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Colorimetric Determination of pH Values using Silver Nanoparticles Conjugated with Cytochrome *c*

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Some of metal nanoparticles have the potential for use as colorimetric assays for estimating solution properties, such as pH and temperature due to localized surface plasmon (LSP) phenomena. This report describes the use of silver nanoparticles (AgNP) conjugated with cytochrome *c* (Cyt *c*) for the colorimetric determination of solution pHs. When the pH of a solution decreases, the Cyt *c* immobilized on the AgNP undergoes a conformational change, leading to a decrease in the interparticle distance between Cyt *c*-AgNP probes and consequent red-shift in LSP. As a result, the color of the Cyt *c*-AgNP probe solution changes from yellow to red and finally to a grayish blue in the pH range from 11 to 3. This gradual color change can be used to determine the pH of a solution over a wide pH range, compared to other colorimetric methods that use gold nanoparticles.

Key Words : Bioconjugates, Colorimetric sensor, Cytochrome *c*, Conformational change, Silver nanoparticle

Introduction

Metal nanoparticles (NPs) have received considerable attention due to their optical properties that originate from charge-density oscillations, which are referred to as localized surface plasmons (LSP).[1,2] These optical properties of NPs have been applied in various biotechnological fields, such as imaging, sensing, and cell targeting.[3-5] Modulating distances between NPs based on conformational changes in biological molecules absorbed to the NPs surface have been attracted considerable interest as a new methodology for the design of powerful optical sensors.[6-8] This can be accomplished because, when NP aggregation occurs, the average particle distance is shortened to a distance below the diameter of the particles, resulting in a color change in the colloidal NP solution.[9] NPs can be conjugated with biological molecules (i.e., proteins and DNAs) and small organic molecules through a variety of interactions including physical adsorption, electrostatic binding, specific recognition, and covalent coupling.[10] Among the various proteins, cytochrome *c* (Cyt *c*) is a well characterized protein that undergoes a conformational change under destabilizing conditions. The heme group of Cyt *c* is sensitive to changes in pH, temperature, ionic strength, and solvent composition.[11,12] The Zare group reported on a colorimetric sensor that takes advantage of the pH-induced conformational change of Cyt *c* absorbed on gold nanoparticles (AuNPs). In this system, an abrupt color change occurred from the original red color of AuNPs to blue at approximately pH 6.[13] However, this steep transition of the optical signals under certain pH conditions has limited applicability for a precise sensing tool. For the precise monitoring of the pH of a solution, the transition region should be more sluggish. In order to achieve this purpose, we designed a new type of colorimetric pH probe by using silver nanoparticles to determine the pH of a solution over a wide pH range, from 3 to 11, as shown in Figure 1. It is generally known that Cyt *c* undergoes a conformational change in solution with a change in pH, when the axial ligand Met80 is replaced with Lys73 and Lys79.[11,14] When the solution pH is decreased, the Cyt *c* on the surface of silver nanoparticles (AgNPs) partially unfolds and can interact with the residues of Cyt *c* that are protonated by H^+ ions in the solution.[15] As a result, this interaction causes the network to be extended through cross-coupling between conjugates.[13] After exposure to various pH conditions, each color change of Cyt *c*-AgNP probes was characterized by the naked eye and the consequent spectral shift was also measured by UV-vis spectroscopy. These results were further verified by electron micrographs and light scattering images of the resulting probe aggregation.

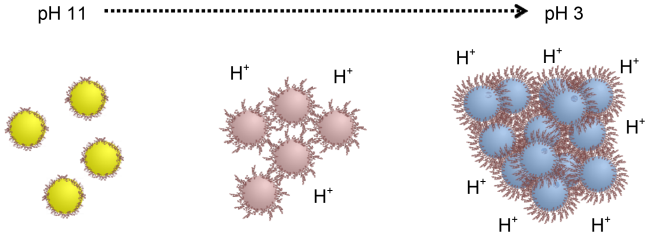

Figure 1. Schematic diagram of the colorimetric change of Cyt *c*-AgNP probes with decreasing pH.

Experimental Section

Materials. Silver nitrate ($AgNO_3$), sodium bis(2-ethyl-

hexyl)-sulfosuccinate (Na-AOT), trisodium citrate, and sodium borohydride ($NaBH_4$) were all purchased from Sigma-Aldrich, and used without further purification. Cyt *c* (from horse heart, Sigma-Aldrich) was dissolved in 25 mM potassium phosphate buffer (PBS, pH 7.2). H_2O was purified to above 18 MΩ using a Milli-Q water system (Millipore).

Preparation of Cyt *c*-AgNP Probes. The colloidal Ag particles were fabricated by the reduction of $AgNO_3$ in the presence of Na-AOT.[16] A 0.01 M of $AgNO_3$, 0.03 M of trisodium citrate and 95 mL of DI water were mixed and stirred for 30 min in an ice bath. While the solution was being vigorously stirred, a 0.05 M of $NaBH_4$ was rapidly injected into the solution and 1 ml of Na-AOT (5 mM), a surfactant, was then added to the solution. The as-prepared solution was aged overnight in the dark to permit the growth of spherical particles. In order to prepare Cyt *c*-AgNP probes, a 0.1 mg/mL solution of Cyt *c* (from horse heart) in PBS buffer (pH 7.2) was injected into the AgNP colloidal solution at a 1:300 ratio. The pH of the AgNP solution was adjusted to 9.5 and this pH was maintained throughout the reaction sequence to prevent the denaturation of Cyt *c*. The solution was allowed to stand for 5 hr to ensure full surface coverage.

Characterization of Cyt *c* -AgNP Probes at Various pH Values. Before exposure to various pH conditions, we measured the pH value of Cyt *c*-AgNP probe solution. Then, the pH of the probe solutions were adjusted from 11 to 3 by adding 1 M HCl solution and 1 M NaOH solution at room temperature, in order to observe color changes of them depending on the pH values. The pH value was determined with pH meter (ORION 920A, USA). The absorption spectrum of Cyt *c*-AgNP probes at various pH values was measured by UV-vis absorbance spectrometry (Spectra academy, K-mac) using quartz cuvettes with a 1 cm path length. The hydrodynamic diameter of the Cyt *c*-AgNP probes was determined by electrophoretic light scattering spectrophotometry (ELS-8000, Otsuka Electronics).

Morphological Analyses of Probe Aggregation. Slide glasses, cleaned in a piranha solution, were immersed in a 5 mM ethanolic solution of 11-mercaptoundecanoic acid (MUA) for 24 hr at room temperature. The slides were sonicated for 30 min and then rinsed with ethanol, DI water and dried with N_2. Cyt *c*-AgNP probes at different pH values were immobilized on the slides that had been functionalized by MUA overnight. The resulting samples were mounted on a dark-field microscope (Axio Observer Z1, Carl Zeiss) and light-scattering images were collected using a CCD camera (AxioCam MRc 5, Carl Zeiss). A transmission electron microscope (JEM 1010, JEOL) was used for morphology analysis.

Results and Discussion

The narrower bandwidth and more intense resonance peaks in the case of AgNPs, compared to AuNPs, allow the refractive index changes in the surrounding medium to be easily detected, because the sensing resolution is dependent upon the plasmon bandwidth and the absolute magnitude of the plasmon intensity.[17,18] Therefore, AgNPs would be expected to be superior to conventional nanostructures such as AuNPs[13] as a probe for detecting small changes in the pH of a solution by measuring the shift in the position of the plasmon bands and changes in the band intensity. The surface of the bare AgNPs was capped with a layer of amphiphilic AOT, a surfactant. The surrounding water interacts with the polar head of AOT through H-bond interactions that maintain the spherical AgNP structure. As Cyt *c* is added to the AgNP solution, the positively charged 'lysine patch' of Cyt *c* interacts with the negatively charged sulfonate head group of the AOT surfactant immobilized on the Ag surface.[19] As shown in Figure 2, the average hydrodynamic diameter of the bare AgNPs measured by electrophoretic light scattering spectrophotometry (ELS) was 31.4 ± 3 nm. The hydrodynamic diameter of AgNPs increased to 58.3-251.8 nm. This broad hydrodynamic diameter range of the Cyt *c*-AgNP probes can be attributed to the slight aggregation of the probe solution at initial pH condition (pH 9.5) as well as the formation of the Cyt *c* layer on the AgNP surface. Slightly clustered Cyt *c* molecules in the probe solution can interact with Cyt *c* on the AgNPs.[15] Therefore, hydrodynamic diameters of structures in the probe solution vary from 58.3 to 251.8 nm, according to the clustering amount of Cyt *c* on the nanoparticle.

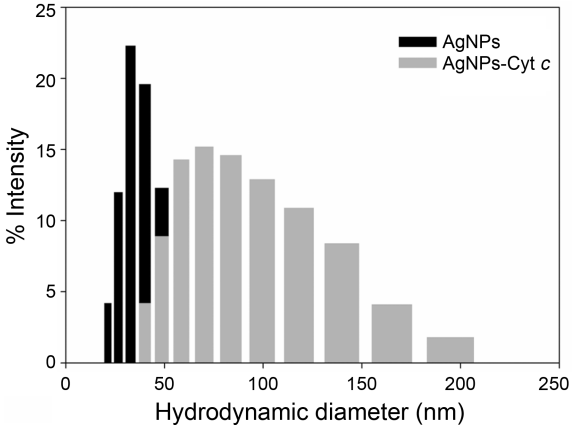

Figure 2. Comparison between the hydrodynamic diameter of AgNPs and that of Cyt *c*-AgNP probes, as measured by ELS.

Figure 3 shows the color change for bare AgNPs and Cyt *c*-AgNP probes with decreasing pH values from 11 to 3. The pH of the solution was adjusted from 3 to 11 by adding 1 M HCl solution and 1M NaOH solution. No color change was observed for the bare AgNP colloidal solution with pH change (Figure 3(a)). On the other hand, the color of the Cyt *c*-AgNP probes gradually changed from yellow to red and finally to a grey-blue (Figure 3(b)).

This color change in the Cyt *c*-AgNP probes was clearly supported by UV-vis spectroscopy, as shown in Figure 4. The maximum absorbance band of bare AgNPs in the absence of Cyt *c* appeared at 394 nm, which is a characteristic of spherical AgNPs.[20] The $peak_{FWHM}$ (full width at half-maximum) of bare AgNPs was 61 nm, indicating a narrow size distribution. After the AgNPs were conjugated with the

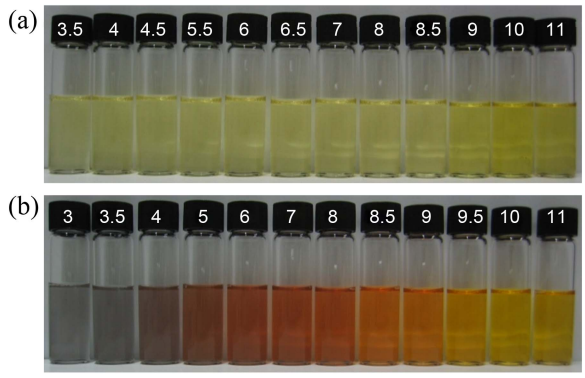

Figure 3. Color change of (a) bare AgNPs colloidal solution, and (b) Cyt *c* coated AgNPs solution with decrease in pH value from 3 to 11.

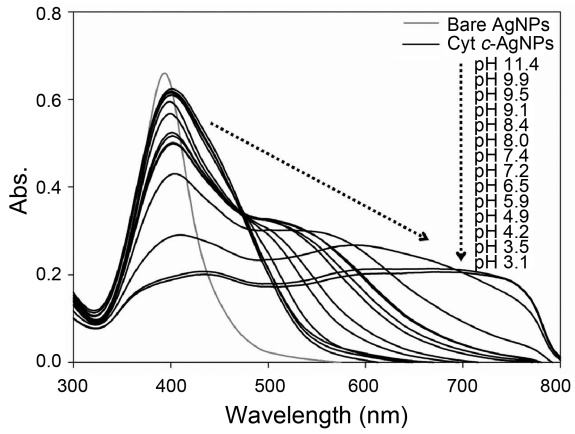

Figure 4. Representative UV-*vis* adsorption spectra of bare AgNPs at pH 9.8 (gray line) and Cyt *c*-AgNPs conjugates (black line) at different pH values.

Cyt *c*, the absorbance peak was shifted from 394 nm to 401 nm. This red-shift between bare AgNPs and Cyt *c*-AgNP probes is related to alterations in the refractive index of AgNPs by the Cyt *c* layer.[21] The $peak_{FWHM}$ for the conjugates at pH 9.5 was relatively broadened to 123 nm, indicating that aggregates were formed by Cyt *c* absorbed on the Ag surface during the conjugation process. As the pH of the solution was decreased, the absorbance peak at 401 nm disappeared and a broad peak appeared newly in the range between 500 nm and 750 nm. The red-shift in the UV-vis spectrum as the result of decreasing the interparticle distance of NPs has been demonstrated through experimental results[22] and theoretical modeling.[23]

The absorbance ratio between the aggregated and non-aggregated conjugates can be identified by comparing the increment of the absorbance peak at λ = 550 nm with the decrement of that at λ = 401 nm, as shown in Figure 5. This absorbance ratio increased gradually with decreasing pH from 11 to 3 and the shoulder, considered to be an intermediate state, over the pH range from 7.5 to 5.5 appeared (Figure 5(a)). This tendency also was observed from the increase in the average hydrodynamic diameter of probes induced by the aggregation (Figure 5(c)). Through the

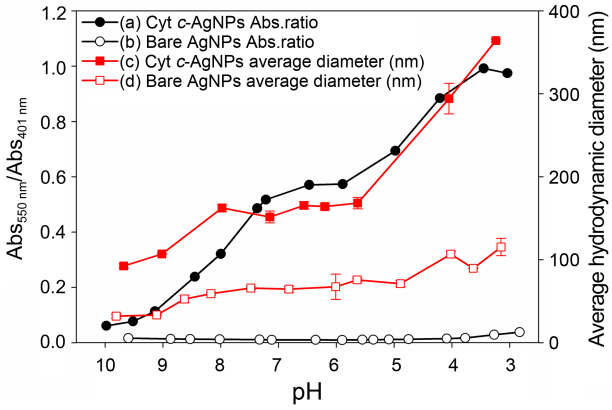

Figure 5. The absorption ratio of Cyt *c*-AgNP probes between 401 nm and 550 nm (a) and bare AgNPs (b). Average hydrodynamic diameter of Cyt *c*-AgNP probes (c) and bare AgNPs (d) at each specified pH. The lines that connect the data points are inserted as a visual guide.

absorbance ratio displayed in Figure 5, it should be noted that the gradual color change of the Cyt *c*-AgNP probes is clearly different from the pH behavior of Cyt *c*-AuNP probes, in which a sharp color transition occurs at a specific pH value.[21] A slight increase in absorbance and the average diameter of bare AgNPs (Figure 5(b) and 5(d)) from pH 5 induced by the instability of AgNPs. However, this color change was not detectable by the naked eye. Individual Cyt *c*-AgNP probes at specific pH values were characterized using dark-field microscopy and TEM, as shown in Figure 6. Figure 6a-d shows light scattering images of representative Cyt *c*-AgNP probes at different pH values. At pH 9.5

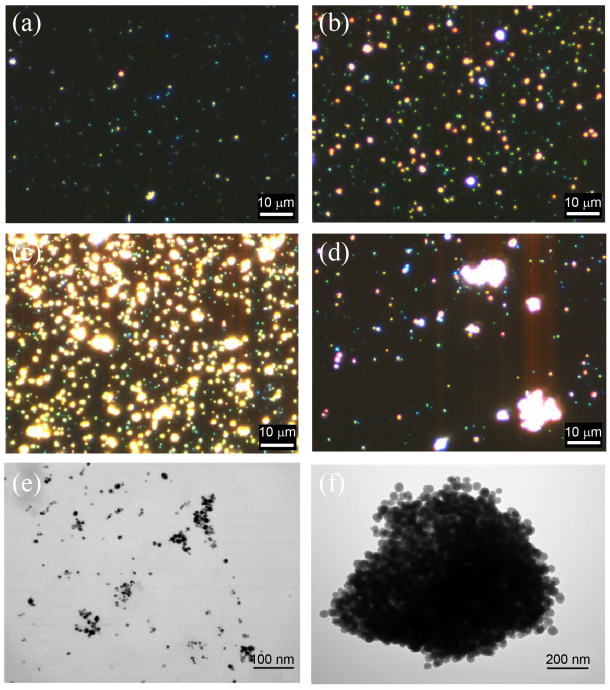

Figure 6. Light-scattering images of Cyt *c*-AgNP probes; (a) at pH 9.5 (b) pH 6.9 (c) pH 5.1 (d) pH 3.3. TEM images of Cyt *c*-AgNP probes; (e) at pH 9.5 (f) pH 3.3.

(Figure 6(a)), the majority of particle color was blue and green. The color index for blue, green and red generally correspond to a size index of 50 ± 10, 70 ± 10, and 90 ± 10 nm.[24] Therefore, the size of the Cyt *c*-AgNP probes can be presumed to be in size range between 40 nm and 80 nm. This discrepancy between the sizes measured individually by microscope and the hydrodynamic diameter measured by the ELS (Figure 5) is due to the extensive hydration of the probes in solution.[25] At pH 6.9, large probe aggregates derived from the pH-induced conformational change in Cyt *c* appeared as a red color (Figure 6(b)). As shown in Figure 6(b)-(d), larger aggregates were formed at lower pH values. More clear morphology images of probe aggregates at pH 9.5 (Figure 6(e)) and pH 3.3 (Figure 6(f)) were obtained by the TEM analysis of Cyt *c*-AgNP probes in solution. In the case of pH 9.5 the morphology of the sampled probes is nearly uniformly spherical and the average diameter of aggregate was 87 ± 38 nm (n = 32) (Figure 6(e)). On the other hand, the Cyt *c*-AgNP probe sampled at pH 3.3 indicated the presence of a largely assembled aggregate form (Figure 6(f)).

Conclusions

In conclusion, an effective colorimetric method for detecting the pH of a solution over a broad pH range from 11 to 3 using an AgNP probe conjugated with Cyt *c* is described. The pH-induced conformational change of Cyt *c* immobilized on AgNPs allows the modulation of interparticle distance between AgNPs, which leads to a change in the color of the probe solution. A gradual and distinguishable color change, from yellow to red to grey-blue with changes in pH values, can be easily observed with the naked eye. This results in an achievement of pH determination in a broad pH range, considering that conventional colorimetric assays for the determination of pH values are reliable only in a narrow pH range. The proposed sensing probe is expected to be useful as a simple, rapid, and sensitive optical pH probe, which would be applicable to the field of monitoring environmentally relevant media (i.e., cells and drinking water).

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