Dispersed Gold Nanoparticle Array Produced by Apoferritins Utilizing Biomineralization and Chemical Conversion

Kazuyuki Nobusawa,† Naofumi Okamoto,‡ Karen Siew Ling Chong,§ Xi Lin,‖ Kenji Iwahori,‡ and Ichiro Yamashita‡,*

†Graduate School of Materials Science, Nara Institute of Science and Technology, 8916-5 Takayama, Ikoma, Nara 630-0192, Japan
‡Graduate School of Engineering, Osaka University, 8-1 Mihogaoka, Ibaraki, Osaka 567-0047, Japan
§Institute of Materials Research and Engineering, Agency for Science, Technology and Research, 2 Fusionopolis Way, Innovis, #08-03, 138634 Singapore
‖Biomedical Sciences Institute, Agency for Science, Technology and Research, 61 Biopolis Drive, #03-12 Proteos, 138673 Singapore

ABSTRACT: A new method for producing a dispersed gold nanoparticle (Au NP) array to anchor probe DNAs onto a DNA-sensing electrode has been developed. A homogenous gold sulfide (Au2S) core (precursor of Au NP) was biomineralized in the cavity of a mutant apoferritin (K98E) with enhanced negative outer-surface charges. We employed a slow chemical reaction system utilizing a stable cationic gold complex. K98E could attract the gold complex, and Au2S NPs were synthesized. K98E enabled dispersed placement of the synthesized Au2S core onto a cationic 3-aminopropyltriethoxysilane (APTES) layer on a substrate. UV–ozone treatment eliminated the protein shells and APTES layer. X-ray photoelectron spectroscopy confirmed that the Au2S core was reduced to Au NPs under the same treatment. Atomic force microscopy (AFM) clearly showed that the combination of apoferritin versatility, chemical system design, and UV–ozone treatment successfully produced a dispersed Au NP array on the substrate.

INTRODUCTION

In the field of genetic diagnostics based on DNA hybridization, a probe-DNA array with specific spatial regularity and controlled density is ideal.1–5 An excessively dense probe-DNA array prevents hybridization and results in a low detection efficiency. An appropriate distance between the probe DNAs provides a high hybridization efficiency. Controlled density leads to a reproducible signal-to-noise ratio (S/N), a low error rate, and quantitative analysis of DNA in practical sensing timelines. However, conventional approaches toward probe-DNA array fabrication, such as DNA solution droplet spotting systems, fail to regulate the number of probe DNAs on each spot.4 A new fabrication method for a probe-DNA array with specific interdistance and density is needed.

Gold nanoparticle (Au NPs) are widely used in the field of biological detection due to their versatility and biological inertness. It was also reported that Au NPs could anchor probe DNA through the formation of sulfide bonds between Au and thiol-terminated DNA.5–7 Therefore, it is highly desirable to develop a new process to place homogenous Au NPs on an electrode, with specific interdistances, to anchor probe DNA. The Au NP size should be as small as possible to control the number of attaching probe DNAs. A large electrostatic repulsive force among the probe DNAs would limit the number of attaching probe DNAs; ultimately, only one probe DNA attaches onto one Au NP.

In previous work, we produced a recombinant apoferritin (rFer), K98E, with enhancing negative outer-surface charges.8 K98E was composed of 24 subunits of a deletion mutant, which includes 9–175 amino acids of the L-chain apoferritin subunit and the 98th positively charged lysine residue, that was genetically replaced by negatively charged glutamic acid. We proved that K98E could synthesize homogenous iron oxide NPs internally. Taking advantage of the enhanced negative charges, we designed an elaborated electrostatic interaction system between K98E and a lithographically patterned 3-aminopropyltriethoxysilane (APTES) nanodisk array on a SiO2 substrate, 45 nm in diameter, with 200 nm intervals.1 Under specific solution conditions and Debye lengths, each K98E with an iron oxide NP was successfully placed one by one on the APTES nanodisk. Therefore, if Au NPs can be synthesized in K98E, K98E could deliver its accommodating homogenous Au NP to the APTES layer or nanodisk, as designed.

There are several works that report that native apoferritin (heterogeneous supramolecule)9 and exterior- and interior-surface-modified apoferritin10 produce Au NPs using anionic chloroauric ions and Au coordinated by four Cls as a gold source. However, it was hard for K98E, with enhanced negative...
charges, to attract anionic gold complexes. A new chemical system is required to realize Au NP synthesis.

In this work, we report an alternative method to place independent Au NPs less than 5 nm in diameter on the substrate. First, gold sulfide (Au$_2$S) NPs were synthesized in the K98E cavity using cationic instead of anionic gold complexes. Second, the obtained K98Es with Au$_2$S NP cores were allowed to adsorb as a dispersed array on an APTES-covered substrate. Finally, UV–ozone treatment was conducted to eliminate the APTES layer and protein shell and extract sulfur from Au$_2$S NPs simultaneously. The new method successfully produced a dispersed monolayer array of Au NPs.

**RESULTS AND DISCUSSION**

Apoferritin biomineralizes excessive iron ions into an iron oxide core inside the cavity and stores them until use, in vivo. Native apoferritin surface charges are thus located to attract positive ions around threefold channels connecting the outer solution and inner cavity. Positive iron ions are introduced through the narrow threefold channels, and the incorporated ions start crystallizing at collected negatively charged residue sites on the inner surface. This elucidated NP synthesis mechanism is generally accepted and the introduction of positively charged ions is the first key process. We reported that more than 20 kinds of NPs could be successfully formed in vitro by mimicking native biomineralization. On the contrary, preceding works on Au NP synthesis in native ferritin used a negative gold complex, Au coordinated by four Cls. It was plausible that the complex overcame local charge arrangement on the native ferritin outer surface. However, it was considered difficult for the K98E with enhancing negative outer-surface charges to attract the negative gold complex.

In our previous work, we synthesized Au$_2$S NPs in native apoferritin in vitro, where a new reaction system, the slow chemical reaction system (SCRY [CdS, CuS]), was introduced. In the SCRY, a stable cationic gold complex$^{15}$ with the formula Au[CS(NH$_2$)$_2$]$^+$ was used to suppress nucleation outside apoferritin. AuCl$_4^-$ ions and thiourea produces Au[CS(NH$_2$)$_2$]$^+$, which should be stable enough to remain in a dissolved state during the NP synthesis. It was expected that the negatively enhanced K98E would electrostatically accumulate Au[CS(NH$_2$)$_2$]$^+$, slowly decomposing into Au$_2$S or AuS.$^{12,16}$ The decomposed gold–sulfur ions in the vicinity of the threefold channels would have a chance to enter the cavity. Consequently, the Au$_2$S core would form inside the apoferritin.

The Au complex formation rate is critical for Au$_2$S NP synthesis. The rate depends greatly on the solution pH. We first studied the pH dependence. K98E (0.5 mg/mL), KAuCl$_4$ (2 mM), and thiourea (20 mM) in various buffer solutions at pH 6–9 were incubated overnight. NP synthesis was inspected using 400 nm optical absorbance because NPs show optical absorbance at 400 nm and the protein-shell absorbance was negligible (Figure 1a). The absorbance should be proportional to NP growth. The absorbance increased with higher pH and reached a maximum at pH 8.0 (Figure 1b). This tendency was in good agreement with our previous work with native ferritin. This is reasonable because the rates had no relation with the ferritin surfaces.

Second, the other important factor for the Au complex formation rate, thiourea concentration, was investigated. Thiourea stabilized the gold complex and slowed the decomposition rate. K98E (0.5 mg/mL), KAuCl$_4$ (2 mM), and thiourea (20 mM) in various buffer solutions at pH 6–9 were incubated overnight, and the core formation was checked. The NP formation increased in proportion with the thiourea concentration (Figure 1c) and saturated over 30 mM. A moderately excess amount of thiourea stabilized the gold complex and suppressed nucleation in the bulk solution, which
led to an increase in NP formation. In the case of a much higher concentration, the decomposition of the complex was too slow to synthesize NPs overnight. From these results, we concluded that the most appropriate synthetic conditions were 0.5 mg/mL K98E, 2 mM KAuCl₄, and 30 mM thiourea in TAPS, pH 8.

Figure 2 shows transmission electron microscopy (TEM) images of the synthesized NPs inside K98E. K98E stained with aurothioglucose shows dispersed black dots and surrounding white rings, 10 nm in diameter. As aurothioglucose could not enter the cavity, the black dots should be the synthesized inorganic NPs, Au₂S. The white ring diameter agreed well with the K98E protein diameter (12 nm) and thus these white rings were considered to be negatively stained K98E protein shells (Figure 2a). Bare Au₂S NPs quickly aggregate in aqueous solutions, but there were no NP aggregates, which assures that the NPs are protected by a protein shell. Figure 2b shows the area in which ferritin was highly condensed. Inorganic NPs were separated by the protein shell (invisible because of no staining). This indicates that NPs would disperse even in highly dense ferritin areas and that protein-shell elimination would produce a dispersed two-dimensional NP array. A high-resolution TEM image without staining shows that the NPs were generally spherical but had various shapes (Figure 2c). Additionally, a clear lattice fringe at some regions of the NPs was observed (Figure 2c). The lattice fringe did not appear across the whole area. This strongly indicates that the NPs were a collection of single crystals with some parts in an amorphous-

Figure 3. XPS spectra of Au₂S–K98E deposited on an APTES-modified Si wafer before/after UV–ozone treatment in the (a) Au 4f, (b) S 2p, (c) C 1s, and (d) N 1s regions. The spectra represent (i) as-prepared and (ii) UV–ozone-treated samples.
like state. It is generally accepted that there are many nucleation sites on the inner surface and nucleation occurs simultaneously. Some of the nuclei grow into a core. Therefore, it was plausible that the artificial Au2S synthesis had multiple nucleation events. There were no NPs larger than 7 nm, and the average diameter was determined from the nonstained TEM images as roughly 6 nm. The same sample was used in the following X-ray photoelectron spectroscopy (XPS) and atomic force microscopy (AFM) measurements.

XPS measurements were performed to evaluate the elemental compositions of the synthesized NPs. XPS analyzes the elemental composition and chemical or ionic binding states of elements in a material. Because XPS can detect a depth of only several nanometers, we fixed the K98E with NPs in a monolayer manner on a cationic APTES-modified Si wafer. From narrow binding energy range measurements (Figure 3), Au 4f and S 2p signals were clearly observed. The binding energies (BEs) of Au 4f7/2 and 4f5/2 were 83.7 and 87.4 eV, respectively, at which the full width at half-maximum (FWHM) was 1.1 eV. Although the BE and FWHM at the 4f7/2 peak are almost the same as those of Au0, it is uncertain whether the oxidation state of Au inside K98E is assigned to typical Au0. Energy shifts in the oxidation of gold have been reported around +1 to 2.4 eV in relation to zerovalent states, but these data were not in agreement with our results, which were +0.5 eV after UV−ozone treatment.

The signal from sulfur disappeared completely, indicating that Au2S could be decomposed to Au. There was a slight upshift in the Au signal from the initial state after UV−ozone treatments. The Au 4f7/2 BE was positioned at 84.2 eV. One of the possible reasons is due to the oxidation of Au. UV−ozone or O2 plasma treatment could oxidize Au clusters, NPs, and flat surfaces and mostly caused the formation of Au2O3.22,27 Energy shifts in the oxidation of gold have been reported around +1 to 2.4 eV in relation to zerovalent states, but these data were not in agreement with our results, which were +0.5 eV after UV−ozone treatment. Therefore, the slight shift in Au 4f spectra could not be attributed to the production of oxidized Au. On the other hand, it is known that the relative BE shift of Au depends on the type of support substrates and particle size.14,26 Oxides such as SiO2 and TiO2 cause positive shifts; for instance, the BEs of Au0 and Au3+ in 6 nm Au NPs placed on TiO2 were shifted by +0.35 and +0.97 eV, respectively, in bulk metallic gold.22 For the size effect, BE shifts of Au NPs from 1.5 to 6 nm in size on different substrates were observed. In our study, the produced Au contacted the SiO2 surface directly and the elimination of S atoms by UV−ozone treatment reduced its size. Therefore, it is reasonable to attribute the slight positive shift to surrounding circumstances and size reduction.

The distribution of NPs after UV−ozone treatment was studied by AFM observation (Figure 4). Using the APTES layer for the placement of Au2S−K98E, ferritin was electrostatically adsorbed and high-density adsorption was realized. The ferritins were isolated independently on the substrate (Figure 4a). The AFM image height profile showed the maximum height difference was ca. 10 nm, which indicates that the ferritin consisted of a monolayer without forming large

Figure 4. AFM images of Au2S−K98E on an APTES-modified Si wafer (a) before and (b) after UV−ozone treatment.
agglomerates. After UV–ozone treatment, the NPs remained independent and there was no NP aggregate. The height profile showed that the particle size was reduced to less than 5 nm (Figure 4b). The results clearly showed that a dispersed monolayer array of Au NPs was produced.

The obtained SiO₂ substrate with a Au NP array was used to anchor single-stranded DNA (ssDNA). A thiol-terminated 19-mer ssDNA solution was incubated overnight on the substrate. A SiO₂ substrate without a Au NP array was also underwent the same procedure as a control. After removing unreacted ssDNA, XPS measurements of both substrates were performed to check DNA fixation. The XPS measurement results are shown in Figure 5. In the P 2p region, it showed that there was a peak for the substrate with the array at around 134 eV, and the substrate without the array had a background noise level peak. The result suggested that Au NP array anchored ssDNA, which has phosphate backbones. N 1s signals, which come from bases, showed a clear difference. The substrate with the array showed a peak at around 400 eV, and the peak from the substrate without the array is hard to distinguish. These two results confirmed that the ssDNAs were fixed on the substrate with a Au NP array. The results demonstrated that probe DNAs can be fixed using the process described in this contribution.

### CONCLUSIONS

For applications in DNA sensing, precisely regulated placement of Au NPs acting as anchorages for probe DNA is a key technique. The rFer K98E could place an inner NP onto lithography-patterned cationic nanodisks. However, the negatively charged K98E could not synthesize Au NPs using a typical anionic gold complex. We employed the SCRY and introduced a stable cationic gold complex with thiourea, SC(NH₂)₂, as a sulfur ion source, which also acts as a ligand. The basic reaction conditions were set as follows: 0.5 mg/mL K98E, 2 mM KAuCl₄ and 30 mM thiourea in 50 or 100 mM TAPS buffer solution at pH 8. The effect of solution pH on core formation was investigated using phosphate, 2-(N-morpholino)ethanesulfonic acid, and Tris buffer solutions. The solution mixture containing KAuCl₄ and thiourea was slowly added into the K98E buffer solution, and the resultant homogenous solution was incubated overnight at room temperature. After incubation, precipitates were discarded by centrifugation (15000g, 15 min), and the supernatant was purified by size-exclusion chromatography (SEC), with 50 mM Tris (pH 8.0) as an elution buffer. After SEC, the fraction was further purified by density-gradient centrifugation (DGC), with sucrose density gradients of 15, 30, and 60%. The samples were stored at 4 °C.

After SEC, the solution pH and thiourea concentration dependencies on core formation in K98E were investigated. The concentrations of K98E after SEC were set at 0.4 mg/mL using the Bradford assay method, and the optical absorbance at 400 nm (cell length = 1 cm) was measured by UV–vis spectroscopy. For XPS and AFM analyses, the ferritin (apoferitin with core) with the eluents after DGC were used, for which the estimated absorbance at 400 nm was over 4, as the concentration of K98E was 0.4 mg/mL. The second core-formation reaction was also processed after SEC purification of the first core reaction with about half the amount of reagents compared to that in the first reaction. The samples after the first and second core-formation reactions showed no obvious difference in the absorbance at 400 nm.

The quality of NPs in K98E was studied by TEM (JEOL JEM-2200FS and JEM-3100FEF). The ferritin molecules were stained with aurothioglucose, which cannot enter the cavity. This staining method can distinguish ferritin with an Au₂S core from apoferritin. High-resolution measurements were also carried out to observe core lattice fringes.

**Characterization of the Synthesized NPs.** XPS (Shimadzu Kratos Axis I65) was employed to analyze the
elemental compositions of the synthesized NP cores. Mono Al Kα X-rays at 120 W were used to excite the photoelectrons. A hemispherical analyzer with a pass energy of 40 eV was used. All spectra were calibrated using C 1s = 284.8 eV as a reference. For quantitative analysis of the peak positions, line widths, and relative areas for the elements, the spectra were fitted with Gaussian functions after linear background subtraction, using original instrument software.

**Ferritin Adsorption and NP Array Production.** An APTES monolayer to fix Au₂S-K98E was prepared by the vapor-deposition process. A Si wafer with a thermally oxidized layer was cleaned by UV–ozone treatment for 10 min at 115 °C (SAMCO UV-1) and subsequently left in a container filled with APTES vapor with less than 10% relative humidity for 5–6 h. APTES-deposited substrates were rinsed with ethanol and milliQ water and then baked for 3 min at 110 °C. APTES-deposited substrates were rinsed with ethanol and milliQ water. NP placement was confirmed by AFM observation (SII SPI3800N/SPA400).

Protein-shell removal from Au₂S-K98E was performed by UV–ozone treatment. Au₂S-K98E-dispersed APTES substrates were set in the ozone generation chamber and exposed to UV–ozone treatment for 10 min at 115 °C. DNA immobilization onto Au NP Array. DNA immobilization was carried out using 5'-thiol-modified 19-mer ssDNA with a six-carbon linker. After the activation of the thiol group by tris(2-carboxyethyl)phosphine at pH 7.5, the reaction solution was purified using Bio-spin 6 chromatography columns (Bio-Rad). The resultant droplet (20 μL) was placed on the SiO₂ substrate with a Au NP array. The sample was incubated overnight at room temperature in a small container to avoid solution evaporation. After incubation, the substrate was immersed twice in the buffer solution for 20 min each, followed by rinsing four times with pure water to remove unreacted ssDNA completely. As a control, a substrate prepared using apoferritin without Au₂S NPs was also treated using the same procedure. Sample characterization and DNA fixation were performed by XPS measurements.

**AUTHOR INFORMATION**

Corresponding Author
*E-mail: yamashita@pmdp.arl.eng.osaka-u.ac.jp.*

ORCID
Ichiro Yamashita: 0000-0002-0010-6214

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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