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Continuously Evolving 'Chemical tongue' Biosensor For Detecting Proteins

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ABSTRACT

We developed a unique continuously evolving colorimetric sensor array based on AuNPs decorated by two single-stranded oligonucleotides with different molar ratios for protein discrimination. The number of differential receptors in this sensor array could be easily extended by adjusting the molar ratios of two DNA, resulting in continuously improved discrimination ability. The continuous response data of target samples against our sensing system could be easily obtained and exclude abnormal signals. The sensing system could discriminate twelve proteins at the concentration of 200 nM in the presence of 50% human urine with accuracy of 100%, showing feasible potential for diagnostic applications. Remarkably, HSA at various concentrations, the pure Lys and HSA, and the mixture of these two proteins with different molar ratios had been successfully discriminated in LDA plot as well in the presence of human urine sample. This novel strategy will be very promising for the design of cheaper and

more reliable sensor arrays for target samples.

In this study, we proposed a novel strategy to prepare differential receptors by simply mixing two kinds of DNA on gold nanoparticles surface, which could drastically reduce the design and synthetic efforts.

Keywords:

Continuous response pattern, AuNPs decorated by two oligonucleotides, Colorimetric sensor array, Protein discrimination

1. Introduction

Chemical sensor arrays, also known as 'artificial nose/tongue', apply the differential binding of analyte and a set of cross-reactive receptors to generate a characteristic pattern for each target sample [1-9]. As a result, none of the receptors in the array need to be highly specific for any given analyte. The time-consuming design and synthesis of high specific receptors for each analyte, which is required in conventional biosensors with a 'lock-and-key' recognition principle, may be circumvented. Up to now, various receptor systems have been reported to construct sensor array, including substituted porphyrins [10], polymer-nanoparticle [11], polymers [12], quantum dots [13-15] and nanoparticles [16-18] and these sensor arrays have been successfully used to detect small molecular compounds [19-22], biological molecules [23-25], cells [26], and even drug mechanisms [27]. However, as the discrimination ability of a sensor array is critically depend on the number of sensing elements, the design and synthesis of a large number of receptors is still required for detecting various types of analytes.

Recently, a novel strategy of using only one or two sensing material to prepare a collection of differential receptors was proposed to solve this problem, which could drastically reduce the design and synthetic efforts. For example, an electronic nose was fabricated by depositing different amounts of porous In_2O_3 microtubes on Al_2O_3

substrate for the discrimination of 14 volatile organic compounds [28]. By mixing two dye encapsulating nanoparticles of different polarity in different mixture ratios, a colorimetric sensor array with a polarity gradient was built to discriminate 7 volatile primary amines [29]. A collection of gold surfaces, generated by the self-assembly of two disulfide molecule with various proportions, could identify 4 proteins in solution using SPRi detection [30]. Although these efforts have been done, the application of this strategy is still very rare, especially in biosensing. It is highly needed to apply this strategy to a more universal sensing system for practical application.

In this report, we proposed a new sensing system by simply mixing different DNA in varying molar proportions and allowing them to assemble on gold nanoparticles surface, which lead to a collection of combinatorial cross-reactive receptors with evolutionary properties for protein recognition (**Scheme 1**). Under high concentration of salt, these receptors exhibited different aggregation behaviors in the presence of different proteins or their mixtures, resulting in various colorimetric signals that could be detected by UV-vis detector or even naked eye. More importantly, by increasing the number of sensing elements, the discrimination power of this sensor array to proteins could be improved in both buffer and complex matrix. As AuNPs and DNA with different sequence could be commercially synthesized with low cost, high purity and robust stability, this novel strategy will be very promising for the design of cheaper and more reliable sensor arrays for target samples.

2. Experimental section

2.1 Materials and instruments

All of the oligonucleotides were purchased from Sangon Biotech Co., Ltd. (Shanghai, China): 5′ - GGT TGG TGT GGT TGG -3′ (TBA), 5′ - TTT TTT TTT TTT TTT -3′ (T15). Chloroauric acid ($HAuCl_4$) and sodium chloride were purchased from Sinopharm Chemical Reagent Company (Beijing, China). All of the proteins were purchased from Beijing Biodee Biotechnology Co., Ltd. (Beijing, China). Sodium citrate was purchased from Xilong Chemical Co., Ltd. (Shantou, China). Other reagents and chemicals were at least analytical reagent grade. Absorption spectra were recorded on SpectraMax^{R}M2e Multi-Mode Microplate

Reader at room temperature (Molecular Devices, California, USA). The 96-Well micro titer plates were produced from Costar (3590, USA). The water used throughout all experiments was purified by a Milli-Q system (Millipore, Bedford, MA, U.S.A.).

2.2 Sensing protein studies

Synthesis of AuNPs: The water-soluble AuNPs with the diameter of 13 nm were synthesized according to the literature procedures [31]. Citrate-stabilized AuNPs (13 nm) were prepared as described in previous literature. In brief, trisodium citrate solution (38.8mM, 20 mL) was added to a boiling, rapidly stirred solution of $HAuCl_4$ (1.0 mM, 200 mL). The solution was kept boiling at 15 min. After being naturally cooled to room temperature, the solution was stirred in all experiment and the AuNPs were filtered (with 0.22 μM Millipore membrane filter).

Proteins Discrimination: Using TBA and T15 at final concentration of 100 nM, five types of mixtures with TBA molar ratios of 0, 20, 50, 80, and 100% was prepared. 100 μL of AuNPs was added into 50 μL of mixture with five different concentrations of TBA and T15, respectively. The five types of AuNPs-DNA were incubated with vigorous shaking for 30 min to fabricate five sensing elements. 50 μL of target protein stock solution (final concentration of 50 nM) and 250 μL of deionized water was successively added and shaken mildly. After 30 minutes, the protein was detected in the presence of 50mM NaCl. Then, absorbance values of the mixture at both 520 and 620 nm were read by a Multi-Mode Microplate Reader. The raw data were first normalized (A/A_0) to eliminate the potential bias caused by the difference in the original signal intensity of sensing system. The colorimetric patterns resulted from the aggregation of AuNPs were subjected to linear discriminant analysis (LDA). For practical application, urine sample was gathered from the morning urine of healthy people and it centrifuged for 5 min (12, 000 rpm) to remove the insoluble matrix. Pure urine diluted one time was used for proteins discrimination without adding NaCl.

3. Results and discussion

As a proof-of-principle colorimetric sensor array, we employed two single-stranded

DNA, aptamer TBA15 with high affinity and T15 with weak affinity on AuNPs, with different molar ratios as receptors toward protein recognition. We anticipated that variation of the TBA15 to T15 ratio on the surface of AuNPs should provide differential binding affinities. At a constant total concentration of 0.1 μM, five DNA mixtures with [TBA]/([TBA]+[T15]) molar ratios of 0, 20, 50, 80 and 100% were added into water-soluble 13 nm AuNPs solution, serving as five receptors solution. We found that the five receptors solution gave different absorbance spectra and color to the same Lys protein in the presence of 50 mM NaCl (**Fig. 1A**). The color of sensing solution got darker with higher TBA content in different receptor. The signal change value was defined as A/A_0, where A and A_0 were UV-Vis adsorption ($A = OD_{620nm}/OD_{520nm}$) of the mixture colloid solution in the presence and absence of the target proteins, respectively. The response value (A/A_0) increased with increased molar ratios of TBA, indicating that the aggregation degree of AuNPs also gradually increased. Because of the structured TBA possess weaker binding affinity for AuNPs than the linear oligonucleotide T15 [32, 33], the bound TBA15 strands were more easily released into the solution as a result of the competitive displacement of proteins. The observed response behavior of the differential receptors demonstrated the possibility for binding affinity-based analyte discrimination achieved by simple gradient mixing of two types of the single-stranded oligonucleotides.

The responses of more sensing elements to more proteins were further investigated. Using TBA and T15, at a constant final total concentration of 0.1 μM, eleven cross-reactive sensing elements were prepared from AuNPs decorated by two oligonucleotides with TBA molar ratios of 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100%. Four pure proteins including acid (Pep), natural (Con-A), and base proteins (Try and Lys) at 50 nM were tested by using our system. As shown in **Fig. 1B**, for each protein, the different sensing elements displayed different response values (A/A_0). More importantly, for a given receptor composition, the response values were dependent on the sensing target protein, indicating that colorimetric sensor array with eleven receptors responded differently toward each protein. The response values (A/A_0) of target Pep against eleven differential receptors were no significant changes

and less than 1.0, which demonstrated the stability of the colloidal solution became much better. Their corresponding to photographs showed that the sensing Pep system still remained bright red (**Fig. S1**). The pH value of the sensing system was about 7.0. The acid protein Pep with lower pI (～2.5) could be negative charged and difficult to bind AuNPs. In contrast, base protein Lys with higher pI (～11) could be positive charged and easily bind to AuNPs, resulting in destroying the stability of AuNPs-DNA. The response values (A/A_0) of target Lys greatly increased with increasing of TBA content in differential receptors, suggesting that the aggregation degree of AuNPs appeared enhancement. In the presence of Lys, the colloid solution color appeared blue. This substantial difference in colors was sufficient for unaided eye discrimination in target sample or blank sample (**Fig. S1**). While the response values for another base protein Try were almost 1.0, indicating that the sensing elements did not be disrupted. The color of sensing elements for Try was not obviously changed with that of their blank experiments, displaying wine red (**Fig. S1**). In addition, with the TBA content increase of differential receptors the response values (A/A_0) of netural protein Con-A also displayed gradually upward trend. These results demonstrated that sensing elements responded differently to target proteins by various interactions such as electrostatic interaction, hydrogen bonding, and π–π stacking.

These results demonstrated that it was feasible to establish simple colorimetric sensor array for multiple proteins, which consisted of AuNPs modified by two types of oligonucleotides with the molar ratio of continuous changes. More importantly, the response of a certain sensing element to a given protein was not a simple linear addition of the responses obtained from pure TBA15 and T15. That revealed each combinatorial receptor could provide additional information for sensing and the discrimination ability of the sensor array with more receptors might become higher. Moreover, the response was also linked and closed to that obtained from its neighbors. Therefore, the response signal for each protein offered by the colorimetric sensor array could be considered as continuous evolution. One of the advantages of this kind of sensor array over the traditional array approaches giving uncorrelated orthogonal

data was that the signal of one receptor correlated with the others and abnormal signals could be identified and excluded.

After the discrimination of pure analyte, the next challenge of sensor array was to analyze complex mixtures. The mixtures with Lys/Try (named as Mix1) and Lys/Pep (defined as Mix2) molar ratios of 50/50 (Lys, Try, and Pep at total concentration 50 nM) were chosen as model analytes. As illustrated in **Fig. 1C**, the continuous evolution signal was achieved from our colorimetric sensor array and was able to distinguish between Mix1 and the individual proteins. Furthermore, the response values of Mix1 were close to the average values of two pure proteins with Lys and Try, suggesting the additive behavior with respect to the signals obtained from the pure proteins. The same phenomenon of Mix2 was also observed in **Fig. 1D**. These results confirmed that the ability of the continuous evolution pattern of the mixture analysis was provided by a simple linear addition of the pure analytes.

To obtain visual examination of the continuous evolution pattern of target analytes, all data of the response pattern was subjected to linear discrimination analysis (LDA). As shown in **Fig. 2A**, the pure and mixed protein samples could be discriminated using three given receptors with TBA content accounting for 0, 50, and 100%, respectively. But all sample clusters were located in compacting area. The same samples were identified using six given receptors including three original and three additional receptors, resulting in increased dispersion degree of clusters in **Fig. 2B**. Euclidean distance (the length of the line segment connecting the points) between two samples was continuously increased when the number of differential receptors was up to eleven in **Fig. 2C**. Euclidean distance of two samples including pure and mixed proteins was all increased with increasing the number of transducers as illustrated in **Fig. 2D**. The larger Euclidean distance became, the higher the discrimination power of training and unknown samples was achieved.

To further explore the application of this sensor array to real samples, it was used to distinguish target proteins at 200 nM spiked in 50% human urine. Notably, it did not need to add extra salt (NaCl) into the samples. Initially, these proteins were analyzed using this colorimetric sensor array with two transducers, which included TBA

accounting for 0 and 100% to generate a 2D plot. As illustrated in **Fig. 3A**, only 9 of the 13 samples were clearly identified with the accuracy of 94.0%. But two groups of proteins (Con-A and IgG, EA and TRF) still exhibited significant overlap and remained unidentified with 95% confidence ellipses (marked in the red square), suggesting the limited discrimination ability. Then, another transducer with TBA content 50% was added to obtain three sensing elements, only two proteins (EA and TRF) still exhibited overlap. The results showed that the discrimination accuracy was improved from 94.0% to 97.0% in **Fig. 3B**. We further added one transducer (TBA content 20%) to our colorimetric sensing platform including four sensing elements, which properly identified all proteins without overlap and completely separated in **Fig. 3C**. When five sensing elements (TBA accounting for 0, 20, 50, 80, and 100%) were employed in **Fig. 3D**, compared with four elements system, the Euclidean distance between EA and TRF increased from 0.416 to 0.4839 (**Fig. S5**). These results confirmed that the Euclidean distance between samples was increased with the number of sensing elements, indicating the increase of discrimination power. We then tested this colorimetric sensing system against unknown samples generated from 13 kinds of proteins. The unkown samples were ranked in terms of Euclidean distance to the groups from the training matrix and clusted the nearest samples to the respective groups. In this work, 39 unknown samples were identified with the accuracy of 100% (**Table. S5**).

For a sensing array system, it was challenging to discriminate different proteins at various concentration levels and identify a mixture of proteins. The potentials of practical applications of the present sensing system, e.g., the identification of HSA at five concentrations in the presence of a 50% human urine sample, i.e., 50nM, 100nM, 250nM, 500nM, and 1000nM, were demonstrated. As shown in **Fig. 4A**, 5 groups of samples were located explicitly in 5 isolated clusters in the canonical score plot. We found that the LDA plots of various concentrations respond to certain patterns and could be well differentiated from each other. The results indicated that the LDA plots of various concentrations were not random, but rather responded to certain patterns and could be well differentiated from each other. Then, we used this system to detect

the unknown concentration of HSA, according to the results of 500 nM and 1000 nM, which embodies the practicality of the system. The linearity of the dose-response curve suggests that the interactions between the sensing element and the protein species were homogeneous and stable (**Fig. 4B**). After training the sensing system, the discrimination accuracy of 14 unknown protein samples was observed to be 100% (**Table S7**).

Further experiments proved that the recognition capability of the multidimensional sensing system was also highly effective when applied to protein mixtures, as demonstrated by the mixtures of Lys and HSA at 400 nM with Lys/HSA molar ratios of 60/40, 40/60 and 20/80 as well as pure Lys and HSA in the presence of a 50% human urine sample. **Fig. 5** indicated that these protein mixtures were clearly distinguished from each other in the LDA plot, by proper arrangement of the order of ratios in the dimension of the first factor. Additionally, the mixture with high content of HSA (Lys/HSA=20/80) was located near the sample of pure HSA. The discrimination accuracy of 11 unknown samples was found to be 100% after training of the sensor array (**Table S9**). These results demonstrated that a potential ability of this platform would allow simultaneously semiquantitative analysis of a single protein concentration level as well as complex composition. Furthermore, HSA, had been considered as the marker of end stage renal disease [34], thus, the sensing system it could be a practical method for quantitation or detection of urinary human serum albumin.

4. Conclusion

In conclusion, we have developed a new continuously evolving colorimetric sensor array for protein recognition. By varying the molar ratios of two single-stranded oligonucleotides on AuNPs surface, differential receptors could be easily added to the electronic tongue, providing additional information and improved discrimination ability. Both pure proteins and mixed proteins could be discriminated by this sensor array. Twelve proteins spiked in human urine were identified. Furthermore, the sensor had the potential to simultaneously discriminate different concentrations of the same protein and the mixture of proteins at various molar ratios. Compared with the

traditional protein discrimination sensor arrays, our sensor array provided at least three obvious advantages: 1) the number of differential receptors was able to easily extend by adjusting the mixing ratios of two oligonucleotides; 2) the continuous evolving pattern analysis for target samples could be easily obtained and exclude abnormal signals; 3) Euclidean distance between samples could be regulated by the number of sensing elements. Additionally, the configuration of our sensor array was very simple. The novel sensor strategy presented in this study will contribute to the design of cheaper and more reliable sensor arrays for target samples.

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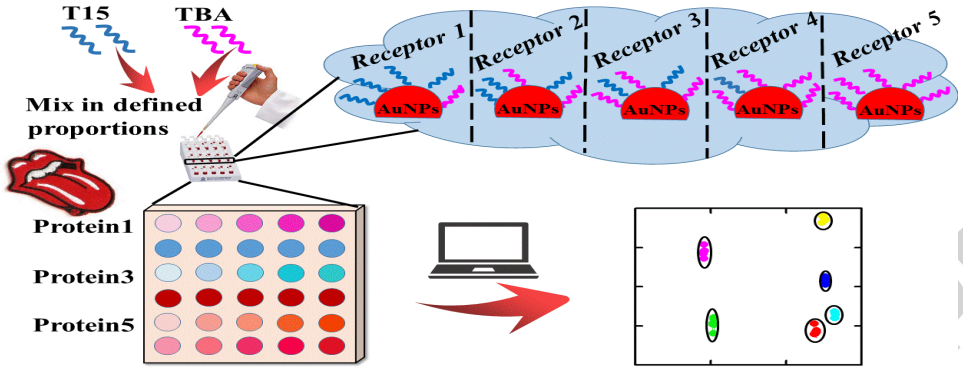

Scheme 1 Schematic illustration of continuous evolution pattern for protein recognition by using colorimetric sensor array based on AuNPs absorbed by TBA and T15 varying their molar ratios.

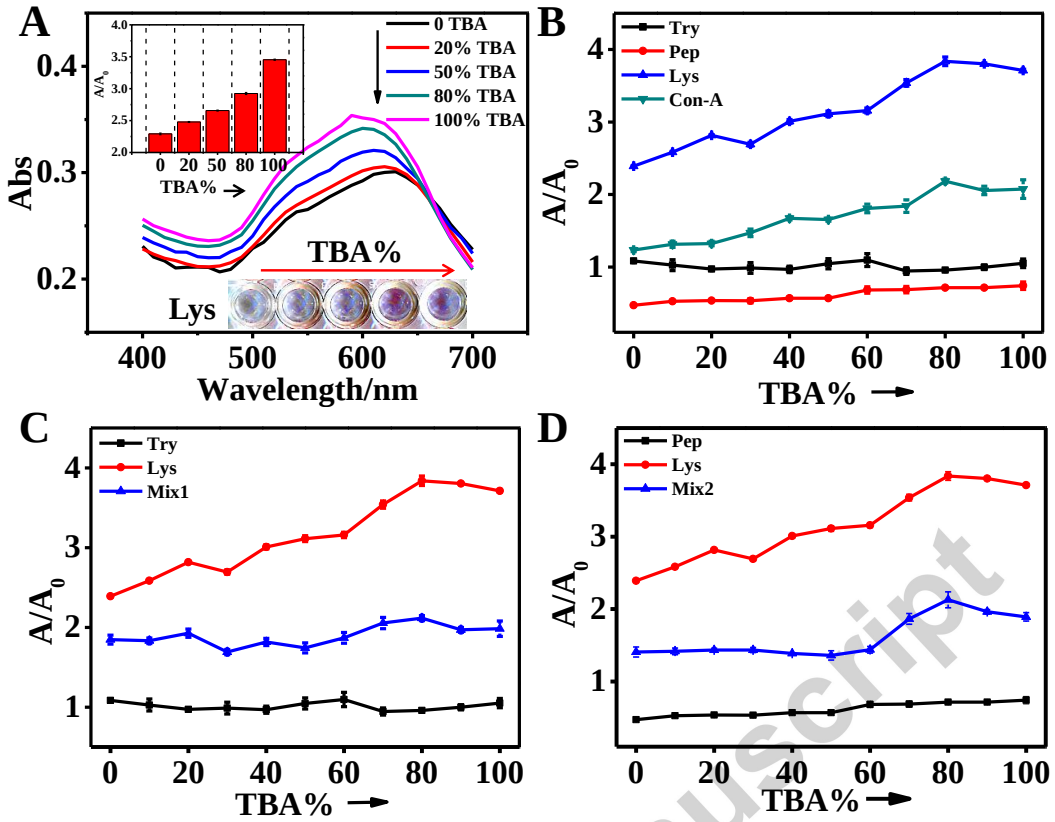

Fig. 1 (A) The absorbance spectra and colorimetric visualization of AuNPs-DNA with different content of TBA. The inset showed that response signal increased with the increasing molar ratios of TBA. The arrow indicated the direction of the TBA content change in sensing elements; Continuous response signal obtained from our colorimetric sensor array for pure proteins and mixtures; (B) Try, Pep, Lys and Con-A; (C) Try, Lys and their Mix1; (D) Pep, Lys and their Mix2. The final concentration of pure proteins and mixtures was 50 nM.

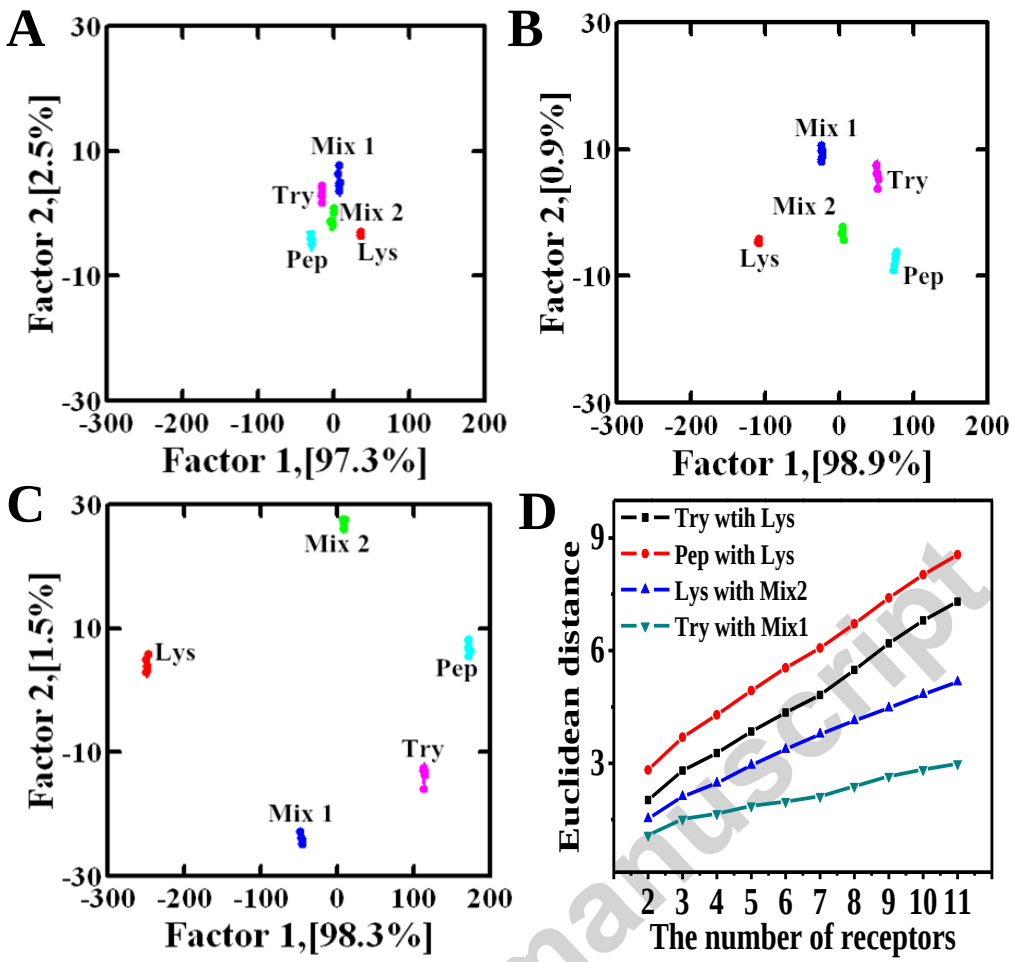

Fig. 2 LDA for pure and protein mixtures including Try, Pep, Lys, Mix1 (50% Try + 50% Lys), and Mix2 (50% Pep + 50% Lys). (A) three receptors with TBA 0, 50, and 100%; (B) six receptors with TBA 0, 10, 20, 50, 70, and 100%; (C) eleven receptors with TBA 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100%. In each case, a total protein concentration of 50 nM was employed. (D) plot of the Euclideance distance versus the number of receptors.

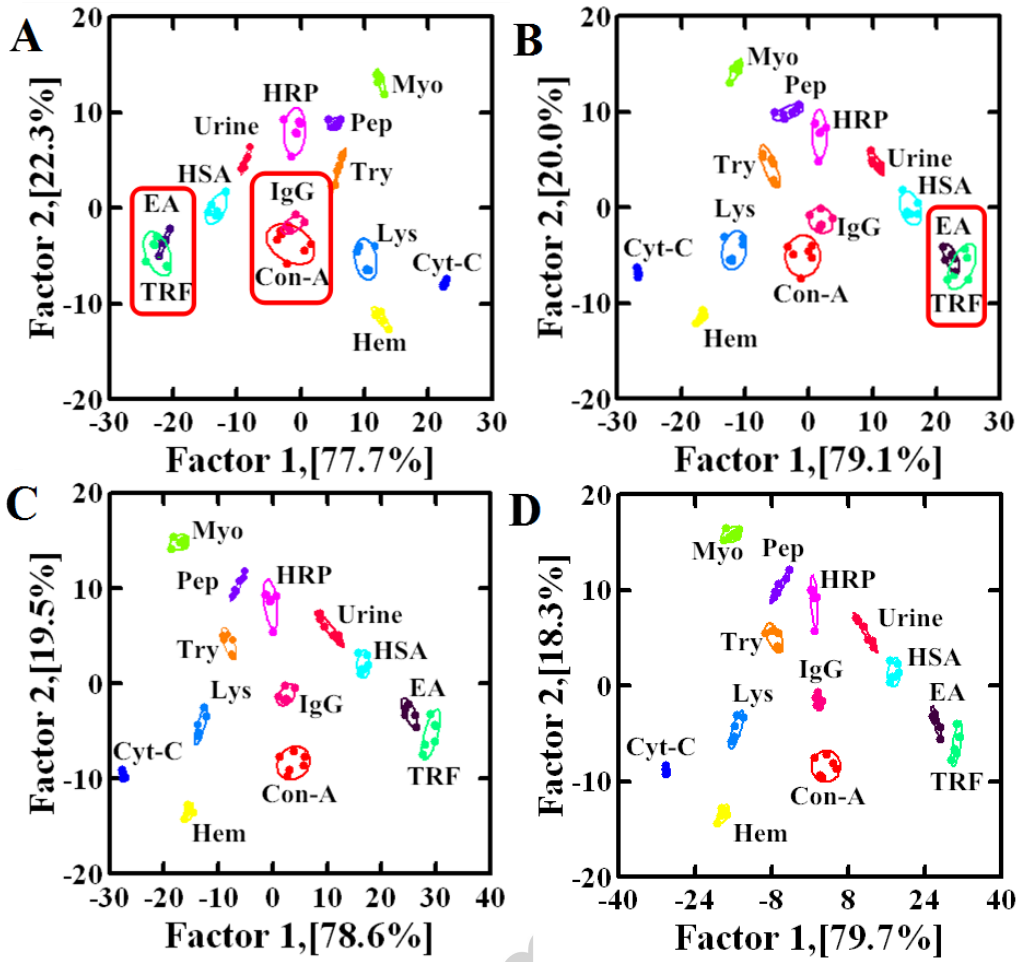

Fig. 3 Canonical score plot for the discrimination of proteins at the 200 nM level spiked in 50% human urine based on AuNPs-DNA conjugates (A) two transducers with TBA 0 and 100%; (B) three transducers with TBA 0, 50, and 100%; (C) four transducers with TBA 0, 20, 50, and 100%; (D) five transducers with TBA 0, 20, 50, 80, and 100%.

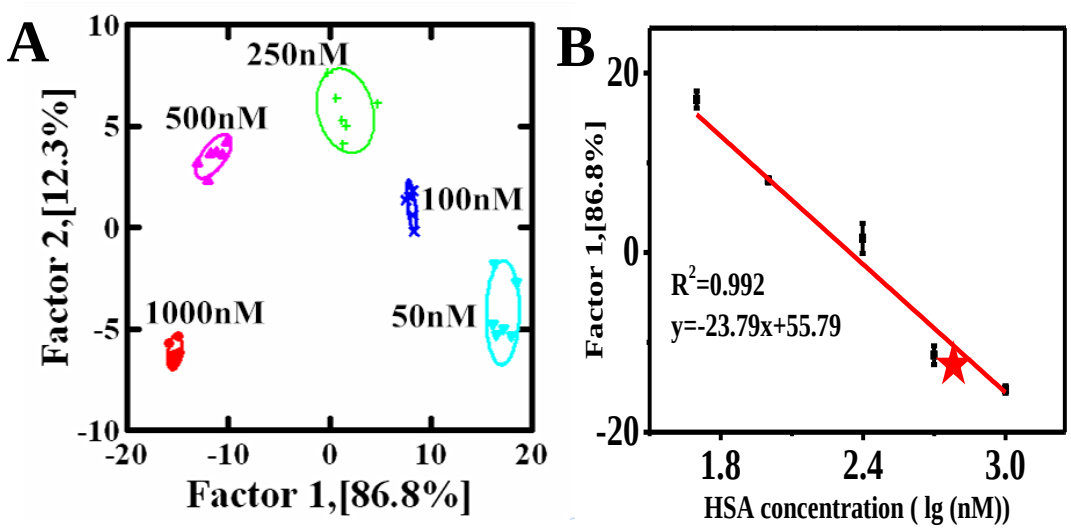

Fig. 4 Identification of proteins at various concentration levels by using the system in 50% human urine. (A) Canonical score plot for the response patterns obtained with the sensing array system against HSA at 50 nM, 100 nM, 500 nM, 1000 nM for six replicate detections. (B) Plot of the discriminant Factor (1) versus the logarithm of the HSA concentration. Red five-pointed star is on behalf of the unknown concentration of HSA.

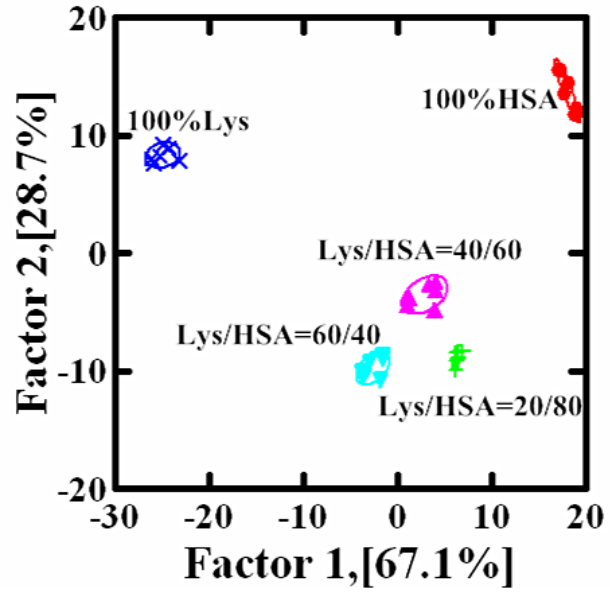

Fig. 5 Canonical score plot for discrimination mixtures of Lys and HSA at different molar ratios in 50% human urine. (total protein concentration: 400 nM)

Highlights

1. A unique continuously evolving colorimetric sensor array based on AuNPs decorated by two single-stranded oligonucleotides with different molar ratios for protein discrimination;
2. The number of differential receptors was able to easily extend by adjusting the mixing ratios of two oligonucleotides, the continuous response data of target samples against our sensing system could be easily obtained and exclude abnormal signals;
3. The sensing system could discriminate twelve proteins at the concentration of 200 nM in the presence of 50% human urine with accuracy of 100%, showing feasible potential for diagnostic applications.

In this study, we proposed a novel strategy to prepare differential receptors by simply mixing two kinds of DNA on gold nanoparticles surface, which could drastically reduce the design and synthetic efforts.