dutton, J. E.; Rogers, L. J. *Biochim. Biophys. Acta* **1978**, *537*, 501−506.
(12) Chao, Y.-Y. H.; Bersohn, R.; Aisen, P. *Biochemistry* **1979**, *18*, 774−779.

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