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An optical biosensing platform for proteinase activity using gold nanoparticles

Yao-Chen Chuang, Jung-Chun Li, Sz-Hau Chen, Ting-Yu Liu, Ching-Han Kuo, Wei-Ting Huang, Chih-Sheng Lin*

Department of Biological Science and Technology, National Chiao Tung University, Hsinchu 30068, Taiwan

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ABSTRACT

The surface plasmon resonance (SPR) wavelength of colloidal gold nanoparticles (AuNPs) can vary when the AuNPs aggregate, have different sizes or shapes, or are modified with chemical molecules. In this study, an optical biosensing platform for a proteinase activity assay was established based on the SPR property of AuNPs. The 13-nm AuNPs were modified with gelatin (AuNPs-gelatin) as a proteinase substrate and subsequently modified with 6-mercaptohexan-1-ol (MCH) (AuNPs/MCH-gelatin). After proteinase (trypsin or gelatinase) digestion, the AuNPs lose shelter, and MCH increases the attractive force between the modified AuNPs. Therefore, the AuNPs gradually move closer to each other, resulting in AuNPs aggregation. The AuNPs aggregation can be monitored by the red shift of surface plasmon absorption and a visible color change of the AuNPs is from red to blue. Such a color change can be observed with the naked eye. For detection, the absorption ratio, A_{625}/A_{525}, of the reacted AuNPs solution can be used to estimate quantitatively the proteinase activity. A linear correlation has been established with trypsin activity at concentrations from 1.25×10^{-1} to 1.25×10^{2} U and matrix metalloproteinase-2 activity at concentrations from 50 ng/mL to 600 ng/mL.

1. Introduction

The degradation of substrates by proteinase plays an important role in most metabolic and pathophysiological pathways. For example, a family of zinc-dependent proteinases, matrix metalloproteinases (MMPs), play key roles in several biological processes, including cell proliferation and migration, inflammation, and tissue remodeling [1,2]. In particular, because of their significant role in promoting cancer processes and cardiac disease [2–5], MMPs have become important targets for clinical tumor and heart disease diagnosis. Therefore, a sensitive and convenient assay for specific proteinase activity is needed for application to clinical diagnosis. Standard assays for proteinase activity include those based on substrate zymography, radioisotopes, or fluorogenic substrates. However, these techniques are time-consuming and require specific instrumentation; furthermore, labeled-substrates are expensive and inconvenient. Therefore a label-free and convenient method should be established for the assays of proteinase.

Nanoparticles, special gold nanoparticles (AuNPs), are the materials of interest in a rapidly developing area of biosensor [6–8]. AuNPs exhibit strong surface plasmon resonance (SPR) that depends on the size of AuNPs and the relative distance between AuNPs [9–11]. A colorimetric assay using the aggregation of AuNPs is a simple and sensitive method that can be used to detect biomolecules such as proteins, nucleic acids, enzymes, and cells [12–15]. Spherical AuNPs with an interparticle distance larger than the average particle diameter appears red in color. These particles' color can change from red to blue when the interparticle distance becomes smaller than the average particle diameter due to aggregation [16,17].

Currently, most of the AuNPs-based colorimetric assays for nucleic acids and enzymes detection are mainly dependent on the properties of enzyme catalysis, DNA hybridization [18,19], antigen–antibody reaction [20,21] and molecule interactions (such as protein–ligand interaction) [22–24] to induce a change in the AuNPs dispersion or AuNPs aggregation. Most of the AuNPs-based diagnoses using detection of enzyme activity are mainly dependent on enzyme catalysis to induce the change in AuNPs aggregation [25,26]. However, the use of AuNPs to establish a platform for the detection of enzyme activity faces some difficulties. The colloidal stabilization/aggregation phenomena of functionalized-AuNPs are rather complicated. For example, the charge and structure of proteins must be considered when discussing the stabilization/aggregation of protein-modified AuNPs. Additionally, the ion concentration in the reaction solution can affect enzyme activity

* Corresponding author. Department of Biological Science and Technology, National Chiao Tung University, No.75 Po-Ai Street, Hsinchu 30068, Taiwan. Tel.: +886 3 5131338; fax: +886 3 5729288.
E-mail address: lincs@mail.nctu.edu.tw (C.-S. Lin).

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and AuNPs stability. Therefore, in applying an AuNPs-based biosensing platform for the detection of enzyme activity, the modification process could promote monodispersed AuNPs to aggregate, in addition to the cations, which are supplied by the reaction buffer could also induce the aggregation of AuNPs.

It has been reported that macromolecules can be grafted onto the surfaces of colloidal AuNPs [27,28]. In this research, macromolecules were functionalized onto the AuNPs as the substrate and also provided a steric repulsion effect to prevent the AuNPs from coming into close contact [28,29]. Although the macromolecules protected the AuNPs stability, they also lowered the sensitivity of the AuNPs-based sensing methods. To overcome these serious problems, we have designed an AuNPs-based platform to assay the proteinase activity. In the present study, a biomolecule element was modified onto the AuNPs as a substrate, which kept the modified AuNPs stably suspended in solution. In addition, molecules of 6-mercaptohexan-1-ol (MCH) were modified on the AuNPs and played a role as an "inducer" to increase the attraction among the AuNPs. MCH served not only to block the surface space to avoid peptide absorption on the AuNPs but also to increase the attraction among the AuNPs. With the MCH modification, the AuNPs-based biosensing platform is more efficient when applied in an enzymatic assay of proteinase activity.

2. Experimental section

2.1. Chemicals

Sodium citrate was obtained from Merck (Darmstadt, Germany). Tris–HCl was purchased from Chemicon (Invitrogen, San Diego, LA, USA). Agarose and 10× Tris–Borate–EDTA buffer were purchased from Amresco (Cleveland, OH, USA). Sodium chloride (NaCl), calcium chloride ($CaCl_2$), MCH, Triton X-100, Tris–HCl, type A gelatin and hydrogen tetrachloroaurate (III) ($HAuCl_4 \cdot 3H_2O$) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Trypsin, MMP-2, MMP-7 and MMP-9 were purchased from Sigma–Aldrich and MMP-1 was purchased from ProSpec (Rehovot, Israel). All chemicals were of analytical grade. Nanopure water was obtained by passing twice-distilled water through a Milli-Q system (18 MΩ cm; Millipore, Bedford, MA, USA).

2.2. Synthesis and modification of AuNPs

AuNPs were prepared by citrate reduction of $HAuCl_4 \cdot 3H_2O$ according to the reported procedure [30]. A 50 mL aqueous solution consisting of 2.5 mM $HAuCl_4 \cdot 3H_2O$ was brought to a vigorous boil with stirring in a conical flask, and then 38.8 mM trisodium citrate (5 mL) was added rapidly to the solution. The solution was boiled for another 15 min, during which time its color changed from pale yellow to deep red. The solution was cooled to room temperature with continuous stirring. The sizes of the AuNPs were verified by scanning electron microscope (SEM) (JEOL JEM 100 CX electron microscope; JEOL, Tokyo, Japan) and dynamic light scattering (DLS) (BI-200SM; Brookhaven Instruments, Holtsville, NY, USA). Citrate-stabilized AuNPs appeared to be nearly monodisperse, with an average size of 13 ± 1.2 nm. A UV–vis absorption spectrophotometer (Apices Scientific, Boston, MA, USA) was used to measure the absorbance of AuNPs in citrate solutions [8].

2.3. Modification of AuNPs

The modification process for adding gelatin and MCH to the AuNPs was monitored by observing the spectral changes after the addition of gelatin and MCH. The gelatin and MCH were modified onto the AuNP surfaces according to the following procedures. For the preparation of gelatin-modified AuNPs (addressed as AuNPs-gelatin), an aliquot of the aqueous AuNPs solution (950 μL) was mixed with an aqueous gelatin solution (0.1%, 50 μL) and incubated at 37 °C for 2 h. The mixture was then centrifuged for 6 min at 14,000 × *g* to remove the excess gelatin. After two centrifuge/wash cycles, the colloidal AuNPs-gelatin was resuspended in 200 μL NTTC buffer (50 mM NaCl, 50 mM Tris–HCl pH 7.5, 5 μM $CaCl_2$, and 0.05% triton X-100). To prepare gelatin and MCH-modified AuNPs (addressed as AuNPs/MCH-gelatin), AuNPs solution (950 μL) mixed with gelatin (0.1%, 50 μL) was incubated at 37 °C for 2 h, and then MCH (10 μL, 1 mM) was added to the solution and incubated at 37 °C for another 2 h. The mixture was then centrifuged for 6 min at 14,000 × *g* to remove the excess gelatin and MCH. After two centrifuge/wash cycles, the AuNPs/MCH-gelatin colloid was resuspended in 200 μL NTTC buffer. The concentration of modified AuNPs was adjusted to 5 nM for further use in the proteinase activity assay.

2.4. Proteinase activity assay by gelatin-modified AuNPs

For the proteinase activity assay, 50 μL trypsin or MMPs (including MMP-1, MMP-2, MMP-7 and MMP-9) of varying concentration was added into 200 μL of gelatin-modified AuNPs, and the mixture was incubated at 37 °C. All of the solutions were analyzed with UV–vis absorption spectrophotometer, which recorded their spectral profiles and calculated the ratios of absorbance at 625 and 525 nm (A_{625}/A_{525}) after a 10 min reaction time for trypsin and a 30 min reaction time for MMPs.

2.5. MMPs activity assay by zymography

The MMPs activity assay was also performed according to our previously described zymography method [8]. Briefly, MMPs were activated by APMA and then mixed with 2× zymography sample buffer (0.125 M Tris–HCl, pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, and 0.005% bromophenol blue), incubated for 10 min at room temperature, and loaded into each lane of a SDS-PAGE (10%) gel containing 0.1 mg/mL gelatin. After electrophoresis, the gel was washed twice for 30 min in zymogram renaturing buffer (2.5% Triton X-100) with gentle agitation at room temperature to remove SDS, and then incubated at 37 °C for 8 h in reaction buffer (50 mM Tris–HCl, pH 7.4, 200 mM NaCl, and 5 mM $CaCl_2$). After staining with Coomassie brilliant blue, gelatinase activities were identified as clear zones against a blue background.

2.6. Electrophoresis analysis of gelatin-modified AuNPs

Gel electrophoresis analysis modified from Hanauer' protocol [31] was used to check the change in diameter of gelatin-modified AuNPs after proteinase digestion. Agarose gels were prepared with and immersed in 0.5× TBE buffer (Tris–Borate–EDTA buffer, prepared by diluting 10× stock solutions). Before loading the gels with the gelatin-modified AuNPs samples, the gelatin-modified AuNPs were mixed with sodium dodecyl sulfate (SDS), which imparts a negative charge on the gelatin coating of the AuNPs and causes them to run towards the positive electrode [32]. After proteinase digestion for 10 min, each sample of enzyme-treated AuNPs was loaded into one well of the gel. The gels were run in a horizontal electrophoresis system (Mini-Sub Cell GT; Biorad, Corston, UK) for 30 min at 110 V in 0.5× TBE buffer, followed by staining with Coomassie brilliant blue solution for 30 min. Finally, the gel was destained with destaining buffer. After electrophoresis, gel images were taken with a digital camera and processed with only small linear contrast adjustments in order to obtain a true representation of the visual gel appearance.

2.7. Assay of trypsin and MMPs activity by SDS-PAGE

The gelatin digestion activities of trypsin, MMP-1, MMP-2, MMP-7 and MMP-9 were assayed. One microliter of proteinase (100 μg/mL) was added into 20 μL of 0.1% gelatin solution containing 50 mM NaCl, 50 mM Tris–HCl pH 7.5, 5 μM $CaCl_2$, and 0.05% Triton X-100, and the assay mixture was further incubated at 37 °C for 1 h. The final pH of the assay mixtures was 7.5. Following incubation, the assay mixture was heated at 95 °C for 10 min in the presence of electrophoresis sample buffer and subjected to 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were stained with Coomassie brilliant blue R-250.

3. Results and discussion

3.1. Establishment of a colorimetric biosensing platform using AuNPs

AuNPs has been extensively applied in colorimetric biosensing methods for the detection of specific proteins and nucleic acids. Here, we have designed a simple proteinase colorimetric method using functionalized-AuNPs based on the optical property that causes the dispersed AuNPs solution to appear red in color, whereas the aggregated AuNPs solution appears as purple (or black).

Scheme 1 outlines the principal design of functionalized-AuNPs with/without the MCH modification used to assay the proteinase activity. MCH, containing a thiol group (−SH) and a hydroxyl group (−OH), plays an important role as an inducer in the development of the biosensing method. The molecules of MCH connect to the AuNPs through −SH substitution and the −OH exposed on AuNPs surface enhances the attraction force among AuNPs [8,18]. In addition, MCH molecules on the AuNPs also play a role as blockers [33]. MCH molecules cover the surface area of the AuNPs that are not conjugated with gelatin, and therefore the digested peptide will not adsorb on the AuNPs. For the proteinase assay, gelatin was modified onto AuNPs to generate AuNPs-gelatin. After that, AuNPs-gelatin was modified with MCH to produce AuNPs/MCH-gelatin.