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Using Metal Nanoparticles as a Visual Sensor for the Discrimination of Proteins

Haiyan Liu[a], Lin Ma[a], Shenghao Xu[a], Wenhao Hua[b] and Jin Ouyang*[a]

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Protein discrimination is increasingly studied because of its roles in biological chemistry. In this study, we introduced a visual sensor using the metal nanoparticles (NPs) Au, Ag, Cu, Ni and Co to determine the fluorescence (FL) intensity and colour changing patterns to identify ten types of proteins in polyacrylamide gel. The synthesised NPs have little to no fluorescence, and the addition of proteins immobilised by the gel could differentially enhance the FL intensity and change the FL colour of the NPs, thereby producing FL recognition patterns. Based on the different FL enhancement indices of metal NPs with proteins, linear discriminant analysis (LDA) is applied to differentiate the proteins. By altering the synthesis method of metal NPs, a sensor with different FL patterns for protein discrimination is created. In addition, we have applied this method to detect the protein mixtures after one-dimensional (1-D) polyacrylamide gel electrophoresis (PAGE), and to evaluate the serum samples from patients with liver diseases, thalassemia and healthy people. This protein discrimination method is rapid, cost-effective and visual, and it has a "turn on" effect for metal NPs, which may be extended to other proteins or more complex bioanalytes.

Introduction

The discrimination and detection of proteins has recently garnered attention because of its importance in the characterization of pathological conditions and the preparation of biological products.[1-6] Many methods are available for protein detection, such as enzyme-linked immunosorbent assay (ELISA),[7-9] electrophoresis,[10-12] chromatography,[13-15] and mass spectrometry (MS).[16-18] ELISA is utilised to identify certain proteins based on specific antibody-antigen binding, and it has high affinity and specificity. Electrophoresis is used to separate proteins according to the differential migration rate of charged molecules, and the proteins are detected by imaging methods, such as CBB-R250 staining, silver staining and fluorescence imaging. Chromatography is an effective method to separate and detect proteins based on their differences in physicochemical properties, such as partition and adsorption performances. MS is applied to analyse proteins by the charge/mass ratios. These methods all have high selectivity and great sensitivity for the discrimination of proteins in complex biological samples. However, they may require expensive biomarkers, tedious pre-treatment of samples, skilled operators or sophisticated instrumentation, which limits their use in simple and rapid discrimination of proteins.

Sensors and sensor arrays have emerged as a powerful tool for the rapid analysis of biomolecules, which can realize the selectivity and sensitivity of detection through pattern recognition.[19-26] Several successful protein sensors have been reported, for instance, Huo's group used porphyrin, porphyrin derivatives (mainly metalloporphyrins) and chemically responsive dyes as sensing elements for protein identification,[19] Wang and co-workers applied giant magnetoresistive (GMR) sensor arrays for the quantification of protein interactions,[20] Sukwattanasinitt's group set up a fluorescent sensor array using variously charged dendritic phenylene-ethynylene fluorophores for the discrimination of proteins,[21] Rotello's group and Zhang's group expanded 'chemical nose' sensors for protein discrimination using different nanoparticles (NPs).[22-26] Among these methods, fluorescence technique has been widely applied to sensors for sensitive and selective detection, however the application of colorimetric methods remains challenging for visual detection.

Here, we report a novel "turn on" sensor consisting of colloidal metal NPs for protein discrimination, which can provide different fluorescence (FL) intensity and colour changing patterns to realize the selectivity and visuality. The five colloidal metal NPs, Au, Ag, Cu, Co and Ni are protected by poly(N-vinyl-2-pyrrolidone) (PVP), and are synthesised to discriminate among ten proteins (basic properties are provided in Table S1). The FL of the metal NPs could show various degrees of intensity-enhanced and red-shifted when mixed with different proteins immobilised by polyacrylamide gel, which is the basis of our assay. The approach described here has the following advantages: (1) a dual-readout assay based on fluorescence intensity and colour changes, which is selective and visual; (2) it is simple to operate and cost-effective, and only requires the synthesis of metal NPs and immersion of the protein-in gel in the NPs solution; (3) biocompatibility and hypotoxicity of the metal NPs;

and (4) no background interference will be produced because the metal NPs are weakly emissive without the addition of proteins immobilised in gel. Based on these characteristics, our sensor may have a great potential in the field of analytical biochemistry.

Materials and Methods

1. Reagents

All reagents were of analytical-reagent grade and used as received. Water was deionized and further purified with a Milli-Q water purification system (Millipore, Milford, MA). Acrylamide (Acry), N, N'-methylenebisacrylamide (Bis), tris(hydroxymethyl)aminomethane (Tris), aminoacetic acid (glycine), N, N, N', N'-tetramethylethylenediamine (TEMED) were bought from Sino-American Biotechnology (Beijing, China). Glycerine, glycerol, sodium borohydride ($NaBH_4$), sodium citrate and acetic acid (CH_3COOH, 99.5%) were obtained from Beijing Fine Chemical Factory (Beijing, China). Auric chloride acid ($AuCl_3$•HCl•$4H_2O$), sodium hydroxide (NaOH), hydrochloric acid (HCl), ammoniumpersulfate, sodium chloride, sodium dihydrogen phosphate, disodium hydrogen phosphate were purchased from Beijing Reagents Company (Beijing, China). Catalase and hemoglobin human (Hb) were from Sigma-Aldrich (Shanghai, China). Copper (Ⅱ) sulfate anhydrous, poly(N-vinyl-2-pyrrolidone) (PVP (K23-27)), cobalt sulfate heptahydrate, nickelous sulfate, hydrazine hydrate, ethanol, human serum albumin (HSA), egg albumin (EA), papain, trypsin 1:250, human IgG, lysozyme, mucins and γ-globulins were bought from Beijing XinKeZhongjing Biological Technology Co., Ltd (Beijing, China). Silver nitrate was purchased from Alfa Aesar.

2. Instruments

The fluorescent images were formed by a bioimaging system (Vilber Fusion SL4, Beijing Oriental Science & Technology Development Co., Ltd, France), and the data relative to the background, was processed by Bio 1D software. Transmission electron micrographs (TEM) were performed by a JEOL-2010 electron microscope from JEOL Ltd. The fluorescence (FL) spectra were obtained with an LS-55 luminescence spectrometer (PerkinElmer Co., Ltd, USA). The one-dimensional (1-D) polyacrylamide gel electrophoresis (PAGE) were performed by DYY-6B electrophoresis instrument of steady voltage (Liuyi Instrument Factory, Beijing, China). For the discrimination, the data of the FL intensities was processed using classical linear discriminant analysis (LDA) in SPSS v16.0. The process of microwave-heating was operated by ETHOS 1 Advanced Microwave Digestion System (Milestone, USA). The UV-vis absorption spectrum was performed by a TU-1901 from Beijing Purkinje General Instrument Co., Ltd. The isothermal titration calorimetry (ITC) was conducted on a MicroCal VP-ITC calorimeter (Northampton, MA).

3. Preparation of metal nanoparticles

For the preparation of colloidal metal NPs, we used the method of liquid reduction[27-32] with some modifications. The details were shown in supplementary information (Table S2, S3).

4. Preparation of proteins in polyacrylamide gel using 96-well plates and metal NPs-based fluorescence (FL) imaging

The gel solution (7.5%, w/v) was prepared by mixing 4 mL of gel stock solution, 150 μL of $(NH_4)_2S_2O_8$ (10%, w/v), 15 μL of TEMED, and 12 mL of protein (2.67 mg mL^{-1}) in PBS (0.01 M, pH 7.40). Then put 150 μL of gel solution into 96-well plates, after gelatinization, the gel was incubated with 200 μL of colloidal metal NPs on a shaker at room temperature for 3 h. Before removal of 96-well plates, the polyacrylamide gels were washed with deionized water three times to avoid the interference of metal NPs adsorbed on the surface. Using the bioimaging system, the analyte was irradiated for 30 min and the data was gathered adjusting exposure time of 5 s at 655 ± 30 nm as the collected wavelength.

5. Preparation of serum samples

Serum samples from healthy people, hepatoma and β-thalassemia patients were acquired from the Affiliated Hospital of Beijing Normal University, Beijing Ditan Hospital and Guangzhou Children's Hospital (Guangzhou, China), respectively. The serum was isolated from the samples by centrifugation at 2500 rpm three times for 10 min each time. The samples were obtained with informed consent from the human subjects and the Institutional Review Board of Beijing Ditan Hospital approved sample collection.

Results and Discussion

1. Characterisation of the colloidal metal nanoparticles (NPs)

In this study, five colloidal metal NPs (Au, Ag, Cu, Ni and Co NPs), which were weakly fluorescent, were synthesised and characterised by fluorescence (FL) spectrometry and TEM (Figure 1). In Figure 1, it can be seen clearly that under the UV light, the five metal NPs are all weakly emissive (Figure 1A1 and 1A2) and spherical (Figure 1B1). The diameters of each metal NPs are also determined by TEM (see Figure 1B2), and showed that the average diameters of the Au, Ag, Cu, Ni and Co NPs are of 2.23 ± 0.30, 2.06 ± 0.35, 2.21 ± 0.47, 2.39 ± 0.43 and 2.54 ± 0.37 nm, respectively.

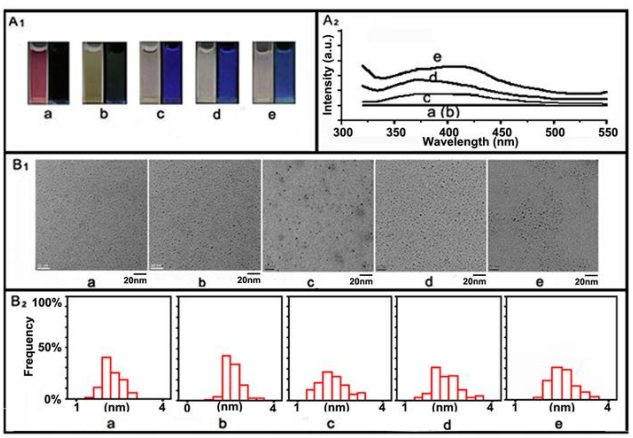

Figure 1. Characterisation of the five colloidal metal NPs (a: Au NPs; b: Ag NPs; c: Cu NPs; d: Ni NPs and e: Co NPs) (A_1) Photographs taken under the sunlight and ultraviolet light at 312 ± 10 nm excitation, respectively; (A_2) Fluorescence emission spectra excited at 312 nm; (B_1) Typical TEM images and (B_2) particle size distributions.

2. The discrimination of proteins through the sensor FL variation of the five NPs

a. The discrimination of proteins using the "fingerprint" maps of the sensor

In this experiment, five metal NPs (Au, Ag, Cu, Co, Ni NPs) are chosen as elements to form a sensor for the discrimination of ten proteins [HSA, papain, catalase, trypsin, lysozyme, hemoglobin human (Hb), egg albumin (EA), mucins, IgG and γ-globulins] with different isoelectric points (pI), according to the pattern variation of the sensor (*i.e.*, the change in the FL intensities and colours of the five NPs). We observed no obvious change in the FL intensity or colour when the NPs are simply mixed with the proteins in solution. However, when the NPs are mixed with proteins that are first immobilised in polyacrylamide gel (Figure 2A), the FL intensities and colours of NPs change greatly. Specifically, the ten proteins are initially immobilised in polyacrylamide gel, and then the entire gel is immersed in the five metal NP solutions for 3 h. Finally the gel is illuminated with a bioimaging system with an excitation of 312±10 nm (Figure 2A).

As shown in Figure 2A, the FL intensities and colours of the five NPs are greatly different from each other for a given protein, and the pattern of the sensor composed of the five NPs is a "fingerprint" map for the protein (see the square frame marked in Figure 2A). It is also observed that the "fingerprint" map of the sensor changes significantly for another given protein, since the FL properties of the NPs have a close relationship with the protein species. Through observation of the "fingerprint" maps for different proteins, which are obtained by the cross response between the proteins and NPs, the proteins can be identified. For example, in the "fingerprint" map for HSA, the Cu NPs are strongly emissive, whereas the other NPs have low FL intensities. Furthermore the FL colours of HSA itself and Ag NPs are pale green, the one of Cu NPs is rose red, the one of Au is bluish violet and the ones of Ni and Co NPs are dusty purple. However, in the "fingerprint" map for papain, the FL intensities of all the NPs are low, and the FL colours of papain itself and Au NPs are grey and the ones of Cu, Ni, Ag and Co NPs are bluish violet.

Similarly, for the specific NPs, the addition of different proteins can also result in diverse changes in the FL intensities and colours. Therefore, the metal NPs can also be differentiated (Figure S1,2). Additional details are provided in the supplementary information.

b. The discrimination of proteins by Linear Discriminant Analysis (LDA)

The raw data of the FL intensity patterns (Figure 2B) are then subjected to LDA based on a multi-dimensional data set (5 NPs × 10 proteins × 5 replicates) to further simplify the protein discrimination. LDA is a linear classical algorithm for pattern recognition, which can project the sample data to either 2-D or 3-D and display the data on a scatter plot.[33] After the projection, the data of the same sample combine to form a cluster (Figure 2C), and the clusters of different samples are well-separated from each other. In this way, the separation of the samples is enhanced.[34-35] As shown in the 2-D plot in Figure 2C, where the spheres represent the sample data transformed from the FL intensity patterns of the proteins, the data of a given protein is visualised as a spherical-cluster and is well-separated from the other proteins. According to the position of the clusters in the plot, qualitative information regarding the proteins can be obtained. Compared with other analysis methods, such as principle component analysis (PCA) and independent component analysis (ICA), LDA can select the most appropriate projecting direction for the sample data to maximise the ratio of between-class scatter and within-class scatter, resulting in the best classification of the samples.[36-37]

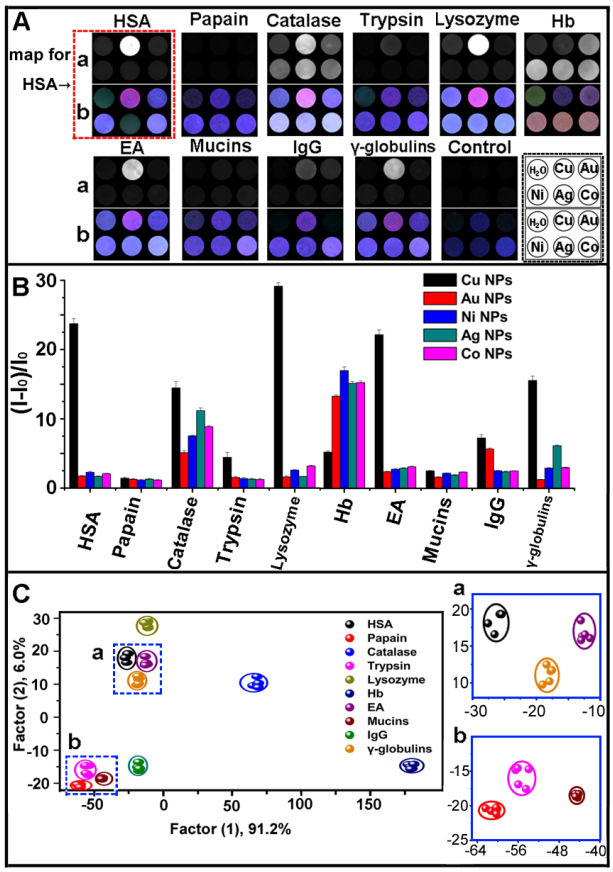

Figure 2. (A) FL intensity (a) and colour (b) difference maps for the ten proteins, with and without the addition of metal NPs. The concentration of each protein is 2 mg mL^{-1}, and the excitation wavelength is 312±10 nm. (B) FL intensity change patterns of the ten proteins in the presence of five metal NPs (the change patterns are acquired as an average of five parallel measurements). I_0 represents the average FL intensity values of the background, and I represents these of protein-NPs.(C) Canonical score plot of the first two factors of FL intensity response patterns, obtained through the sensor against ten target proteins; the insert figures (Ca, Cb) show the partial enlargement of the plot.

However, this technique has some limitations because there is much overlap in the sensor patterns when larger sets of proteins are analysed. For example, the discrimination of proteins with similar properties (such as HSA and BSA) and the distinction between proteins and their denatured ones remain a challenge (Figure S3).

The quantitative analysis experiment is also carried out to determine the sensitivity of the sensor (Figure S4), and the details are supplied in supplementary information. Moreover, we have changed the synthetic method for Au, Ag, Co and Ni NPs; and the four NPs together with the previously synthesised Cu NPs are used as a new sensor for the discrimination of proteins (Figure S5). The new sensor has a different response to the same protein from the sensor mentioned above, which provides us with a

broader view for the recognition of proteins. More details can be seen in supplementary information.

In addition, we have done the experiments of Cu NPs-in gel incubated in protein solutions (such as HSA, lysozyme, catalase and EA), which would add extra value to the work in clinical diagnostic applications. As shown in Figure 3A and 3B that the FL intensities are enhanced to a certain extent, and the FL colours are also changed similar to those of the protein-in gel immersed in Cu NPs solutions. By comparison, it is not as well as that of our former experiments (Figure 3C), and the more discussion on clinical diagnostic applications need to be further studied.

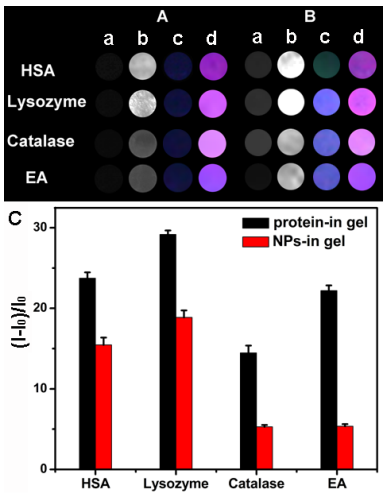

Figure 3. (A) FL images of Cu NPs-in gel incubated in water (a, c) and in the protein solutions (b, d). (B) FL images of protein-in gel incubated in water (a, c) and in the Cu NPs solutions (b, d). The concentration of each protein and Cu NPs is 2 mg mL^{-1} and 2.5 mM, respectively; and the excitation wavelength is 312 ±10 nm; line a and b are FL intensity images, line c and d are FL colour images, line a and c is the control sample of line b and d, respectively. (C) Comparison of the FL intensity changes of protein-in gel incubated in the Cu NPs solutions and Cu NPs-in gel incubated in the protein solutions (the data are acquired as an average of five parallel measurements). I_0 represents the average FL intensity values of the background, and I represents these of protein-NPs.

3. Possible mechanisms

a. The mechanism of the interaction of metal NPs with proteins

In order to understand the interaction of the metal NPs with the proteins, isothermal titration calorimetry (ITC) experiments are conducted to quantify the thermodynamics of metal NPs binding to proteins, with Cu NPs and HSA as examples. Figure 4A shows the ITC profile for the binding of NPs to proteins and the thermodynamic parameters, such as enthalpy changes (ΔH), entropy changes (ΔS) and binding affinity constant (K). Here, the thermodynamic parameters ΔH, ΔS and K are used to describe the binding affinity between the NPs and proteins, which is an indication of the interaction between the NPs and proteins.[38-39] When ΔH>0 and ΔS>0, the interaction primarily depends on the hydrophobic force; whereas when ΔH<0 and ΔS<0, the interaction is controlled by hydrogen bonding and van der Waals interactions and when the absolute value of ΔH≈0 and ΔS>0, the interaction is mainly attributed to electrostatic interactions.[40-41] In this assay, ΔH1>0, ΔS1>0, K1=210 M^{-1}, and ΔH2<0, ΔS2<0, K2=6.22E^4 M^{-1}, it indicates that the interaction of the metal NPs with the proteins is non-specific and mainly manipulated by hydrogen bonding and van der Waals interactions with weak hydrophobic force. The details of ITC experiments are shown in the supplementary information.

b. The mechanism of the variation in the FL properties of the metal NPs

The metal NPs have little to no FL intensity, either in solution or in gel; and no significant FL of the NPs is observed with the addition of proteins. The increased FL intensity and change in the FL colours of the NPs can only be observed when the NPs are added to the proteins immobilised in a gel (proteins-in-gel). The mechanism is discussed in the following section.

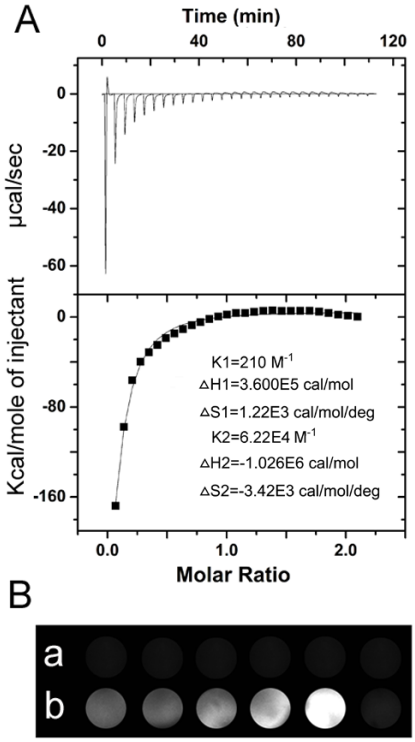

Figure 4. (A) Isothermal titration calorimetry (ITC) data for Cu NPs titration into HSA at 298 K. The concentration of Cu NPs and HSA is 0.5 mM and 0.1 mM, respectively. (B) FL imaging of HSA-in-gel incubated in water (a) and in Cu NPs solution (b), with gel pore diameters of different sizes.

It is already known that the NPs can combine with proteins and form protein-NP complexes, thus it is hypothesised that the NPs can also be immobilised in a gel through the interaction with the proteins-in-gel, which changes the surrounding environment of the NPs. First, by immobilising the NPs in a gel, the collisions between protein-NPs and water and those between inter-protein-NPs are reduced and thus the FL intensity is enhanced.[42-43] Second, the distance of the inter-NPs and between the NPs and proteins may be changed via the immobilisation. It is reported that the localized surface plasmon (LSP) of metal NPs is influenced greatly by the distance between the NPs,[44-45] which may lead to the FL enhancement of the NPs. To verify the mechanism, gels with different pore sizes are synthesised, and the proteins are immobilised in these gels (the concentration of protein is constant); then the metal NPs are added into the gel with HSA and Cu NPs as examples. From the results shown in Figure 4B, significant differences in the FL intensities can be observed, indicating that the distance between the NPs has a close relationship with the FLvariation of the NPs. Third, the change

in the distance between the NPs and proteins may also lead to the fluorescence resonance energy transfer (FRET) from the proteins to the metal NPs, resulting in increased FL intensities of the metal NPs.[46-47] Additional details regarding this mechanism are provided in the supplementary information. Since the sizes and inherent properties of each protein are distinct, the diameter of the protein-NPs in the gel and the distance between the protein-NPs will also be different, which may contribute to the various FL intensities and colours of the same NPs binding to different proteins. The difference in the FL of the various NPs is attributed to the different inherent properties of the NPs. In summary, it is postulated that the change in the local environment of the NPs upon binding to the proteins-in-gel has generated the variation in the FL properties of the NPs.

Gel components are also excluded (see supplementary information), and this mechanism requires additional studies.

4. Application of the metal NPs based on gel for the detection of mixed proteins and serum samples

a. The detection of mixed proteins after one-dimensional polyacrylamide gel electrophoresis (1-D PAGE)

To evaluate the potential application of the metal NPs, the detection of protein mixture (catalase, Hb and HSA) by three metal NPs (Cu, Ni and Co NPs) is performed. The protein mixture is separated after 1-D PAGE, which is a powerful separation technique for complicated biological samples.[48] The gel is then divided into four pieces and immersed in water and the three NPs, respectively. The proteins labelled with the NPs are visible under UV illumination (Figure 5) . As shown in Figure 5, the three proteins are well separated and show different FL intensities, which is in consistent with the results shown in Figure 2A. However, the FL colour changes are not completely consistent with the results shown in Figure 2A, which may be attributed to the effect of the protein tailing, band broadening and electrophoresis buffer.

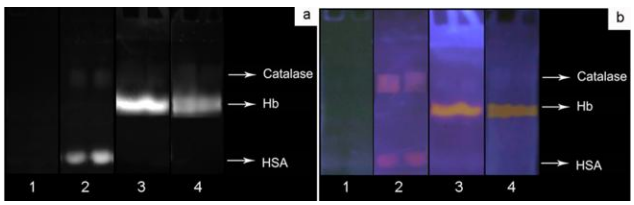

Figure 5. Metal NPs-based FL imaging for the detection of a protein mixture after 1-D PAGE, illuminated with a bioimaging system with an excitation of 312 ±10 nm (1: distilled water; 2: Cu NPs; 3: Ni NPs and 4: Co NPs) (a) FL intensity images (b) FL colour images.

b. The discrimination of serum samples using the metal NPs

In this experiment, the gel-based metal NPs are used to assist the discrimination of hepatocellular carcinoma (HCC) patients from healthy men using serum. The HCC patients have already been diagnosed by the standard method of chemiluminescent microparticle immunoassay (CMIA). Here the sera are first immobilised in gels, and the gels are then immersed in solution of Cu, Ni or Co NPs; the variation of the FL intensity and colours of the NPs is observed under the illumination of a UV transillumination table. As shown in Figure 6, the FL colours of the HCC patient serum-NPs are significantly different from those of the healthy serum-NPs; although no significant difference in the FL intensities are observed. To make a better distinction, the data for the FL of the NPs are then subjected to LDA (Figure S8). The result provided by our sensor is in accordance with that obtained by the standard method, which proves that our sensor can be an auxiliary method for the clinical standard method for the distinction between HCC patients and healthy men. Clinically, many proteins in the serum of HCC patients will change greatly compared to the healthy men,[49-50] for example, haptoglobin (Hp),[51] Hb,[52-53] α L-fucosidase (AFU), γ-glutamyltranspeptidase isoenzymes Ⅱ (GGT-Ⅱ)[54] and alpha fetoprotein[55]. So these protein differentiation might be the possible reason for the differential FL properties between HCC patient serum-NPs and healthy serum-NPs, and the mechanism needs to be further studied. Furthermore, we use this sensor to discriminate thalassemia patients from healthy men. Additional details are provided in the supplementary information.

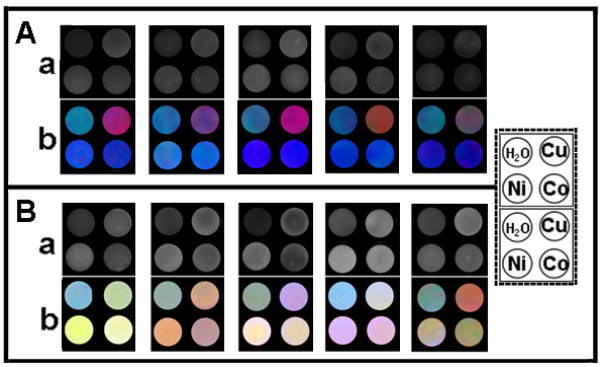

Figure 6. FL intensity (a) and colour (b) difference maps for sera from healthy men (A) and HCC patients (B), with and without the ddition of metal NPs.

Conclusions

In this study, a fast and simple sensor composed of five metal NPs for protein discrimination is fabricated. This sensor is easy to operate, visualise, and has a low cost. Using the variation in the FL intensities and colours of the metal NPs, the detection and distinction of ten proteins and human serum from hepatocellular carcinoma (HCC) patients can be accomplished. To the best of our knowledge, this is the first time that the polyacrylamide gel has been utilised in a sensor. Because of the good biocompatibility and diversity of the metal NPs, this sensor will broaden the application of metal NPs for protein identification or may be used to identify the components of even more complex biological samples, such as bacteria and cells.

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Notes and references

[a] *Key Laboratory of Theoretical and Computational Photochemistry, Ministry of Education, College of Chemistry, Beijing Normal University, Beijing 100875, P.R. China. Fax: +86-10-62799838; Tel: +86-10-58805373; E-mail: jinoyang@bnu.edu.cn*

[b] *Department of Clinical Laboratory,Beijing Ditan Hospital, Capital Medical University,Beijing, 100015, P.R. China*

†Electronic Supplementary Information (ESI) available: [preparation of one-dimensional polyacrylamide gel electrophoresis (1-D PAGE) and metal NPs-based FL imaging, basic properties of proteins, all the fluorescent data, preparation of five colloidal metal NPs, the emission fluorescence spectra and the UV-vis absorption spectrum, the differentiation of NPs, quantitative analysis of the proteins, FL change profiles of NPs in the presence of gel components, the discrimination of serum samples by the metal NPs based on gel]. See DOI: 10.1039/b000000x/

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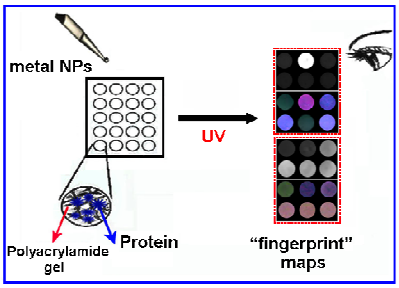
Using Metal Nanoparticles as a Visual Sensor for the Discrimination of Proteins

Haiyan Liu[a], Lin Ma[a], Shenghao Xu[a], Wenhao Hua[b] and Jin Ouyang*[a]

[a] Key Laboratory of Theoretical and Computational Photochemistry, Ministry of Education, College of Chemistry, Beijing Normal University, Beijing 100875, P.R. China.

[b] Department of Clinical Laboratory, Beijing Ditan Hospital, Capital Medical University, Beijing, 100015, P.R. China



The fluorescence of metal NPs are changed differently upon a protein-in gel, forming a visual sensor for protein discrimination.