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# Enzyme Amplified Array Sensing of Proteins in Solution and in Biofluids

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## Abstract

We have developed an enzyme-nanoparticle sensor array where the sensitivity is amplified through enzymatic catalysis. In this approach cationic gold nanoparticles are electrostatically bound to an enzyme (β-galactosidase, β-Gal), inhibiting enzyme activity. Analyte proteins release the β-Gal, restoring activity and providing an amplified readout of the binding event. Using this strategy we have been able to identify proteins in buffer at a concentration of 1 nM, substantially lower than current strategies for array-based protein sensing. Moreover, we have obtained identical sensitivity in studies where the proteins are spiked into the complex protein matrix provided by desalted human urine (~1.5 μM total protein; spiked protein concentrations were 0.067% of the overall protein concentration), demonstrating the potential of the method for diagnostic applications.

## Introduction

Irregular protein concentration levels in biofluids, e.g. serum, urine, and saliva provide essential information for the early diagnosis of many pathological conditions such as hypoalbuminemia,[1] cancers,[2] Alzheimer's disease,[3] prostatisis,[4] HIV,[5] and other disease states.[6] The development of strategies for monitoring protein levels remains a major issue in medical diagnostics, pathogen detection, and proteomics.[7] Substantial efforts have been devoted to develop precise and efficient methods for protein sensing,[8] including enzyme-labeled immunoassays,[9] electrophoresis methods,[10] and analytical techniques.[11]

The "chemical nose/tongue" approach[12] presents a potential alternative to specific recognition and separations techniques. In this approach, a sensor array is generated to provide differential interaction with analytes via *selective* receptors, generating a stimulus response pattern that can be statistically analyzed and used for the identification of individual target analytes[13,14] and also analysis of complex mixtures.15 Over the past few years, this technology has been successfully applied for protein detection using array-based approaches, including porphyrins,

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**Supporting Information Available**. Experimental section, synthesis of ligands, synthesis of gold nanoparticles, $^{13}$C NMR and $^{1}$H NMR spectra, fluorescence titration curves, training matrix, protein purification, gel electrophoresis, zeta potential, and dynamic light scattering. These materials are available free of charge via the Internet at http://pubs.acs.org.

16 oligopeptide-functionalized resins,17 and polymers.18 In a real world example, a single functional conjugated polymer poly(thiophene) has been successfully applied as a food freshness sensor to detect biogenic amines in fish associated with food poisoning (e.g. histamine) with increasing concentrations from 22.5 μM (2.5 ppm) to 4.5 mM (500 ppm) to build a fish matrix.19 Recently, we have developed nanoparticle-GFP based "chemical nose" strategy for protein detection in biofluid that is highly sensitive (500 nM)20 as compared to other reported similar approaches (1–350 μM).16,17,18c,d We have also developed a sensor array composed of gold nanoparticles and fluorescent polymers that can identify proteins,21 bacteria,[22] and cancerous cells[23] through a fluorophore-displacement mechanism. This sensor array achieved detection limits of 215 nM for low $M_w$ proteins.

The increased sensitivity required for many diagnostic uses[24] presents a challenging goal for array-based sensors because the detection process generally relies on fluorescence responses that are restricted by the inherent emissivity of the fluorophores used. To overcome this limitation, we have explored the use of enzymes to provide array-based sensors with enhanced sensitivity. In this Enzyme Amplified Array Sensing (EAAS) approach, the sensitivity of the array is amplified through an enzymatic reaction. This approach couples the signal amplification process of ELISA with the versatility of the "chemical nose" approach. We report here the use of this method to sense and identify a range of biomedically relevant proteins at 1 nM in both buffer and desalted human urine.

Our EAAS features three components: a) β-galactosidase (β-Gal) as the enzyme, b) 4-methylumbelliferyl-β-D-galactopyranoside (MUG) as a fluorogenic substrate to provide "turn on" sensing, and c) gold nanoparticles (AuNPs) as the receptors to provide differential protein affinity, and hence discrimination. In practice, cationic AuNPs electrostatically bind the anionic β-Gal, inhibiting the enzyme without denaturation.[25] Displacement of the particle by analyte proteins restore β-Gal activity, generating a fluorescent readout signal (Figure 1) that is amplified through enzymatic catalysis.

## Results and Discussion

The anionic tetrameric enzyme β-Gal (17.5×13.5×9 nm, pI = 4.6, $M_w$ = 465 kDa), was chosen as the amplifying element due its stability to a wide range of temperature, pH, and ionic strength conditions.26,[27] Gold nanoparticles (~2 nm core diameter) with a positive surface charge were used to bind efficiently to the anionic β-Gal through electrostatic complementary and electrostatic charge interactions (see Figures S29, S31, and S33 for zeta potential and DLS measurements). These particles feature a large surface area with size comparable to that of proteins, allowing these systems to mimic protein-protein surface interactions,[25] an excellent staring point for sensor design. These AuNPs feature a tetraethylene glycol unit in the ligand shell to minimize the denaturation of the bound enzyme/analyte protein and variable terminal functionality to generate the differential affinity required for sensing (Figure 2, Figure S29, and Table S7).[28]

As a starting point, we focused on the optimization of the binding ratio between **AuNP**s (**NP1–NP6**) and β-Gal through inhibition activities in phosphate buffer. We conducted an activity assay of β-Gal-catalyzed hydrolysis at various concentrations of nanoparticles (see Figure S26). Typically, a concentration of 0.5 nM of β-Gal in phosphate buffer solution (5 mM, pH = 7.4) was incubated with various concentration of **NP1–NP6** for 30 minutes and 1 mM of the fluorogenic substrate (MUG, $\lambda_{exc}$ = 455 nm) was added to **AuNP**-enzyme complexes for the inhibition and enzyme-substrate reaction studies. As a control, the enzyme inhibition was also studied using neutral tetra(ethylene glycol) functionalized nanoparticles. The normalized first-order rate of fluorogenic substrate hydrolysis was plotted versus the ratio of nanoparticles to β-Gal, and showed a tendency to decrease upon addition of nanoparticles, as shown for **NP2**

in Figure 3. This result clearly indicates that activity of β-Gal is inhibited by nanoparticle binding. This inhibition of β-Gal activity depends on subtle structural changes of peripheral ligands on the **AuNP**s with the linear end group (**NP1**) exhibiting less suppression than a branched isomeric structure (**NP2**). Control experiments with non-charged NPs ($\mathbf{NP_{TEG}}$) were carried out and no interaction or inhibition of the enzyme was observed (see Fig. S35).

A total of nine proteins of various sizes, surface charges, molecular weights, and isoelectric points were chosen to test generality and limitations of our sensor, (Table 1, see Table S6 for zeta potentials, $r_h$ values and extinction coefficients at 280 nm of the analyte proteins). Fluorogenic substrate hydrolysis for the β-Gal/**AuNP** conjugates against individual proteins in buffer is summarized in Figure 4. The individual target proteins generated distinguishable and highly reproducible rates of the fluorogenesis, indicating the potential for protein discrimination.

All proteins were tested using a fluorescence displacement assay of six β-Gal/**AuNP** assemblies array for six replicate measurements, providing a data set as a 6 × 6 × 9 matrix. The resulting data was analyzed through linear discrimination analysis (LDA) using SYSTAT software (version 11)[29] and transformed into five canonical factors. This statistical analysis method is used to recognize the linear combination of features that differentiate two or more classes of objects or events. The five canonical factors contain 42.2%, 34.1%, 12.1%, 5.8%, and 4.9% of the variation, respectively. The canonical score plot of the first three factors is presented in Figure 5, where each dot represents the fluorescence response pattern of a single protein target to the β-Gal/**AuNP** sensor array. The canonical plot reveals nine distinct clusters corresponding to individual target proteins that give rise to a 100% classification accuracy obtained from a jackknifed matrix in LDA. This result demonstrates that the β-Gal/**AuNP** sensor array is sensitive enough to differentiate target proteins in the 1 nM range, significantly more sensitive than prior methods (1–350 μM),[16,17,18c,d] including our previous fluorescent polymer-nanoparticle conjugates system (215 nM for the low $M_w$ proteins).[21]

The high sensitivity of our β-Gal/**AuNP** sensor array can be attributed to signal amplification through the enzyme-substrate reaction of β-Gal. Significantly, the same training matrix analyzed using only one nanoparticle structure gives rise to classification accuracies of 33%, 44%, 37%, 31%, 44%, and 35% for **NP1** to **NP6** respectively, indicating almost equal ability of each particle to discriminate between protein targets (Table S3).

To investigate the robustness identification accuracy of the β-Gal/**AuNP** sensor array, we prepared sixty unknown protein samples at 1 nM randomly chosen from the training set for identification. The fluorescence response patterns obtained for each unknown against the sensor array were analyzed through LDA analysis. The resulting patterns were classified through the canonical score plot by the first two factors of simplified fluorescence patterns based on the Mahalanobis distances of unknowns to the centroid of the respective protein clusters in the canonical score plot. An identification accuracy of 92% (55 correct out 60) demonstrates reproducibility of our enzyme-nanoparticle sensor system for identification.

Sensing of proteins in real world biofluids such as protein in human urine provides a far more demanding test than sensing in simple buffer solutions. The overall protein content (>1.5 μM, 0.150 g/L) and the multianalyte nature of the human urine (>1500 proteins as competing biomolecules) generate a complex matrix that is challenging for sensor design.[30] An additional complication is variation in ionic strength, an issue that is addressed biomedically through desalting using spin column chromatography. We employed this technique in our studies but we are aware that as with current analytical methods this desalting adds an additional step to the analytical procedure. (see Figures S27 and S28). [30,31]

The complexation between β-Gal and cationic AuNPs in desalted human urine (Bioreclamation Inc.) was determined by the hydrolysis of MUG by β-Gal in the presence of various concentrations of AuNPs (Figure 6, additional information see Figures S34 and S35). In this experiment, β-Gal was dissolved in desalted human urine (~1.5 μM, see SI for details) buffered to pH = 7.4 using 5 mM phosphate buffer. This solution was then equilibrated with a stoichiometric amount of nanoparticles for 15 min. Then, an excess amount of the MUG solution (1 mM) was added to initiate the enzymatic reaction. The activity of β-Gal was directly correlated with the AuNPs concentration, indicating that the activity of β-Gal is inhibited by AuNPs complex formation.

Using the optimized conditions (*vide supra*), it was established that 1 nM concentration of spiked proteins was required for reproducible differentiation of the target analytes (Table 1). As before, we created a training matrix (six β-Gal/**AuNP** adducts × nine proteins × six replicates) with β-Gal/**AuNP** adduct and each of the proteins. Each protein in the human urine protein solution generated a distinct fluorescence response. The rates of fluorogenic substrate hydrolysis for β-Gal/**AuNP** pair in the presence of individual protein analytes are summarized in Figure 7, showing that **NP1** and **NP4** exhibit stronger affinity for β-Gal than for other proteins, producing smaller hydrolysis rates and less fluorescence response. As before, this fluorescent response pattern was subjected to further LDA analysis producing a 6 × 6 × 9 matrix. This matrix was transformed into five canonical factors. The five canonical factors contain 62.3%, 20.7%, 9.1%, 4.3%, and 0.9% of the variation, respectively.

The canonical score plot of the first three factors is presented in Figure 8, where each dot represents the fluorescence response pattern of a single protein target to the β-Gal/**AuNP** sensor array. The canonical plot reveals nine distinct clusters corresponding to individual target proteins, giving rise to a 100% classification accuracy based on the jackknifed matrix in LDA. This result demonstrates that the β-Gal/**AuNP** sensor array is sensitive enough to differentiate each of the target proteins at 1 nM in the biofluid matrix (0.067% of the total protein content in urine), comparable with the preliminary study carried out in buffer. This sensitivity is improved as described before $4\text{-}2\times10^2$-fold in comparison with simple fluorophore displacement[21] and it is also comparable with the preliminary study carried out in buffer. The particles in this study are well suited for differentiation: the same training matrix analyzed using a single nanoparticle gives rise to classification accuracies of 33%, 52%, 41%, 43%, 48%, and 31% from **NP1** to **NP6**, respectively (Table S10). This indicates almost an equal contribution of each particle in the discrimination of the examined protein targets.

The accuracy of the β-Gal/**AuNP** sensor array was validated by identifying unknown proteins in the competitive environment of desalted human urine protein solution. Sixty unknown protein solutions spiked at 1 nM were chosen arbitrarily from the training set. The fluorescence response patterns were newly analyzed through LDA analysis, and further classified by the Mahalanobis distances of unknowns to the centroid of the respective protein clusters in the canonical score plot. This process identified 55 out of 60 unknowns correctly, corresponding to a 92% identification accuracy, demonstrating both the feasibility and reproducibility of our enzyme-nanoparticle sensor system.

## Conclusions

In this study, we have demonstrated that the use of enzymatic amplification dramatically increases the sensitivity of the array-based sensing of proteins. Using this EAAS method, we rapidly and reproducibly sensed proteins at concentrations of 1 nM in both phosphate buffer and desalted human urine. These studies demonstrate that sensing can be achieved with high sensitivity in a complex biomatrix, providing an important first step for the creation of array-based biosensors for real-word diagnostic applications. In our ongoing studies, we are

exploiting both new alternative approaches for protein detection and new data analysis strategies to apply this methodology to more complex matrices featuring a large diversity of target analytes.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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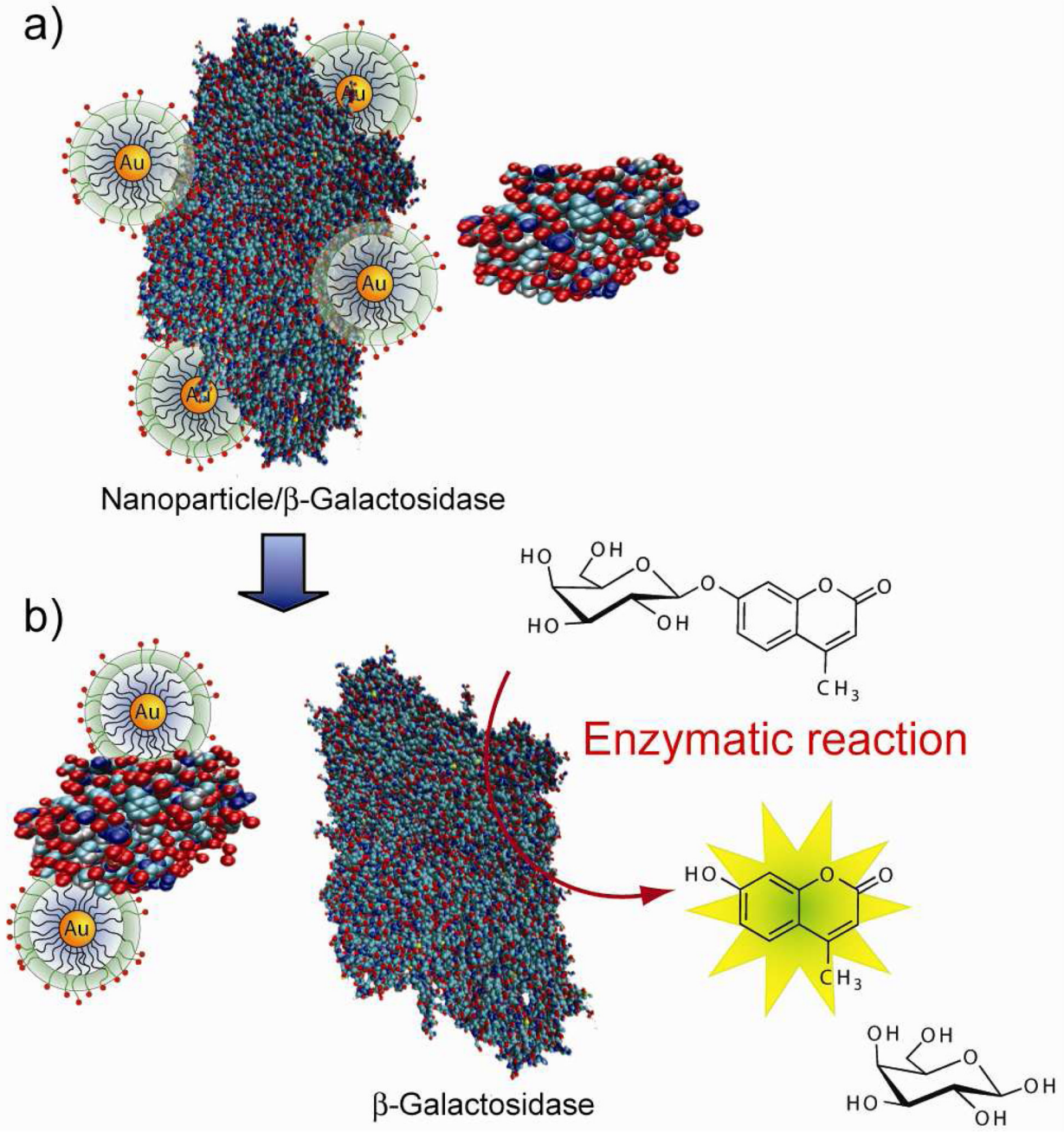


**Figure 1.**

A schematic representation of sensors comprised of β-galactosidase (β-Gal) and cationic **AuNP**s. In a) supramolecular adducts of β-Gal and **AuNP** formed through complementary electrostatic interactions, inhibiting the enzymatic activity of β-galactosidase. As shown in b) β-galactosidase is displaced from the β-Gal/**AuNP** complex by protein analytes, restoring the catalytic activity of β-Gal towards the fluorogenic substrate 4-methylumbelliferyl-β-D-galactopyranoside, resulting in an amplified signal for detection.

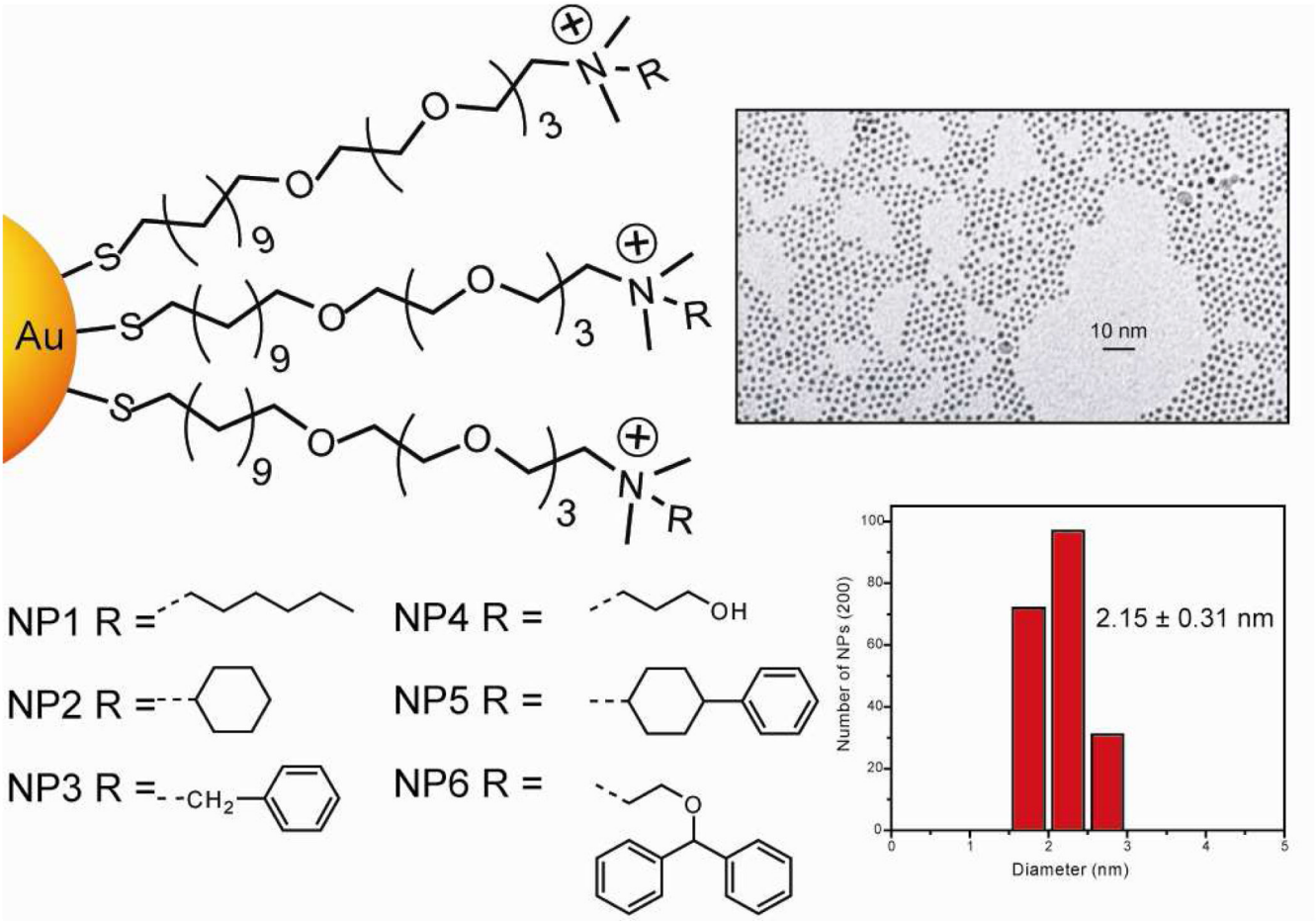


**Figure 2.**

**Structure features of the cationic gold nanoparticles (NP1–NP6)**. The transmission electron microscopy (TEM) and histogram plot show the morphology, monodispersity, and sizes of the metallic core gold nanoparticles.

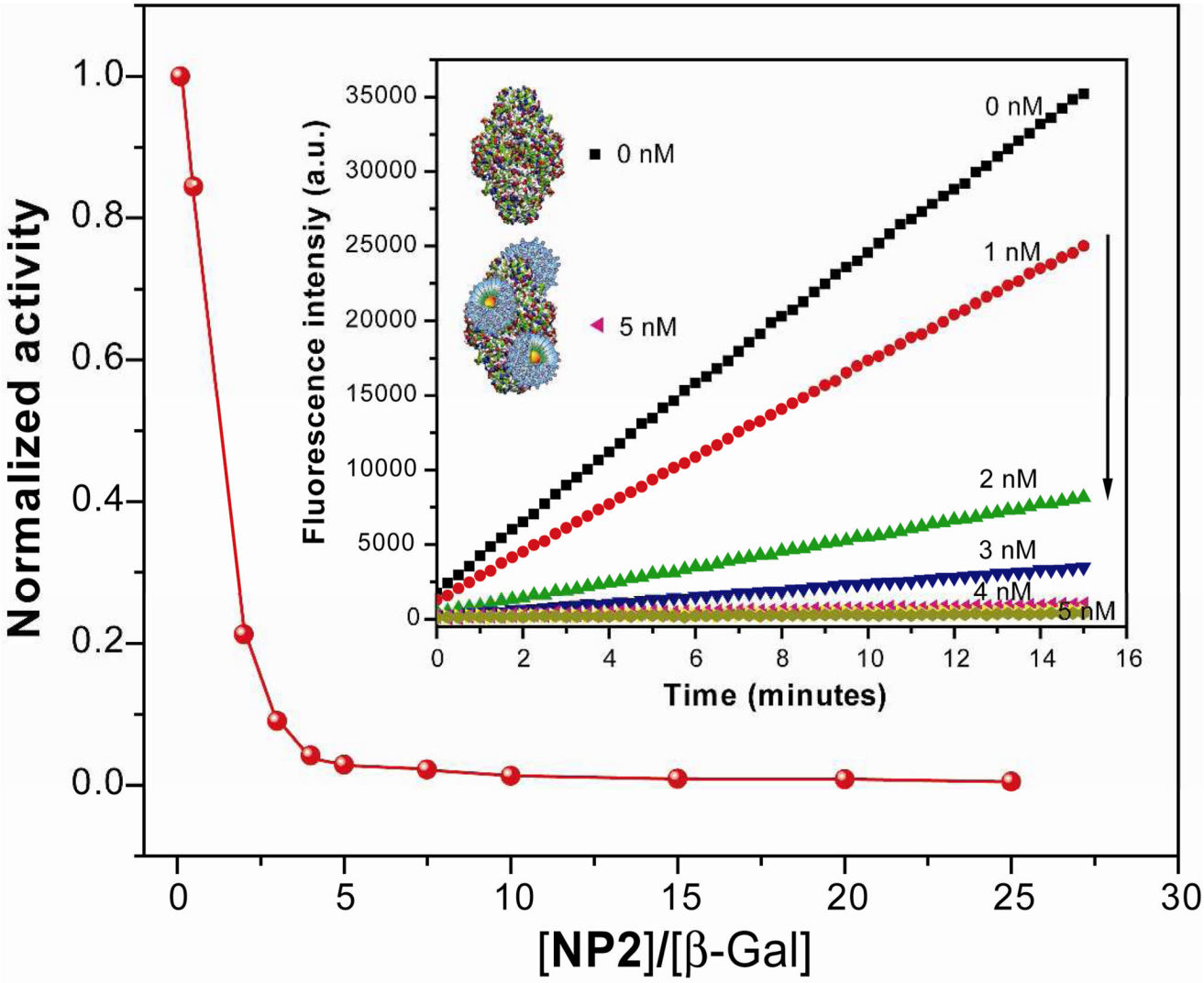


**Figure 3.**

Normalized inhibition activity of β-Gal (0.5 nM) against 1 mM substrate MUG upon addition of cationic **NP2** in 5 mM phosphate buffer. The inset shows the kinetics of the fluorescence spectra before and after addition of **NP2**. The arrow in the inset indicates the direction of activity (0 nM indicates free enzyme and 5 nM indicates inhibited enzyme with NPs).

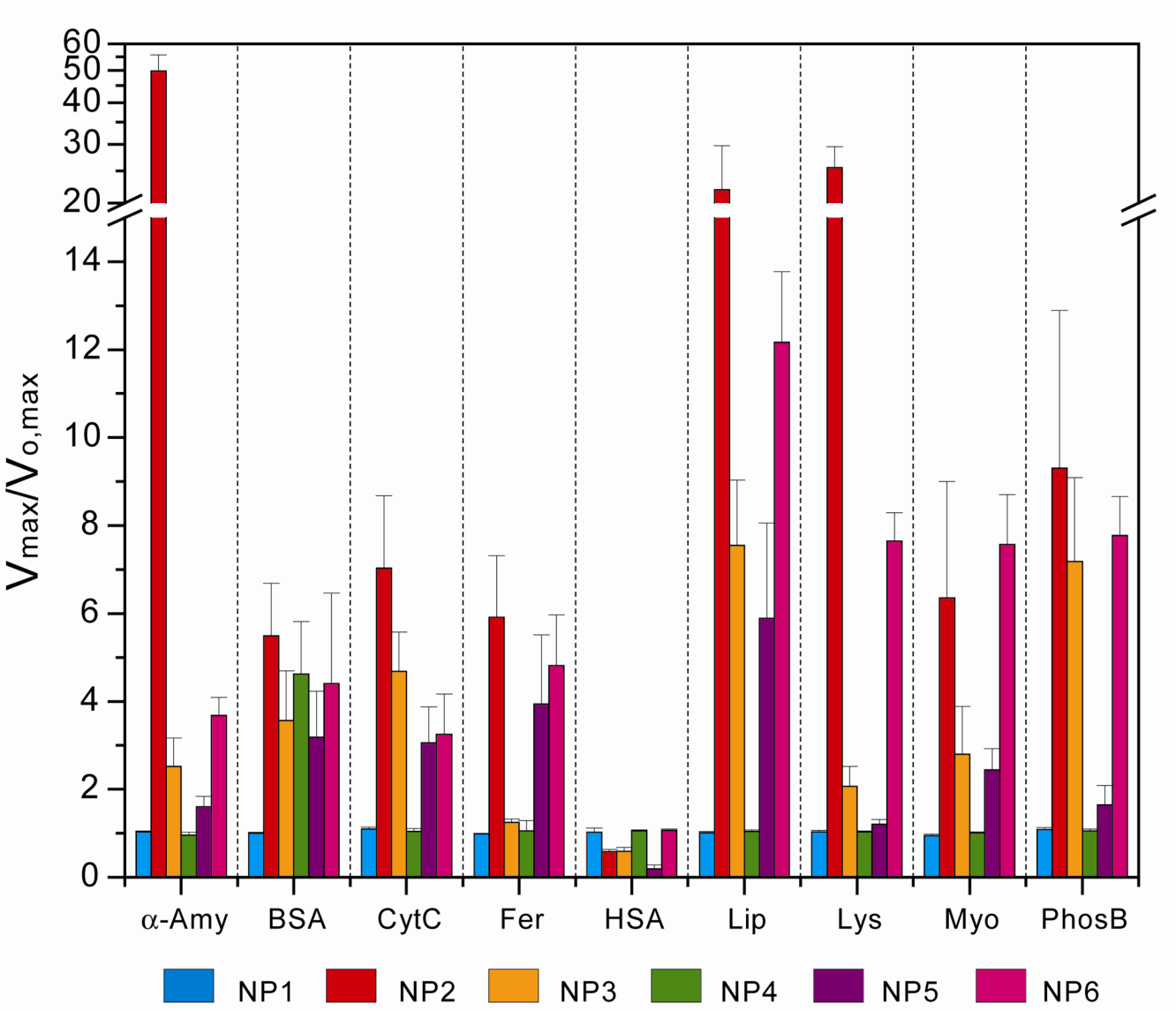


**Figure 4.**

Fluorescence response patterns ratio of β-Gal and six **AuNP** adducts against various target proteins. Each value represents an average of six parallel measurements with standard deviation.

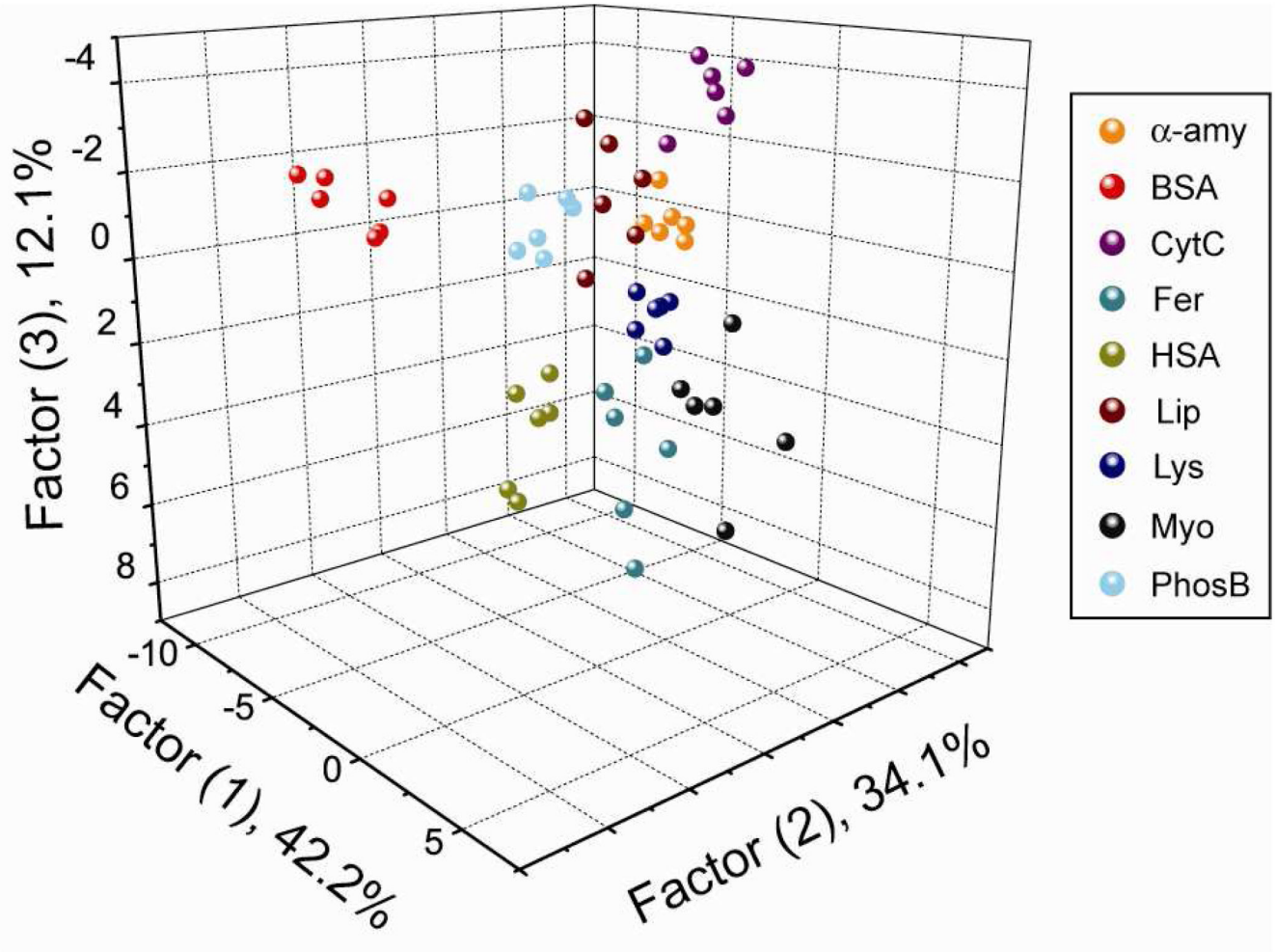


**Figure 5.**
Canonical score plot of the first three factors of fluorescence response patterns obtained through β-Gal/**AuNP** sensor array against nine target proteins in 1 nM concentration.

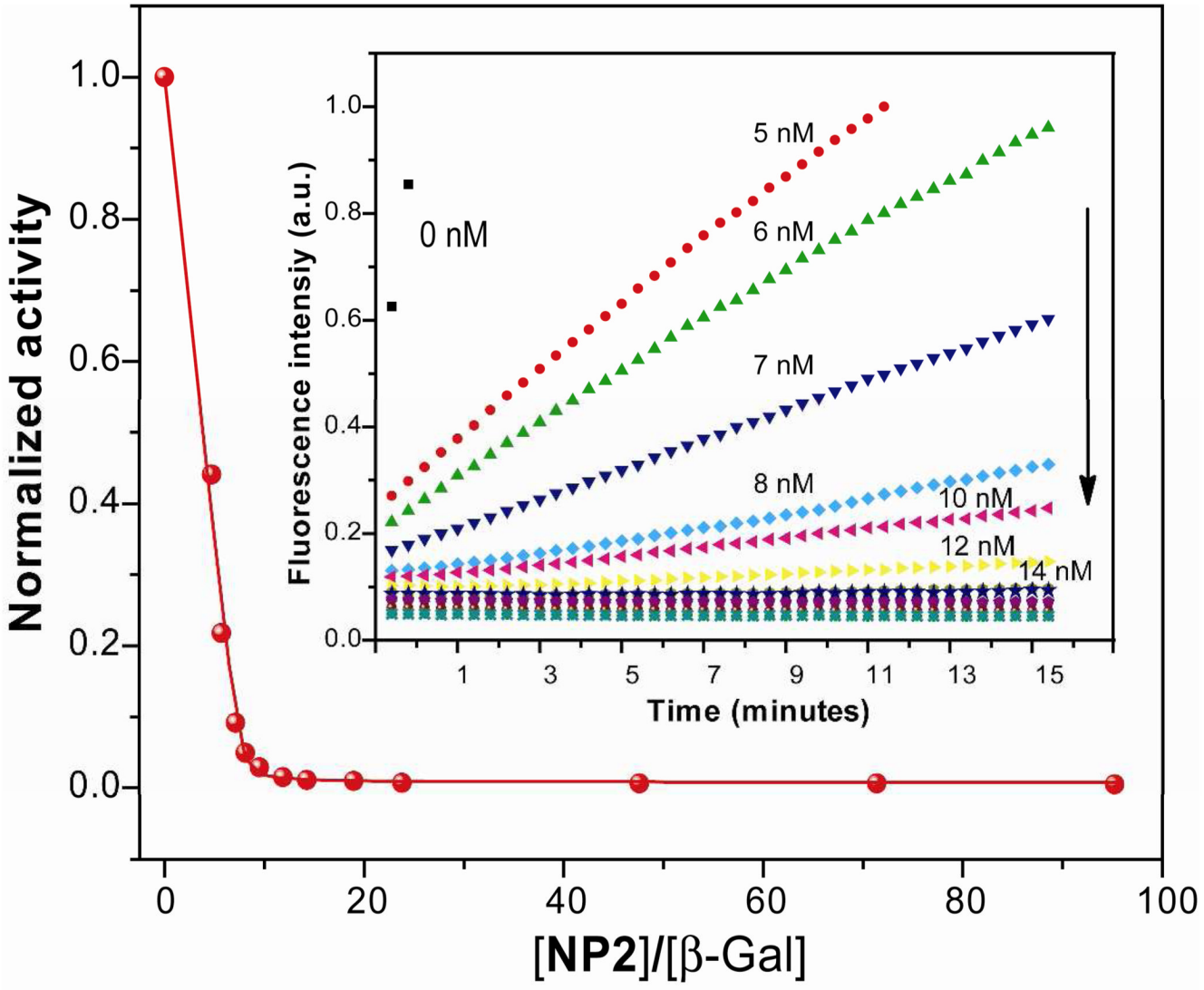


**Figure 6.**

Normalized inhibition activity of β-Gal (0.5 nM) against 1 mM substrate MUG upon addition of cationic **NP2** in the presence of desalted human urinary proteins. The inset shows the kinetics of the fluorescence spectra before and after addition of **NP2**. The arrow in the inset indicates the direction of activity.

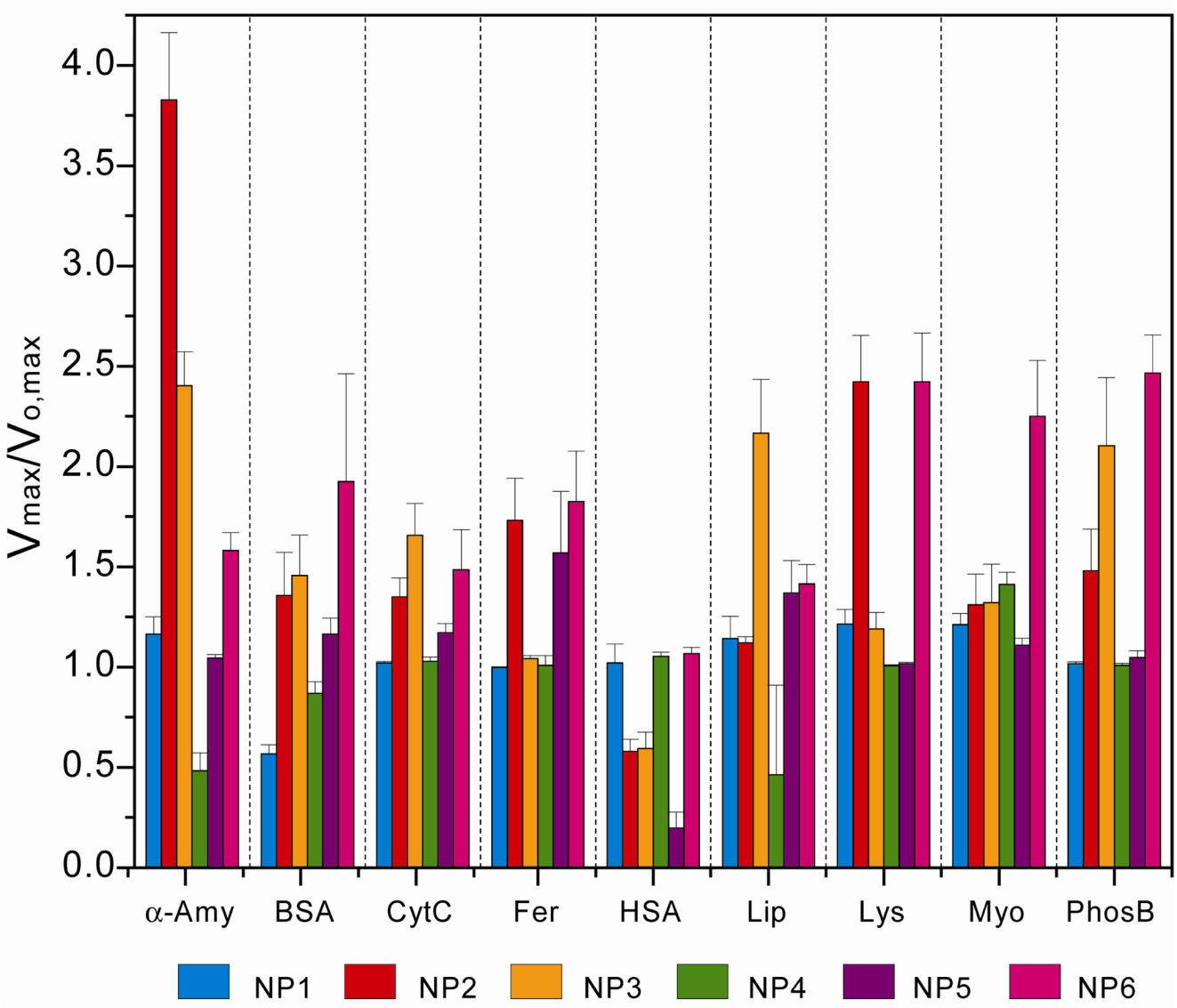


**Figure 7.**
Fluorescence response patterns ratio of β-Gal and six **AuNP** adducts against various target proteins. Each value represents an average of six parallel measurements.

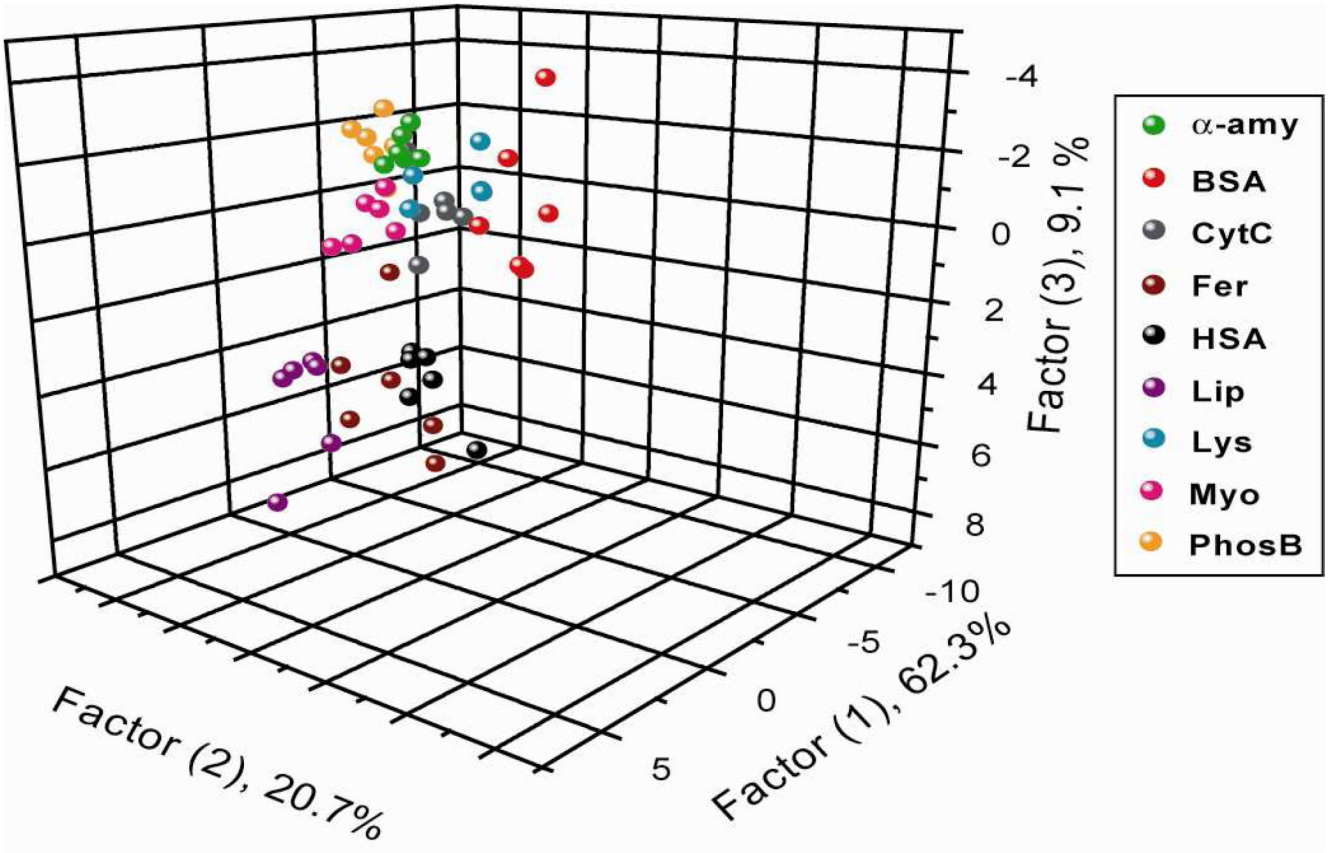


**Figure 8.**
Canonical score plot of the first three factors of fluorescence response patterns ratio obtained through β-Gal/**AuNP** sensor array against nine target proteins at 1 nM in desalted human urine (~1.5 μM total protein content).

**Table 1**

Physical properties of the proteins used as sensing targets in phosphate buffer solution at pH 7.4.[18a]

| Protein‡ | Mw (kDa) | pI |
|---|---|---|
| *α-Amylase (α-Am)* | 50.0 | 5.0 |
| Bovine serum albumin (BSA) | 66.3 | 4.8 |
| Cytochrome *c* (CytC) | 12.3 | 10.7 |
| *Ferritin (Fer)* | 750.0 | 4.5 |
| *Human serum albumin (HSA)* | 69.4 | 5.2 |
| *Lipase (Lip)* | 58.0 | 5.6 |
| *Lysozyme (Lys)* | 14.4 | 11.0 |
| *Myoglobin (Myo)* | 17.0 | 7.2 |
| *Alkaline phosphatase (PhosB)* | 140.0 | 5.7 |

‡Proteins in *italics* are commonly found in human urine.