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Colorimetric Protein Sensing Using Catalytically Amplified Sensor Arrays **

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The detection of proteins at low concentrations is of critical importance in allergy testing,^[1, 2] clinical treatment,^[3, 4] and early diagnosis of many diseases,^[5, 6] requiring development of fast, sensitive, and cost-effective protein sensors aimed at point-of-care applications. Among these sensors, colorimetric methods provide both enhanced instrumental transduction as well as the potential for direct visual readout.^[7] For example, the clearly distinguished color shifts resulting from controllable aggregation of gold nanoparticles have facilitated simple color readout for biosensor applications.^[8]

Array-based sensing uses differential binding interactions with an array of selective receptors, providing an alternative to small molecule^[9]/biomarker^[10] detection. Colorimetric implementation of this methodology has created highly effective small molecule sensors that have been applied to a variety of important analytes.^[11] Application of colorimetric methods to array-based sensing of biomolecules is challenging, however, due to the low concentrations of these materials relative to the amount of signal required for colorimetric transduction. In recent studies we have used enzyme amplification for colorimetric detection of bacteria.^[12] While enzyme-based amplification processes are attractive, protein stability under transport and storage conditions limits the application of this strategy to highly controlled environments.^[13]

Synthetic enzyme mimics^[14] such as macromolecular complexes,^[15] iron oxide nanoparticles,^[16] and cerium oxide nanoparticles^[17,18] feature higher stability than enzymes over a wide range of pH levels and temperatures. The ability of these mimics to catalytically amplify responses has made them useful for biosensing. For instance, Fe₃O₄ NPs that mimic horseradish peroxidase (HRP) activity have been used to replace HRP in traditional immunoassay techniques^[16, 19] and to achieve label-free detection of nucleic acids.^[20] We report here the extension of this approach to array-based sensing of proteins, with Fe₃O₄ NPs serving as both transduction and recognition elements for highly sensitive (50 nM) detection and identification of proteins.

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For our sensing studies, dopamine functionalized Fe₃O₄ NP (Dop-Fe₃O₄ NP) and trimethylammonium functionalized Fe₃O₄ NP (TMA-Fe₃O₄ NP) were fabricated (Figure 1b-c) to afford diverse receptor profiles to probe the difference in protein surface hydrophobicity and size. Differential interactions of analyte proteins with these Fe₃O₄ NPs affected the accessibility of reaction substrates to the NP surface, causing concomitant alteration in catalytic efficiency (Figure 1a). Discern patterns were then generated with colorimetric readouts provided by the Fe₃O₄ NP-catalyzed oxidation of colorless 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), to a green product in the presence of hydrogen peroxide (H₂O₂).

Initial studies focused on optimizing the sensor response. An effective chromogenic response at 420 nm was observed using 25 µg/mL Fe₃O₄ NP with 2.5 mM H₂O₂ and 1 mM ABTS in 5 mM sodium acetate (CH₃COONa) buffer (pH 5.0). Changes in NP catalytic activity were then determined using two model proteins, lipase and lysozyme (Figure 2). In the case of lipase, a dramatic drop in reaction rate was observed, with complete inhibition observed at higher concentrations (50 nM, Figure 2a). By contrast, lysozyme did not significantly affect the colorimetric reaction up to a concentration of 100 nM (Figure 2b). The mechanism for protein differentiation was probed by dynamic light scattering (DLS) measurements of the protein-NP complexes using 10-fold higher concentration of particle and protein to provide reliable DLS signals. Lipase-NP complexes formed large aggregates with both Fe₃O₄ NPs (Figure 2a inset), resulting in intense protein absorption and complete blocking of substrate access to NP surface, consistent with the large change in activity observed. In contrast, no significant changes in particle size were observed after incubation with lysozyme (Figure 2b inset). The difference of activity and size changes between lipase (pI 5.6) and lysozyme (pI 11.0) revealed how surface charge affects protein-NP interaction affinity

Sensing of individual analytes in isolation provides a testbed for array-based sensors, providing insight into their ability to detect small changes in analyte structure. The ability of our sensor to discriminate proteins was tested using ten proteins featuring varying size and charge (Table 1). In the procedure, each protein solution was first mixed with NP (25 µg/mL) to a final protein concentration of 50 nM and incubated at room temperature for 30 minutes followed by the addition of H₂O₂ (2.5 mM) and ABTS (1 mM). After 15 minutes of incubation at room temperature, OD responses (OD/OD₀, where OD₀ is a control without addition of protein) at 420 nm were monitored.

As displayed in Figure 3b, the two particle sensor array generated a range of outputs. [21] As a general trend, proteins possessing lower pIs (α-Am, BSA, HSA, Lip) exhibited relatively stronger inhibition of the NP catalytic activity than those with higher pIs (Lys, CytC, ChT, Myo), indicating surface charge plays an important role in discrimination. Proteins with similar pIs also gave different signal patterns, however, indicating that protein size and surface hydrophobicity also play a role in protein-NP interactions. These protein surface and structural properties can be detected by proper regulation of sensor design. For example, ligand flexibility provided a preference for different sized proteins with similar pIs such as lipase and alkaline phosphatase (PhosB) (Figure 3b). The NP surface functionality was also a key element discriminating proteins with highly similar pIs and size, such as bovine serum albumin (BSA) and human serum albumin (HSA). Each protein can be reproducibly discerned by the OD response patterns. The pattern differences among analytes can be easily detected even by the naked eye (Figure 3a).

Linear discriminant analysis (LDA) was employed to quantitatively differentiate the OD response patterns of the Fe₃O₄ NPs with proteins. All five replicates of the ten proteins are grouped with 100% accuracy according to the jackknifed classification matrix,²² and scores

of the two factors are plotted with 95% confidence ellipses (Figure 3c). All clusters were separate from each other demonstrating the accurate identification of analytes and notably overlapped replicates showed high precision. In the canonical score plot, the distribution of analyte clusters along the factor 1 axis primarily reflects the OD/OD₀ value. The individual response from each of the elements provides reasonable differentiation: TMA-Fe₃O₄ NP itself can differentiate the ten proteins with 90% accuracy and Dop-Fe₃O₄ NP with 78%. It is noteworthy that by the use of only two modified Fe₃O₄ NPs, the sensor can detect and identify ten proteins rapidly and effectively. The detection efficiency was further validated by the identification of unknown samples using the training set in Figure 3c with 95% accuracy (76 of 80) (Table S2).

In summary, we have developed a new platform for sensitive, fast, and effective colorimetric identification of proteins that uses a catalytically active particle for both recognition and signal transduction/amplification. By tuning the surface functionalities, the Fe₃O₄ NPs were able to differentiate ten proteins at a concentration of 50 nM, substantially lower concentrations than prior colorimetric array-based protein sensors (10–350 μM).²³ The robust nature of these synthetic enzyme mimics gives this strategy the potential to provide real-world sensors for both clinical and non-clinical health applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

a. Illustration of the Fe_3O_4 NP enzyme mimetic amplified colorimetric sensing of proteins.

b. Structures of Dop- Fe_3O_4 and TMA- Fe_3O_4 . **c.** Hydrodynamic diameter and zeta potential of Fe_3O_4 NPs in 5 mM CH_3COONa buffer at pH 5.0.

Figure 2.

Normalized OD at 420 nm upon protein addition with fixed NPs concentration (25 µg/mL).

a. Concentration dependent OD curve of lipase. Inset is the hydrodynamic diameter of functionalized Fe₃O₄ NPs and lipase-NP complexes. **b.** Normalized OD curve of lysozyme with modest permutation. Inset: hydrodynamic diameter of functionalized Fe₃O₄ NPs and lysozyme-NP complexes. Results are average of three measurements and error bars are standard deviation. The NP concentration used in the size measurement is 250 µg/mL and the protein is at a concentration of 500 nM.

Figure 3.

Array-based sensing of ten proteins. **a.** Photograph of the color change upon addition of protein solutions at 50 nM. **b.** OD response (OD/OD₀ at 420 nm) patterns in the presence of proteins at 50 nM (responses are an average of five measurements and the error bars are the standard deviation). **c.** Canonical score plot for the OD response patterns as obtained from LDA with 95% confidence ellipses.

Table 1

Physical properties of protein analytes.

Protein	MW (kDa)	pI
α -amylase (α -Am)	50	5.0
bovine serum albumin (BSA)	66.3	4.8
α -chymotrypsin (ChT)	25	8.7
cytochrome c (CytC)	12.3	10.7
transferrin (Tf)	76	5.9
human serum albumin (HSA)	69.4	5.2
lipase (Lip)	58	5.6
lysozyme (Lys)	14.4	11.0
myoglobin (Myo)	17	7.2
alkaline phosphatase (PhosB)	140	5.7