Detection of Gold Nanoparticles Aggregation using Light Scattering for Molecular Sensing

Yuki YANO*, Masamichi NISOUGI**, Yuki YANO-OZAWA*, Tsuyoshi OHGUNI***, Atsushi OGAWA****, Mizuo MAEDA***** and Tamotsu ZAKO*****†

*, Department of Chemistry and Biology, Graduate School of Science and Engineering, Ehime University, 2-5 Bunkyo, Matsuyama, Ehime 790-8577, Japan

**, Department of Materials Science and Biotechnology, Graduate School of Science and Engineering, Ehime University, 3 Bunkyo, Matsuyama, Ehime 790-8577, Japan

***, Department of Chemistry, Faculty of Science, Ehime University, 2-5 Bunkyo, Matsuyama, Ehime 790-8577, Japan

****, Proteo-Science Center, Ehime University, 3 Bunkyo, Matsuyama, Ehime 790-8577, Japan

*****, Bioengineering Laboratory, RIKEN Cluster for Pioneering Research, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan

† To whom correspondence should be addressed.

E-mail: zako.tamotsu.us@ehime-u.ac.jp
Abstract

Gold nanoparticles (AuNPs) have been commonly used in molecular sensing by the observation of the colour change from red to blue of the AuNP solution, caused by target-molecule-induced AuNP aggregation. In this work, the changes in absorbance and scattering spectra caused by AuNP aggregation were studied using thrombin-induced AuNP aggregation as a model. We demonstrated for the first time that scattering spectra is more sensitive to the changes owing to AuNP aggregation than absorbance spectra. Moreover, a digital colour analysis of darkfield images using dark field microscopy (DFM) facilitated a simple method for detection of AuNPs aggregation without the use of spectroscopic analysis. Furthermore, we demonstrated that DFM is useful to detect AuNPs aggregation in a coloured solution, in which colour change by AuNPs aggregation is not visible.

Keywords: Gold nanoparticles, aggregation, molecular sensing, light scattering, dark field microscopy
Introduction

Recently, gold nanoparticles (AuNPs) have been commonly used for molecular sensing applications because of their favourable optical and electrochemical properties. Notably, the properties of AuNP aggregates differ from those of AuNP monomers. For example, the formation of AuNP aggregates can be recognized by a colour change from red to blue/purple. Because of this, AuNPs are often used for the detection of DNA, proteins, metal ions and chemical compounds in molecular sensing applications. For specific detection purposes, the surface of AuNPs have been functionalized with various modifications, including antibodies, DNA and nucleic acid aptamers.

In this study, both absorbance and light scattering spectra are measured to estimate the AuNP aggregate formation. According to Mie theory, light scattering is a more sensitive method for the detection of AuNP aggregate formation. It has been theoretically shown that change in the scattering intensity upon AuNP size change is larger than that in absorbance. Although dynamic light scattering (DLS) is one of the major analytical methodologies based on light scattering, there have been only a few reports focusing on light scattering spectra. Furthermore, to the best of our knowledge, a study comparing the sensitivity of absorbance and scattering in target detection experimentally has not yet been reported.

Herein, the relative sensitivities of absorbance and scattering methodologies are compared. It is shown that the scattering spectra, upon AuNP aggregation, is more sensitive to the change than the absorbance spectra. For the analysis of AuNP aggregates, we then utilized dark field microscopy (DFM), which can detect the scattering light from nanoparticles. It was previously shown that the digital colour of the AuNP spots in the darkfield images can be used to determine the nanoparticle size. We demonstrate that digital colour analysis of darkfield-images of AuNP aggregates can also be used for detecting targets without
Another advantage offered by DFM is that with this methodology, AuNPs aggregates can be observed in coloured solutions. Target detection by change in colour or absorbance spectra is difficult for samples containing dyes, in particular if the colour of the dye molecule is similar to the colour of the AuNPs. However, our results suggest that DFM can be used to detect AuNP aggregation in such a coloured solution.

**Experimental**

**Materials**

AuNPs (40 nm in diameter) were obtained from BBI solutions (Cardiff, UK). Thrombin binding aptamer (Th-apt, 5'-AGT CCG TGG TAG GGC AGG TTG GGG TGA CT-3’) was purchased from Eurofins genomics (Tokyo, Japan). 2-Melcaptethanol (2-ME) and methyl orange was obtained from Wako (Osaka, Japan). 3-Aminopropyltriethoxysilane (APTES) and bovine serum albumin (BSA) were obtained from Tokyo Chemical Industry (Tokyo, Japan) and Bio RAD (Hercules, CA, USA), respectively. Slide glass and cover glass were purchased from Matsunami Glass Industry (Osaka, Japan).

**Preparation of aptamer-modified AuNP**

The stock AuNP solution was sonicated in a sonication bath for 1 min and centrifuged (25ºC, 15000 rpm, 15 min). The supernatant was removed, and AuNPs were re-dispersed with ultra-pure water to achieve a concentration of 0.34 nM. Th-apt (0.6 µM) was added to the AuNP solution (0.3 nM) in TNK-buffer (20 mM Tris-HCl, pH 7.6, 140 mM NaCl and 5 mM KCl), and the mixture was incubated at room temperature for 120 min (apt-AuNP). Before the detection assay, the thrombin and BSA stock solutions were desalted using a dialysis cup (10000 MWCO, ThermoFisher Scientific, Roskilde, Denmark). The concentration of the obtained thrombin and
BSA samples was determined using BCA protein assay kit (Thermofisher Scientific). These desalted samples were diluted to various concentrations with ultra-pure water.

The amount of the aptamer adsorbed on the AuNPs was quantified as previously described. Briefly, the prepared apt-AuNP solution (200 µL) was centrifuged (25ºC, 8000 rpm, 15 min). The supernatant was removed, and 10% 2-ME (20 µL) was added to remove the Th-apt adsorbed on the AuNPs. The solution was incubated at room temperature for 16 h, and then centrifuged (25ºC, 8000 rpm, 15 min). The amount of Th-aptamer on the AuNPs in the supernatant was quantified using QuantiFluor ssDNA System (Promega, Madison, WI, USA).

**Thrombin detection by the aptamer-modified AuNP**

The thrombin solution at various concentrations (2 µL) was added to the prepared apt-AuNP (16 µL), and incubated at room temperature for 10 min. Then, 108 mM NaCl (7 µL) was added, and incubated for 60 min. The final concentration of NaCl was set at 30 mM. This value was determined in advance as the maximum concentration, where the apt-AuNP would remain dispersed. The samples were then used for further analysis. Thrombin-induced AuNP aggregation also occurred in a solution with 1 mg/mL methyl orange, which was used as a model dye for a coloured solution.

**Measurement of absorbance and scattering spectra**

For the measurement of the absorbance and scattering spectra, an IX-71 microscope (Olympus, Tokyo, Japan) equipped with UPlanApo 100× objective lens and U-DCD dark field condenser was used. Absorbance and scattering spectra (400-800 nm) were measured with microcells. The microcells were prepared with slide glass and a silicon sheet (thickness: 0.5 mm) with a hole. The hole had a diameter of 2 mm and volume of 1.5 µL. A AuNP sample was applied into the
microcell. Scattering and absorbance spectra were measured at the focused point. To eliminate the influence of scattered light from scratches or dust on the glass surface, the measurements were performed in solution. The microcell used in this study can measure samples with a volume below 10 µL. The scattering spectroscopy measurement can be performed with concentrations as low as 10 pM of AuNP, which is advantageous as only a small amount of sample is needed.

**Digital image analysis of the DFM image**

DFM images of the AuNP samples with various concentrations of thrombin were obtained using a BX53 microscope (Olympus, Tokyo, Japan) equipped with a UP73 CCD camera, UPlanFLN 60× objective lens, and U-DCW dark field condenser as described 18,19. The APTES treated glass (APTES-glass) is used for dark field imaging in this study 25. To prepare the APTES-glass, a glass slide was soaked for 30 min in 1% APTES solution. The glass was then rinsed with ultra-pure water, and sonicated for 10 min. The glass was dried at room temperature and stored for future use. A AuNP sample (3 µL) was dropped on the APTES-glass and covered with a cover glass. DFM images were taken using CellSens Standard software ver.1.6 with the following parameters: exposure time 50 msec, ISO 200, image resolution 1600×1200 pixel.

For image analysis of the DFM images, ImageJ 26 was used. The whole colour DFM image was separated to three images of Red (R), Green (G) and Blue (B) components. The intensity of each component image was obtained to calculate the RGB component ratio 20.
Results and Discussion

Thrombin detection by measurement of absorbance and scattering spectra

The colour change from red to blue of the AuNP solution has been utilized for biosensing. Molecules that induce AuNP aggregation could be detected by the colour change. Absorbance spectra are mainly used to estimate the AuNP aggregate formation. In this study, a change in the absorbance and scattering spectra, upon AuNP aggregation, was estimated using thrombin-induced AuNP aggregation as a model. A detection scheme is shown in Figure 1a. In brief, AuNPs modified with aptamer are stable because of electric repulsion. The solution remains red, even under the high-salt conditions. Upon the addition of thrombin, the aptamer adsorbed on AuNP surface interacts with thrombin and desorbs from the NP surface. Accordingly, the amount of the aptamer on the surface decreases, resulting in an unstable AuNP dispersion in the presence of salt, leading to the aggregation of the AuNPs.

As shown in Figure 1b, the addition of more than 10 nM of thrombin induced a colour change in the presence of 30 mM NaCl. On the other hand, the solution colour was unchanged after adding 1 pM-5 nM of thrombin to the blank sample (without thrombin). This result indicates that 10 nM thrombin could be detected by the colour change of a AuNPs solution. This is consistent with a previously report. In comparison, when BSA was added as a negative control, a change in the solution colour was not observed, even at 50 nM BSA (Fig.1c). This suggests that the colour change of the apt-AuNP solution is specific to the addition of thrombin.

Absorbance and scattering spectra of apt-AuNPs after the addition of various amount of thrombin were measured by microscope spectroscopy (Fig. 2). Since the absorbance and scattering spectra peak of AuNPs depends on their diameter, the peak is expected to shift to longer wavelengths as the diameter increases. As shown in Figure 2a, the absorbance peak of the AuNP monomer was observed around 525 nm without thrombin. The absorbance peak at
630 nm, associated with AuNP aggregation, increased as the thrombin concentration increased. A plot of the ratio of the absorbance spectra peaks (OD630/OD525) had a linear correlation to thrombin concentration, with a slope of 0.0076.

Figure 2b shows scattering spectra of apt-AuNPs after the addition of various amounts of thrombin. The peak associated with the AuNP monomer was observed at around 550 nm. The peak intensity at 660 nm increased as the thrombin concentration increased. A plot of the ratio of the scattering spectra peaks ((S660-S480)/(S550-S480)) also had linear correlation to thrombin concentration, with a slope of 0.0294. Importantly, in comparison, the slope value of the scattering ratio is approximately four times larger than the absorbance ratio slope value. For the first time, this suggests that a measurement based on scattering is more sensitive for the detection of AuNP aggregates formation. Theoretical study shows that scattering increment of AuNPs become larger than absorbance increments as the particle size become larger. Thus, it is plausible that the scattering increment upon AuNP aggregation is larger than absorbance increment in similar manner.

Thrombin detection by digital image analysis of the DFM images

To simplify the method for estimating AuNP aggregation, we attempted to utilize digital colour analysis of the dark field image as an analytical method, without spectroscopic techniques. When AuNPs were observed with DFM, AuNP monomers and aggregates formed by salt were observed as bright green spots and orange spots, respectively. Figure 3a shows typical DFM images of monomers and aggregates, and the RGB ratio of each image. In the image of the monomers, the RGB proportion followed the order of G > R > B. On the contrary, in the image of the aggregates, the proportion followed the order of R > G > B. These results indicate that the RGB ratios of the monomers and aggregates are different. These results also imply that the
RGB analysis method could be useful for estimating AuNP aggregation. Figure 3b shows the DFM image of apt-AuNP samples after thrombin addition, at the indicated concentrations. The RGB ratios of each DFM image were calculated and shown in Figure 3c. The ratio of the R component increased and the ratio of the G component decreased in a thrombin-concentration dependent manner. A change in the ratio became significant when more than 5 nM of thrombin was added. This result is consistent with the result obtained by scattering spectra assay (Fig. 2b), indicating that the amount of AuNP aggregation could also be estimated based on digital colour analysis of the DFM images.

It is advantageous that AuNP aggregates can be observed in coloured solutions with DFM. Although target detection by colour change observations could be difficult for samples containing dyes, it is expected that the DFM observation of AuNPs would not be disturbed by the presence of dye molecules. Herein, we attempted the DFM detection of thrombin-induced AuNP aggregation in a coloured solution containing methyl orange as a model dye. The colour of AuNPs could not be distinguished by visual observation (Fig. 4a). However, in the case of DFM observation, AuNP aggregation could be clearly observed (Fig. 4b). As shown in Figure 4c, the RGB ratios of each DFM image of the samples containing dye changed in a dose-dependent manner on added thrombin. This result is similar to that without dye (Fig. 3c). This result supports that DFM enables the detection of target molecules in coloured solutions via target-induced AuNP aggregation.

Conclusions

The colour change from red to blue of the AuNP solution has been utilized for molecular sensing. In this study, change in absorbance and scattering spectra upon AuNP aggregation was studied using thrombin-induced AuNP aggregation as a model. In this study, we demonstrated
for the first time that scattering spectra is more sensitive to the change caused by AuNP aggregation than absorbance spectra. It was also shown that RGB analysis of darkfield images provides a simple method for the detection of AuNP aggregation, without a spectroscopic analysis. Furthermore, we demonstrate that DFM is useful to detect AuNP aggregation in coloured solution, in which colour change by AuNPs aggregation is not visible.

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Figure Captions

Figure 1 Thrombin detection using aptamer-modified AuNPs
(a) Sensing mechanism for thrombin detection. (b) Image of thrombin (0, 1, 10, 100 pM, 1, 5, 10, 20, 50 nM) and 30 mM NaCl added AuNP-apt solution. (b) Image of BSA (0, 1, 10, 15, 20, 50 nM) and 30 mM NaCl added AuNP-apt solution (negative control).

Figure 2 The absorbance and scattering spectral change of aptamer-modified AuNPs by thrombin addition
The absorbance (upper left) or scattering (bottom left) spectra, and correlation between thrombin concentration and absorbance ratio (upper right) or scattering ratio (bottom right).

Figure 3 Thrombin detection by digital image analysis of the DFM images
(a) DFM image of dispersed (left) and aggregated (right) AuNP. The colour component ratio is inserted. (b) DFM image of thrombin and NaCl added AuNP-apt solution without methyl orange. The thrombin concentration is written on the image. (c) Correlation between thrombin concentration and RGB ratio of the DFM image of AuNP without methyl orange.

Figure 4 Thrombin detection in coloured solution using DFM
(a) Image of thrombin and NaCl added to apt-AuNP solution with methyl orange. (b) DFM image of thrombin and NaCl added to apt-AuNP solution with methyl orange. (c) Correlation between thrombin concentration and RGB ratio of the DFM image of AuNP with methyl orange. All white scale bars are 10 µm.
Figure 1 Thrombin detection using aptamer-modified AuNPs
Figure 2 The absorbance and scattering spectral change of aptamer-modified AuNPs by thrombin addition
Figure 3  Thrombin detection by digital image analysis of the DFM images
Figure 4 Thrombin detection in coloured solution using DFM
Aptamer-modified gold nanoparticles

Absorbance

Light Scattering

Digital color analysis of dark field image

Molecular sensing