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**A novel approach for mass production  
of Ag nanoparticles by using salmon milt DNA  
as templates**

「サケ白子DNAを鋳型として用いた銀ナノ粒子合成技術の開発」

Thesis Presented for the Degree of Doctor of Hokkaido University  
(Environmental Science)

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## ABSTRACT

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# ABSTRACT

While marine product industry is important in Japan, fishery waste is one of the serious environmental issues. As one of the proposed solutions, the effective use for food and industrial material has been promoted. Salmon milt is not popular for food and almost discarded as industrial waste. The salmon milt contains deoxyribonucleic acid (DNA) as a major component (around 8% wet weight), which can be efficiently extracted. DNA has been widely studied as one of the natural polymer due to its unique structure, which causes interactions with various molecules and ions. In recent years, biopolymers have been researched as a template to prepare nanomaterials such as metal nanowires and nanoparticles. Among the nanomaterials, silver (Ag) nanoparticles have grown in use as a conductive material and antibacterial material, and the market expansion is expected. DNA, which is one of the biopolymers, has a high affinity to Ag ions; and Ag nanoparticles/nanoclusters have been successfully prepared. However, the Ag nanoparticles in previous studies were not so much appropriate for practical use, because synthetic oligonucleotides and plasmid DNA, which are expensive in cost and difficult for mass production, were employed. The present study is aimed at the production of Ag nanoparticles using DNA, which is massively accessible from salmon milt, and evaluation of the particles for possible use as industrial materials.

This dissertation consists of three chapters. In Chapter 1, the research background was described as presented above. In Chapter 2, the method for mass production of Ag nanoparticles using salmon milt DNA and the resultant particle properties were described. Three types of salmon milt DNA, which differs from their molecular weight (ca. 20 000-10 000 000 Da), were examined and comparisons of the obtained nanoparticles under different conditions were executed. The concentrations of

DNA and AgNO<sub>3</sub> solutions and their mixture ratio were considered to prepare the nanoparticles with high Ag content in high concentration. The molecular weight affected the optimum concentration to prepare nanoparticles and their particle size. Then DNA with the lowest molecular weight used led to the Ag nanoparticles with the finest particle size and the highest Ag content,  $5.3 \times 10^{-2}$  mol/L. The resultant Ag nanoparticles were spherical with the diameter mostly less than 10 nm, and contained as high as over 85 wt% Ag. The zeta-potential of the Ag nanoparticles in water was -73.82 mV, which was negatively charged, similar to DNA. The absolute value was higher than that of DNA, -42.17 mV. This result suggested that the surfaces of the Ag nanoparticles were covered with DNA, which led to the high stabilization in aqueous media. The Ag nanoparticles were favored with high dispersion stability in water as long as 6-month room-temperature standing. These results suggest that the Ag nanoparticles obtained in the present method have an advantage in using the properties of fine nanoparticles in the various fields.

In Chapter 3, antibacterial function of the Ag nanoparticles was investigated for industrial use. The Ag nanoparticles had the growth inhibition effect against both *S. aureus* as Gram-positive bacteria and *E. coli* as Gram-negative bacteria with a concentration-dependent manner. The Ag nanoparticles were immobilized on cationized cotton fabric via phosphate group of DNA in the concentration range from 10 to 34 000 ppm as Ag content. The fabric showed enough inhibitory and killing effect against *E. coli* at a concentration of 10 ppm as Ag. These results suggest that the Ag nanoparticles have the sufficient potential to be used as an antibacterial material.

In conclusion, DNA extracted from salmon milt is a viable template for the preparation of stable Ag nanoparticle dispersion. The method presented is inexpensive and simple, highly suitable for mass production, and thus would benefit the utility of Ag

nanoparticles in various fields. In particular, the Ag nanoparticles may have potential to add the antibacterial function to various textile products, which is expected as one of the most important and widely applicable industrial products.

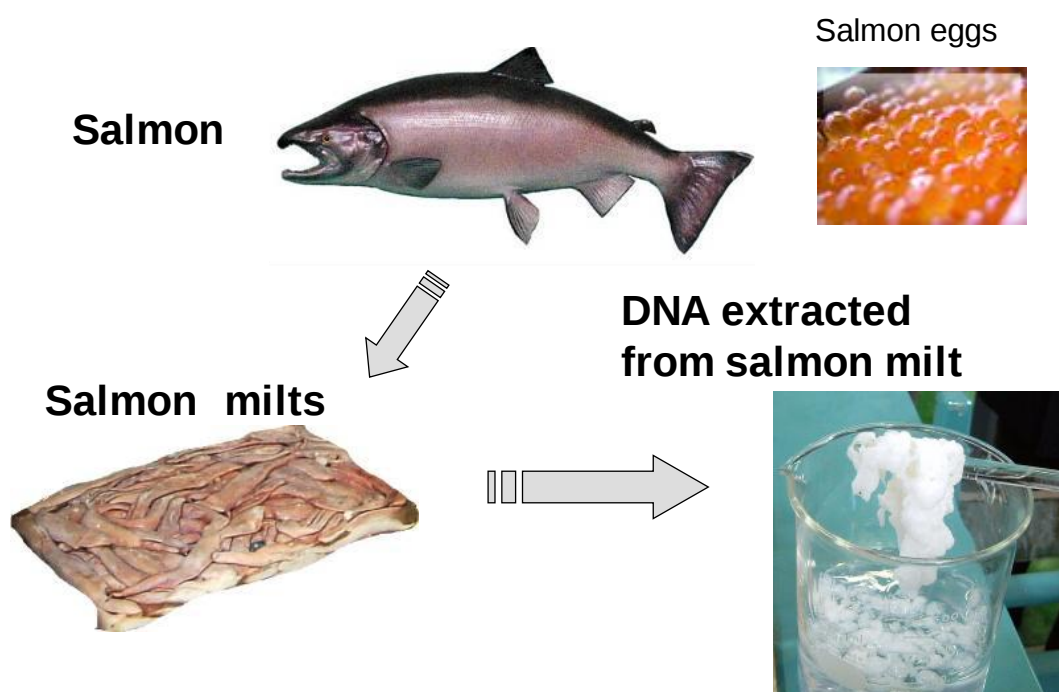
# Chapter 1

## General Introduction

## 1.1 DNA

### Salmon milt DNA

Japan is an island country surrounded by the sea on all sides, and so fishing industry is one of the important industries. On the other hand, fishery waste is one of the serious environmental issues. There are various fishery wastes, such as sea shell, seaweed and internal organs of fish, etc. Then the effective use for food and industrial material have been promoted as one of the solutions to the issue.<sup>1-3</sup> For example, useful biopolymers, such as chitin and chitosan extracted from crab and prawn shells, collagen from fish skin and alginic acid from brown seaweed, have been produced.



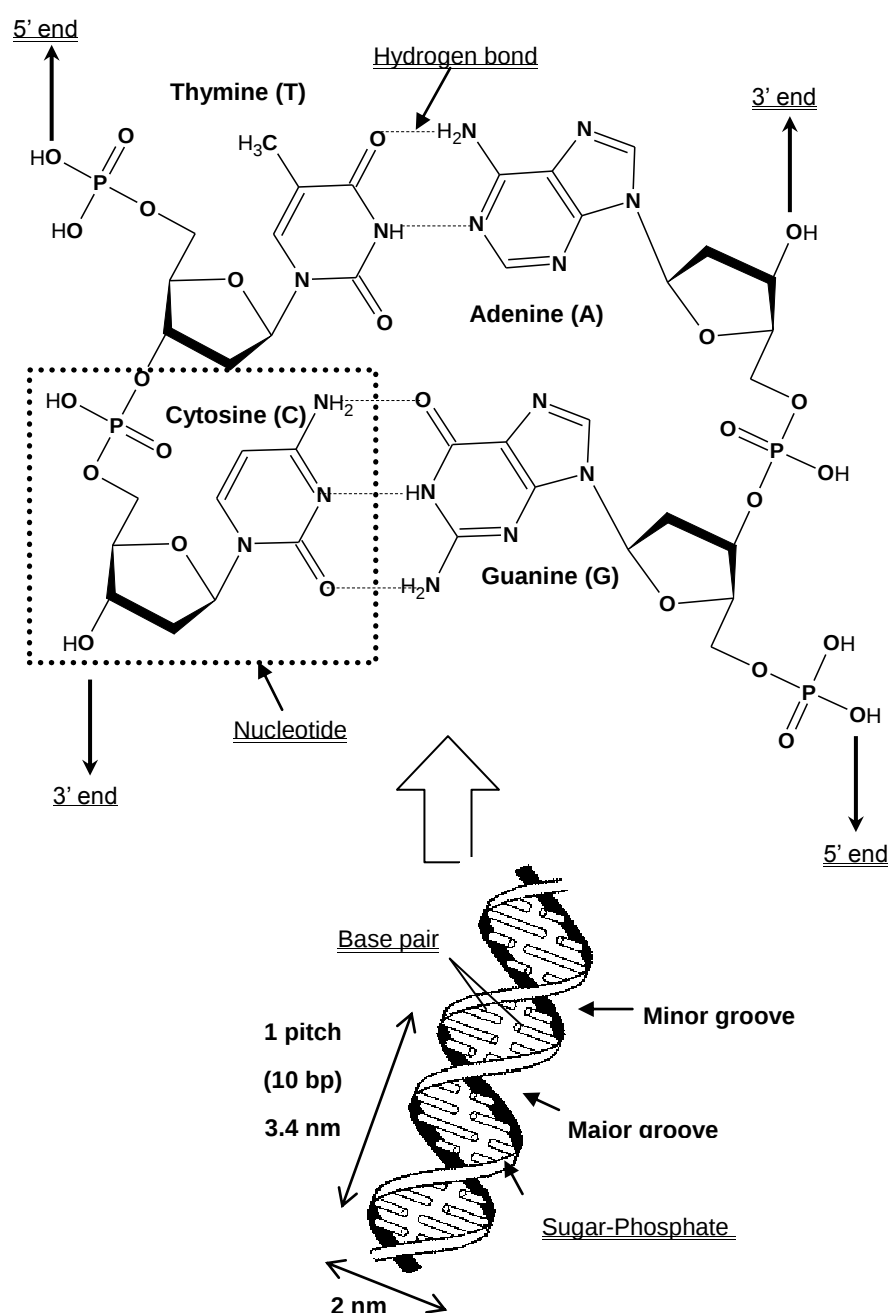
**Figure 1** Extraction of salmon milt DNA from salmon

Salmon is one of the most popular fishes and caught by more than 100 000 ton a year in Japan. Their meat and eggs are useful for food, meanwhile, their milt, which is male reproductive cell, is not popular for food and almost discarded as industrial waste. The salmon milt is obtained over 5 000 ton a year (estimated roughly based on the Statics of Agriculture, Forestry and Fisheries, in 2013) in Japan. Deoxyribonucleic acid (DNA) is one of the major components of milt and can be efficiently extracted. Efficient use of salmon milt DNA may contribute to solve one of the major environmental problems.

### **DNA structure and function**

DNA is the most important genetic components and exists in all lives and all cells as the vehicle of genetic information. Besides that, DNA has been investigated as one of the natural polymers. Since natural polymers often show specific functions which are unique and difficult to mimic by the general synthetic polymers, they have been attracting a great attention as functional materials. To apply DNA to functional materials, its molecular structure is an important key. DNA is a polynucleotide, molecules with backbones made of alternating sugars (deoxyribose) and phosphate groups, with one of four nucleobases: adenine (A), guanine (G), cytosine (C) and thymine (T), attached to the sugars (Figure 2). Nucleobases inside DNA make highly specific base-pairing interactions: A and T, or C and G. Base-pairing can make double-stranded DNA from two complementary single-stranded DNA segments, and the double-stranded structure causes a highly specific molecular interaction via groove binding and intercalation

binding. The negatively charged phosphate backbone allows it to interact with cationic molecules and metal ions.<sup>4-6</sup> Some metal ions can be chelated by the aromatic nucleobases in DNA.<sup>7</sup> Through these interactions, DNA can be used for composite materials,<sup>4-6</sup> adsorbent for various chemicals<sup>8-10</sup> and nanoarchitecture,<sup>11-13</sup> etc.

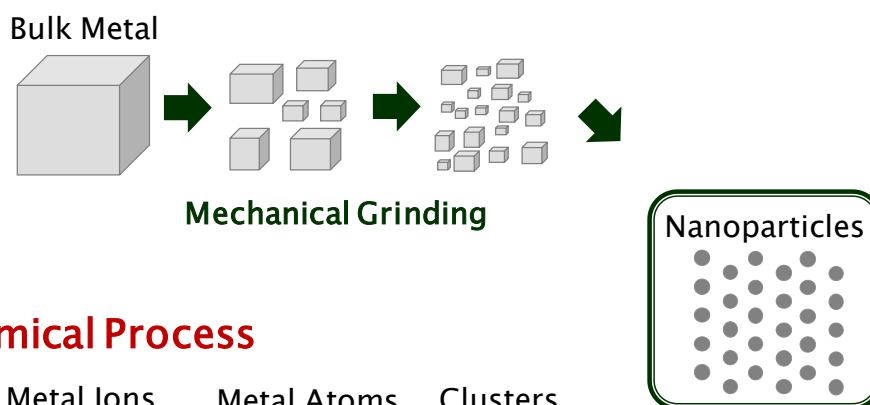


**Figure 2** Structure of DNA

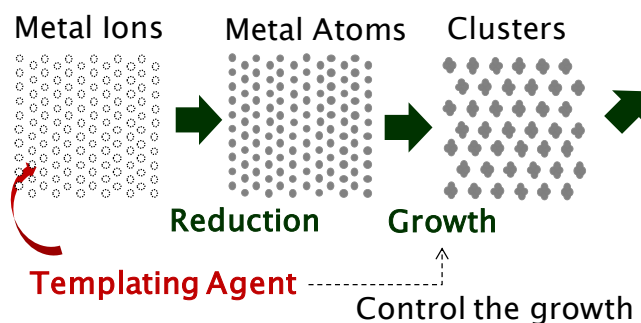
## 1.2 Ag nanoparticles

Metal nanoparticles have attracted much attention due to their optical, physical, and chemical properties that differ from the properties of the bulk metal.<sup>14-16</sup> Among the metal nanoparticles, silver (Ag) nanoparticles have the potential for applications in various areas such as catalysis,<sup>17,18</sup> optics,<sup>19,20</sup> electronics,<sup>21-23</sup> antimicrobial materials<sup>24,25</sup> and the other areas due to the unique properties derived from the quantum size effect in addition to the primary function of silver. Their fine size favors microstructure formation and their high specific surface area causes their efficient reaction.

### Physical Process



### Chemical Process



**Figure 3** Scheme of physical process and chemical process to prepare of metal nanoparticles.

Many methods have been applied to prepare metal nanoparticles (Figure 3). Especially the wet-chemical reduction method has been widely applied because of its ease, low cost and tolerance for a variety of reaction conditions. In the method, the final nanoparticles were prepared by controlling the rate of particle growth after reduction by using metal complex as a precursor and adding a templating agent to metal ions. Due to the high specific surface area and high surface energy of nanoparticles, protective agent is often necessary as a stabilizer to prevent the aggregation of the particles. Since the protective agent exists at the surface of the particles, it contributes to the properties of the particles.

### 1.3 DNA template based Ag nanoparticles

DNA has been studied on the reactions with protons and metal ions in the course of unveiling its intracellular behavior as the hereditary determinant.<sup>7,26</sup> DNA can bind metal ions through different types of interactions. The negatively charged phosphate backbone interacts extensively with positively charged metal cations through simple electrostatic attraction.<sup>12</sup> Alternatively, empty orbitals of the metal cations can accept electrons from phosphate oxygen or from the nucleobase moieties forming coordination complexes.<sup>12</sup>  $\text{Ag}^+$ ,  $\text{Hg}^{2+}$ , and  $\text{Pt}^{2+}$  form a complex exclusively with nucleobases.

Ag nanoparticles have been successfully prepared by chemical reduction of the DNA-Ag(I) complex.<sup>18,27-31</sup> Ag nanoparticles/nanoclusters have been prepared with synthetic oligonucleotides,<sup>18,27-29</sup> plasmid DNA,<sup>30</sup> and bacteriophage T4 DNA,<sup>31</sup> which have the uniform molecular weight and sequence. Various DNA-templated Ag nanoparticles demonstrated the unique properties, such as chiroptical property,<sup>28</sup> optical property,<sup>29</sup> and catalytic performance.<sup>18</sup> The hydrophilic property of the negatively charged phosphate backbone of DNA may act as a protective and dispersing agent for Ag nanoparticles. In addition, the phosphate groups may react with cationic molecules and metal ions to form various DNA composite materials, and can be immobilized to cationic material. Thus DNA is useful not only as a template but also to protect and functionalize the nanoparticles. However, due to the high cost and difficulty in mass production of DNA, the Ag nanoparticles in previous studies were not so appropriate for

the practical use.

The present study is aimed to develop the production of Ag nanoparticles using salmon milt-extracted DNA massively available as industrial materials. Unlike synthetic oligonucleotide/DNA, natural DNA has some distribution in molecular weight, depending on raw materials and extraction conditions. Three types of salmon milt DNA, which differs in their molecular weight, were examined and the comparisons of the obtained nanoparticles under different conditions were executed. The particle properties such as the appearance, composition, crystal structure, thermal behavior, aqueous dispersity and stability were investigated to consider the application as the industrial materials. In addition, the practical utility of the particles for antibacterial materials was evaluated.

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## Chapter 2

Salmon milt DNA as a template for the mass  
production of Ag nanoparticles

## 2.1 Abstract

A wet-chemical approach using DNA extracted from salmon milt as a template to mass produce Ag nanoparticles was developed. Spherical Ag nanoparticles with a main diameter of less than 10 nm were obtained. The concentration of Ag nanoparticles in the as-produced colloidal suspension was as high as  $5.3 \times 10^{-2}$  mol/L. This simple and effective procedure should offer an alternative route to the mass production of Ag nanoparticles for practical applications.

## 2.2 Introduction

Many potential applications for the remarkable electrical and chemical properties of Ag nanoparticles have been proposed.<sup>1-11</sup> The so-called wet-chemical reduction method has been the standard technique for the production of Ag nanoparticles because of its simplicity and high production efficiency. In most wet-chemical reduction methods, biopolymers such as polysaccharides,<sup>11-13</sup> proteins,<sup>14</sup> and nucleic acids<sup>7,15-19</sup> have been used. These biopolymers function as templates for the desired nano-sized structures of Ag nanoparticles. DNA, the most important biopolymer, is also capable of forming Ag nanoparticles through the formation of DNA-Ag(I) complexes. DNAs with a uniform molecular weight and a specific sequence (artificial oligonucleotides,<sup>7,15-17</sup> plasmid DNA,<sup>18</sup> and bacteriophage T4 DNA<sup>19</sup>) have been commonly used. However, there are difficulties in the mass production of these types of DNA.

In previous studies, Nishi and co-workers demonstrated that DNA could be obtained on an industrial scale by using salmon milt as the DNA source.<sup>20-23</sup> In this study, we have evaluated the possibility to use the salmon milt-based DNA as a template for the mass production of Ag nanoparticles. Three types of salmon milt-based DNA were used, each differing in molecular weight. DNA with an average molecular weight of approximately 20 000 Da (single-stranded) proved to be optimal for the reproducible production of Ag nanoparticles. The particle properties such as the appearance, composition, crystal structure, thermal behavior, aqueous dispersity and stability were

also investigated with the expectation as the industrial materials.

## 2.3 Materials and Methods

### Materials

Three types of salmon milt-based DNA were used throughout this study: i) low molecular weight DNA (LMw-DNA; molecular weight approximately 20 000 Da, single-stranded), ii) intermediate molecular weight DNA (IMw-DNA; molecular weight approximately 50 000-100 000 Da, mostly single-stranded), and iii) high molecular weight DNA (HMw-DNA; molecular weight over approximately 10 000 000 Da, double-stranded). These DNAs were obtained from Nisseibio Co., Ltd., Eniwa, Japan. The molecular weights of the DNAs were estimated by HPLC gel filtration for the LMw-DNA and gel electrophoresis for the IMw-DNA and HMw-DNA. The determination of double-stranded or single-stranded DNA was made by measuring the increase in absorbance at 260 nm upon heating the DNA solutions. Silver nitrate ( $\text{AgNO}_3$ , special grade) was purchased from Kishida Chemical Co., Ltd. (Osaka, Japan). Aqueous ammonia (10%), sodium borohydride ( $\text{NaBH}_4$ , chemical grade), and nitric acid ( $\text{HNO}_3$ , for analysis of poisonous metal grade) were obtained from Wako Pure Chemicals Industries Ltd. (Osaka, Japan). Potassium peroxydisulfate (for N and P analysis grade) was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Ultra-pure water (supplied from Milli-Q water purification systems, Merck Millipore, Billerica, MA, USA,  $>18 \text{ M}\Omega$ ) was used in all experiments.

### **Preparation of Ag nanoparticles**

The reactions of DNA ( $5 \times 10^{-5}$  mol/L nucleotide) with Ag(I) at a molar ratio of [nucleotide]:[Ag(I)] of 1:1-1:30 were monitored by UV-Vis spectroscopy (V-560, Jasco, Tokyo, Japan). DNA solutions (0.33-33 g/L, i.e.,  $10^{-3}$ - $10^{-1}$  mol/L nucleotide) were prepared by adding DNA to aqueous ammonia (1.0%). DNA solutions (0.33-33 g/L, i.e.,  $10^{-3}$ - $10^{-1}$  mol/L as nucleotides) were prepared by adding DNA to aqueous ammonia solution (1.0%). Several types of Ag(I)-DNA complexes were prepared by adding aqueous AgNO<sub>3</sub> solutions ( $10^{-2}$ -1 mol/L) to the DNA solutions at the volume ratio of 1:1-1:3, afterwards the states of mixed solutions were observed.

For the Ag nanoparticles preparation, the DNA solution ( $10^{-3}$  or  $10^{-2}$  mol/L) and aqueous AgNO<sub>3</sub> ( $10^{-2}$  or  $10^{-1}$  mol/L) were mixed at a volume ratio of 1:2 (molar ratio of 1:20), and magnetically stirred in an ice bath. Under continuous mixing,  $10^{-1}$  or 1 mol/L of NaBH<sub>4</sub> in aqueous ammonia (10%) was added to the solution; the molar ratio of NaBH<sub>4</sub> to Ag(I) was 1:1. After standing overnight, the supernatant, which included the Ag nanoparticles, was dialyzed using a dialysis membrane (molecular weight cut-off: 14 000, Viskase Companies Inc., Darien, IL, USA) for approximately 24 h.

### **Characterization of Ag nanoparticles**

UV-Vis spectroscopy was used to monitor the reactions between Ag(I), DNA and NaBH<sub>4</sub>. The sample solutions for the UV-Vis measurements were diluted 500-fold with water. The morphologies of the Ag nanoparticles were evaluated by visual observation, UV-Vis spectroscopy, transmission electron microscopy (TEM,

JEM-2000ES, JEOL, Akishima, Japan) and scanning transmission electron microscopy (STEM, HD-2000, Hitachi, Tokyo, Japan). The samples for the SEM/STEM observations were prepared by spotting the diluted dispersion on a lacey carbon film supported by a 400-mesh copper grid (Cu-400CN, Pacific Grid-Tech, San Francisco, CA, USA) and drying at room temperature.

The concentrations of Ag, P, and Na in the dispersion were measured with inductively coupled plasma atomic emission spectrometry (ICP-AES, ICPE-9000, Shimadzu, Kyoto, Japan). The samples were hydrolyzed in the presence of aqueous potassium peroxydisulfate (4.0%) at 120°C and then diluted to an adequate concentration with HNO<sub>3</sub> (10<sup>-1</sup> mol/L). The amount of Ag and DNA in the Ag nanoparticles was calculated from the concentrations measured by ICP-AES and from the solid weight after drying by heating.

The particle size and shape were observed with TEM and atomic force microscopy (AFM, picoscan2500, SII, Chiba, Japan). AFM samples were prepared by spotting the diluted dispersion on exfoliated mica and drying at room temperature. The size distribution was determined by measurement of the height profile of 500 particles in the AFM image. The size distribution of the Ag particles in aqueous dispersion was measured with a dynamic light scattering particles size analyzer (DLS, LB-550, Horiba, Kyoto, Japan), of which particle size range is 1 nm-6 μm. The aqueous dispersion was measured without dilution, and then the scattering light intensity distribution, volume distribution and number distribution were obtained. A detailed observation of the Ag nanoparticles was performed by high resolution observation using field emission

transmission electron microscope (FE-TEM, JEM-2010F, JEOL, Akishima, Japan). The crystal structure and size of Ag nanoparticles were analyzed by X-ray powder diffraction (XRD) measurement with an X-ray diffractometer (RINT 2000/PC, Rigaku Corporation, Akishima, Japan) with CuK $\alpha$  radiation operated at 40 kV and 40 mA.

The zeta-potential of the Ag nanoparticles in water was measured with a particle analyzer (Delsa<sup>TM</sup> Nano HC, Beckman Coulter, Brea, CA, USA). After confirming the dispersion of the samples by dynamic light-scattering, the zeta-potential was determined by electrophoretic light scattering and automatically calculated using the Smoluchowski equation. The sample solution was diluted 10-fold with water, and a flow cell was used for the measurement.

The thermal behavior of the Ag nanoparticles was investigated by thermogravimetry (TG, TG-DTA6200, SII) from 25°C to 1000°C at a heating rate of 5°C/min under an air atmosphere. In addition, the decomposition behavior of the DNA in the Ag nanoparticles was examined by a stepwise heating, maintaining each step for 4 h at 200°C, 300°C, 400°C, 500°C, and 600°C.

### **Long-term stability of the Ag nanoparticles in water**

An aqueous dispersion of Ag nanoparticles was stored without dilution in a plastic tube wrapped with aluminum foil to protect from light for 6 months at room temperature. The sample was then evaluated visually and by UV-Vis spectroscopy.

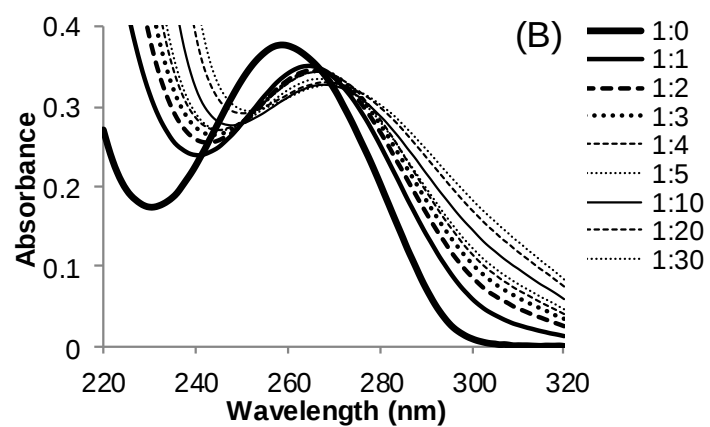
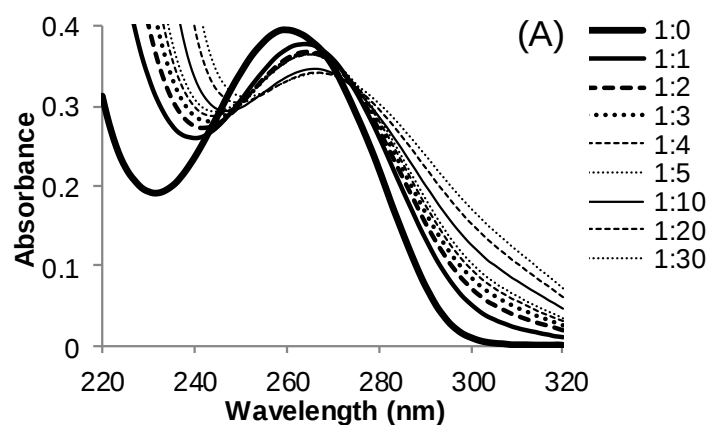
## 2.4 Results and discussion

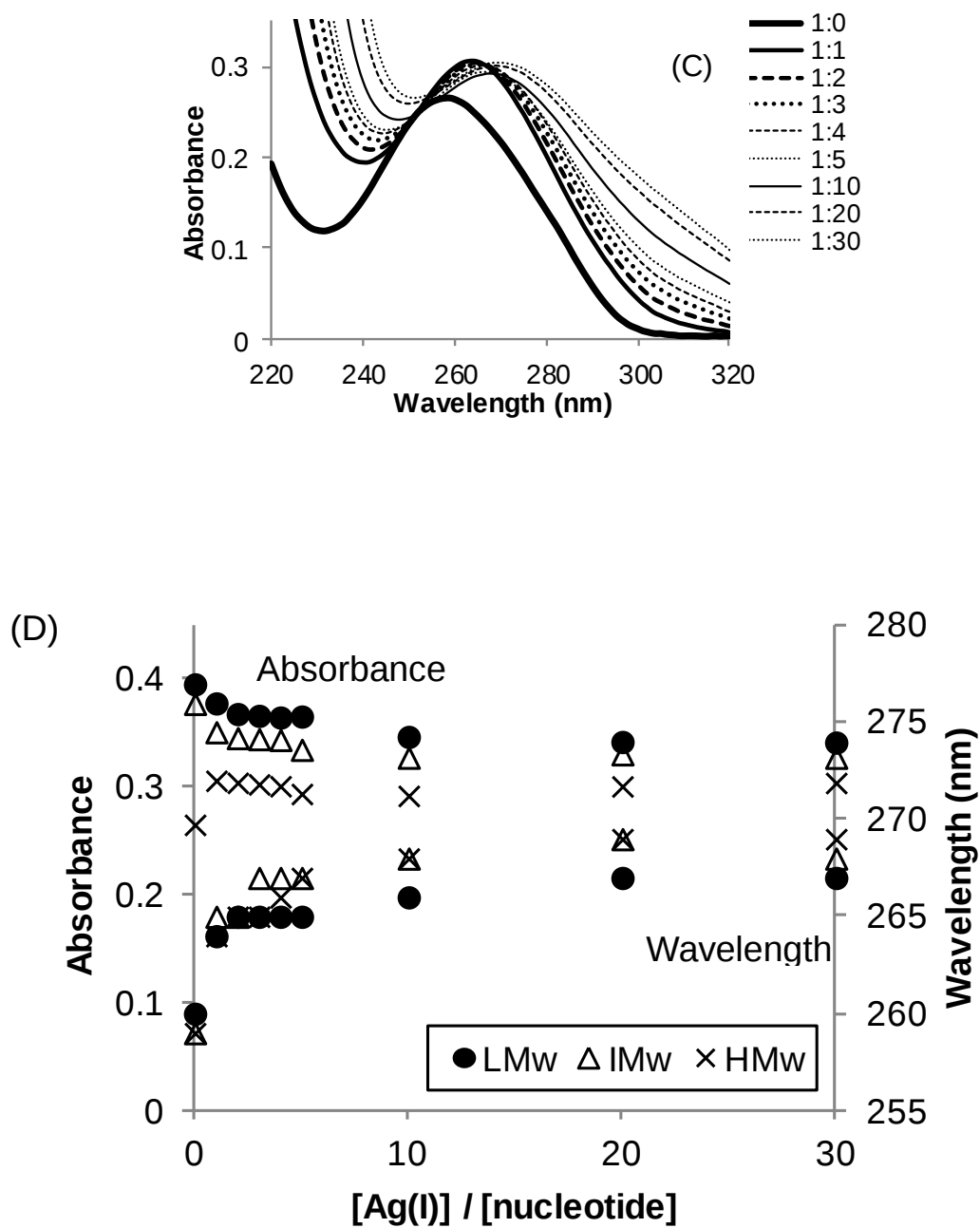
### Ag nanoparticles with salmon milt-based DNA as the template

DNA is capable of selectively binding Ag(I) with their nucleotide bases; this binding changes the wavelength of the maximum absorption of DNA.<sup>15,24,25</sup> In this study, the change in the absorption at 260 nm upon addition of Ag(I) (Figure 1) reveals the formation of the DNA-Ag(I) complex. The increase in absorbance observed only for the HMw-DNA (Figures 1C and 1D) was assumed to be induced by the denaturation of the double stranded DNA.<sup>24</sup> The absorption peak of the DNA shifted with increasing concentration of Ag(I), and then remained unchanged (Figure 1D). These results suggest that the binding of Ag(I) to DNA did not increase as the [Ag(I)]/[nucleotide] ratio rose from 10-fold to 20 or 30-fold; that is, only the amount of unbound Ag(I) increased.

To prepare homogeneously dispersed nanoparticles by addition of reductant, the mixed solution of DNA and AgNO<sub>3</sub> should be homogeneous without change from original state of DNA solution. Various changes, for example, the formation of turbidity or gelation, were induced by the addition of AgNO<sub>3</sub> (Table 1). Previously some conformational changes of DNA, for both single-stranded and double-stranded, were reportedly induced by adding metal ions.<sup>19,26-28</sup> Zinchenko and coworker reported that T4 DNA molecules (166 000 base pairs, 10<sup>-8</sup> mol/L) remained in an unfolded coil state with AgNO<sub>3</sub> up to a concentration of 10<sup>-6</sup> mol/L, and then a further increase in the AgNO<sub>3</sub> concentration to 10<sup>-4</sup> mol/L induced a gradual and significant shrinking of DNA, which observed by the fluorescence microscopy images.<sup>19</sup> Although, the case with

AgNO<sub>3</sub> above 10<sup>-4</sup> mol/L was not done due to release of dye molecule as an indicator from double helix, it was assumed that much conformational change was induced with increasing the concentration. Similar results were also reported for other metal ions.<sup>26-28</sup>





**Figure 1** Evolution of the maximum absorption of DNA in the presence of different concentrations of Ag(I): (A) LMw-DNA, (B) IMw-DNA, and (C) HMw-DNA. (D) Plot of the absorbance and wavelength of the maximum absorption vs. the molar ratio  $[Ag]:[nucleotide]$  from 1:1-1:30.

In our experiments, conformational change of DNA seemed to be induced in high concentration of DNA or high-molecular-weight DNA since the white turbidity and gelation were observed. Aqueous ammonia (1.0%) was used to dissolve DNA well and make the complex well above pH 7 as suggested.<sup>25</sup> That brownish gray turbidity, occurred in higher molar ratio to DNA, was assumed silver(I) oxide, and the unreacted Ag(I) transformed into diamine silver complex in mixed solution. The possible highest concentration of AgNO<sub>3</sub> was estimated to be 10<sup>-1</sup> mol/L because all DNA solutions were visually changed in the case of 1 mol/L AgNO<sub>3</sub>. Meanwhile a lower concentration of DNA solution is desirable to prepare Ag nanoparticles with a higher Ag content. Preparation with 10<sup>-1</sup> mol/L AgNO<sub>3</sub> solution and 10<sup>-3</sup> mol/L LMw DNA solution at the volume ratio of 1:1 was performed as a preliminary examination: a large amount of precipitate was produced, and the solution color got a little changed, indicating that most Ag(I) precipitated without forming the nano-sized particles. It suggested that DNA amount as mentioned above was not enough. Thus the required concentration of DNA would be 10<sup>-2</sup> mol/L or even more with 10<sup>-1</sup> mol/L AgNO<sub>3</sub> to prepare Ag nanoparticles.

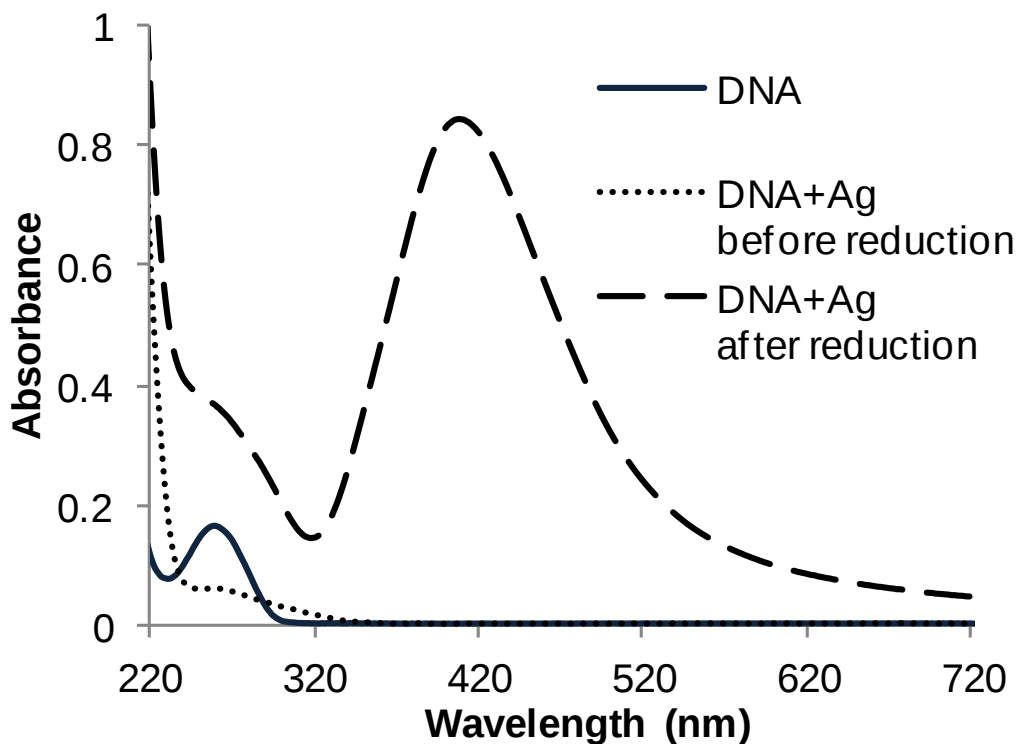
Next, to optimize the volume ratio, 10<sup>-2</sup> mol/L LMw-DNA solution and 10<sup>-1</sup> mol/L AgNO<sub>3</sub> solution were mixed at volume ratios of 1:1, 1:2, and 1:3, and then 1 mol/L NaBH<sub>4</sub> solution was added in a molar equivalence to Ag(I). The solution with a volume ratio of 1:2 changed from clear to dark brown in color with very small precipitates. UV-Vis absorption spectra of the DNA solution, the solution of DNA and AgNO<sub>3</sub>, and the solution after the reduction are shown in Figure 2. The maximum absorption at approximately 260 nm is shifted after the addition of Ag(I), and the peak

at 408 nm, the typical plasmon resonance band of Ag nanoparticles, appeared after the reduction. In the case of the volume ratio of 1:3, a large amount of precipitation was observed, and a very small amount of Ag nanoparticles was produced.

**Table 1.** State of the aqueous DNA- AgNO<sub>3</sub> mixed solutions

|     |                       | Concentration of AgNO <sub>3</sub> (mol/L) |     |     |                  |     |     |     |
|-----|-----------------------|--------------------------------------------|-----|-----|------------------|-----|-----|-----|
|     |                       | 10 <sup>-2</sup>                           |     |     | 10 <sup>-1</sup> |     |     | 1   |
|     |                       | 1:1                                        | 1:2 | 1:3 | 1:1              | 1:2 | 1:3 | 1:1 |
| DNA | Concentration (mol/L) |                                            |     |     |                  |     |     |     |
| LMw | 10 <sup>-3</sup>      | -                                          | -   | -   | -                | -   | -   | ×   |
|     | 10 <sup>-2</sup>      | -                                          | -   | -   | -                | -   | -   | +   |
|     | 10 <sup>-1</sup>      |                                            |     |     | -                | -   | -   | ++  |
| IMw | 10 <sup>-3</sup>      | -                                          | -   | -   | -                | -   | ×   | ×   |
|     | 10 <sup>-2</sup>      | -                                          | -   | -   | -                | -   | -   | ×   |
|     | 10 <sup>-1</sup>      |                                            |     |     | -                | -   | +   | ++  |
| HMw | 10 <sup>-3</sup>      | -                                          | -   | -   | -                | -   | -   | ×   |
|     | 10 <sup>-2</sup>      | -                                          | -   | -   | +                | +   | +   | ×   |
|     | 10 <sup>-1</sup>      |                                            |     |     | +                | +   | ++  | ++  |

-, transparent liquid (without change); +, white turbidity; ×, brownish gray turbidity; ++, gelation.



**Figure 2** UV-Vis absorption spectra of the DNA and the DNA-Ag mixed solutions (before and after adding  $\text{NaBH}_4$ ).

Ag nanoparticles were also prepared using solutions of IMw-DNA ( $10^{-2}$  mol/L) and  $\text{AgNO}_3$  ( $10^{-1}$  mol/L), and HMw-DNA ( $10^{-3}$  mol/L) and  $\text{AgNO}_3$  ( $10^{-2}$  mol/L). The molar ratio of [nucleotide]:[Ag(I)]:[ $\text{BH}_4$ ] was chosen to be 1:20:20. Ag concentrations in the mixed solutions were calculated to be  $6.25 \times 10^{-3}$  mol/L when using the  $10^{-2}$  mol/L  $\text{AgNO}_3$  solution and  $6.25 \times 10^{-2}$  mol/L when using the  $10^{-1}$  mol/L  $\text{AgNO}_3$  solution. As denoted in Table 1, homogenous dark brown solutions were obtained under all conditions. Moreover, a slight turbidity was observed without clear phase separation

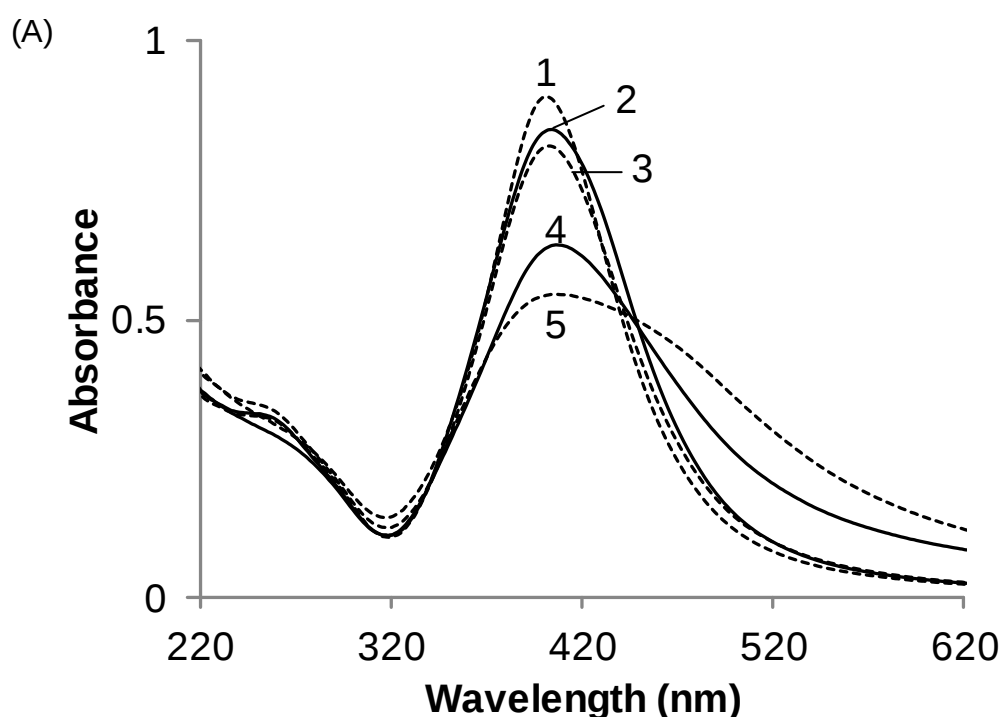
in the solutions with  $10^{-2}$  mol/L IMw-DNA (Table 2, no. 4) and  $10^{-3}$  mol/L HMw-DNA (Table 2, no. 5). Precipitation also occurred in the  $10^{-2}$  mol/L IMw-DNA solution (Table 2, no. 4), and the Ag concentration in the supernatant was above 46% (confirmed by ICP-AES). There was little precipitation in the  $10^{-2}$  mol/L LMw-DNA solution (Table 2, no. 2), and the Ag concentration was only approximately 3%.

**Table 2.** DNA and AgNO<sub>3</sub> solutions employed for the preparation of Ag nanoparticles and the states of the resultant solution after reduction.

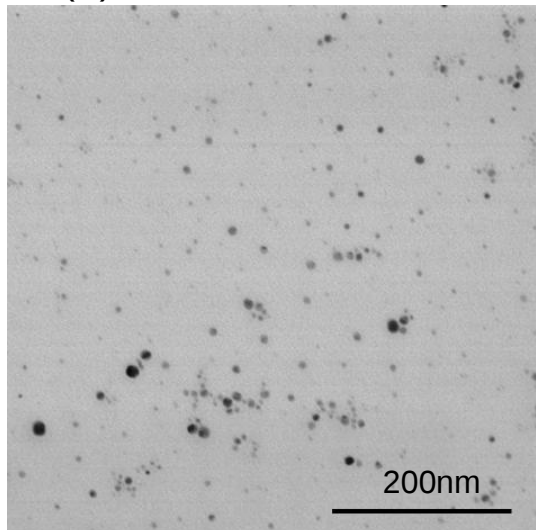
| No. | DNA  |                  | AgNO <sub>3</sub> | State of resultant solution                                       |
|-----|------|------------------|-------------------|-------------------------------------------------------------------|
|     | Type | Conc.<br>(mol/L) | Conc.<br>(mol/L)  |                                                                   |
| 1   | LMw  | $10^{-3}$        | $10^{-2}$         | homogenous dark brown solution                                    |
| 2   | LMw  | $10^{-2}$        | $10^{-1}$         | homogenous dark brown solution<br>slight precipitation            |
| 3   | IMw  | $10^{-3}$        | $10^{-2}$         | homogenous dark brown solution                                    |
| 4   | IMw  | $10^{-2}$        | $10^{-1}$         | homogenous dark brown solution<br>slight turbidity, precipitation |
| 5   | HMw  | $10^{-3}$        | $10^{-2}$         | homogenous dark brown solution<br>slight turbidity                |

The dark brown supernatants were purified by membrane dialysis, a simple method amenable to mass production. After purification, all the obtained solutions were dark brown and homogeneous in appearance. The Ag concentrations as estimated by ICP-AES were  $3.9 \times 10^{-3}$ ,  $4.0 \times 10^{-3}$ ,  $4.3 \times 10^{-3}$ ,  $5.3 \times 10^{-2}$ , and  $3.6 \times 10^{-2}$  mol/L for the  $10^{-3}$  mol/L LMw-DNA,  $10^{-3}$  mol/L IMw-DNA,  $10^{-3}$  mol/L HMw-DNA,  $10^{-2}$  mol/L

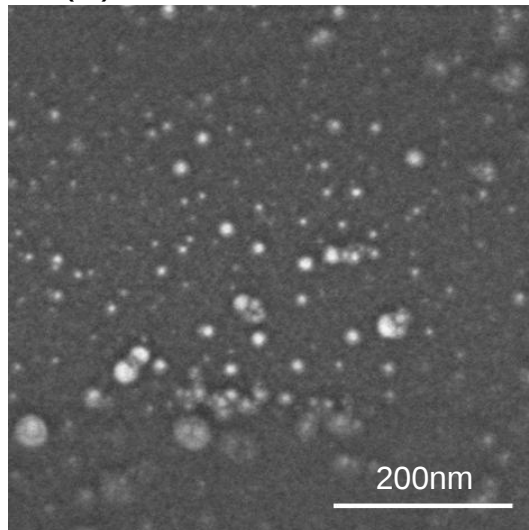
LMw-DNA, and  $10^{-2}$  mol/L IMw-DNA solutions, respectively. The UV-Vis absorption spectra of Ag colloids have been reported to be influenced by the particle size distribution; for example, the absorption peak shifts toward longer wavelengths as the particles become larger,<sup>29</sup> and small aggregates cause a decrease in the peak along with the appearance of a long tail on the high wavelength side.<sup>30</sup> At the same Ag concentration, the UV-Vis absorption spectra showed a similar shape for  $10^{-3}$  and  $10^{-2}$  mol/L LMw-DNA solutions (Figure 3A, no. 1 and 2), and  $10^{-3}$  mol/L IMw-DNA solution (Figure 3A, no. 3), suggesting that these Ag particles possessed a similar size distribution.



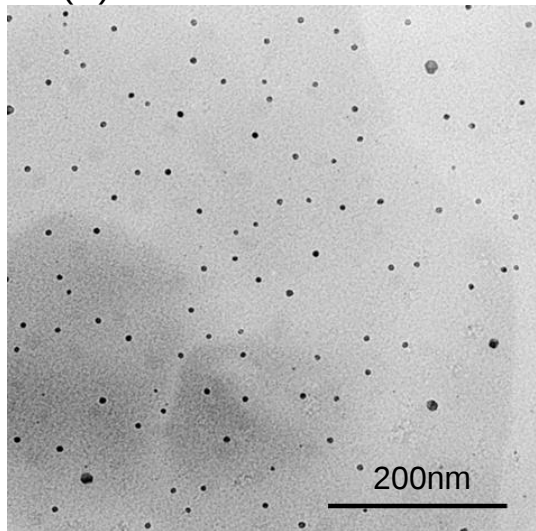
1 (B)



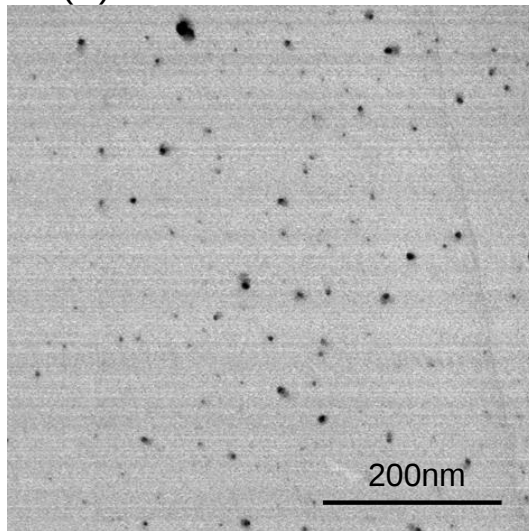
1 (C)

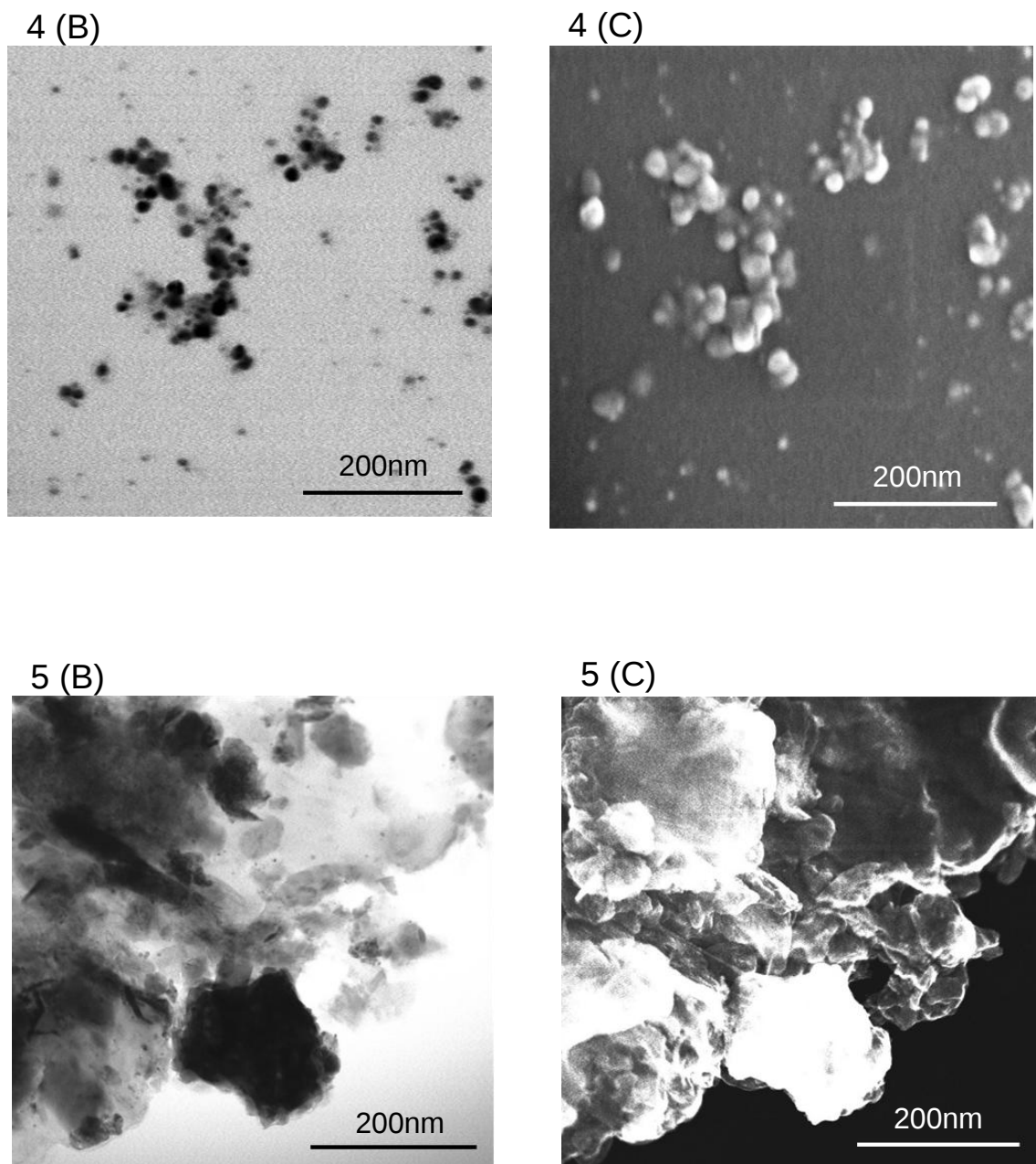


2 (B)



3 (B)



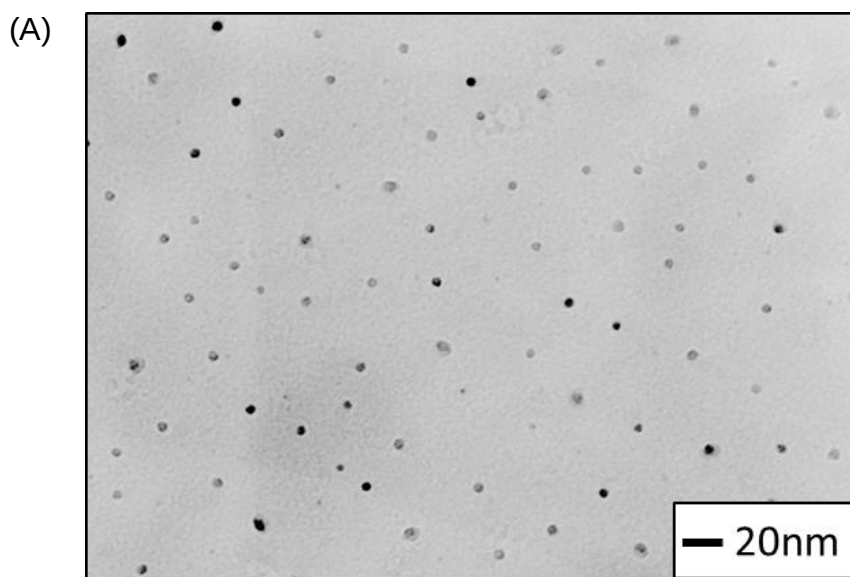


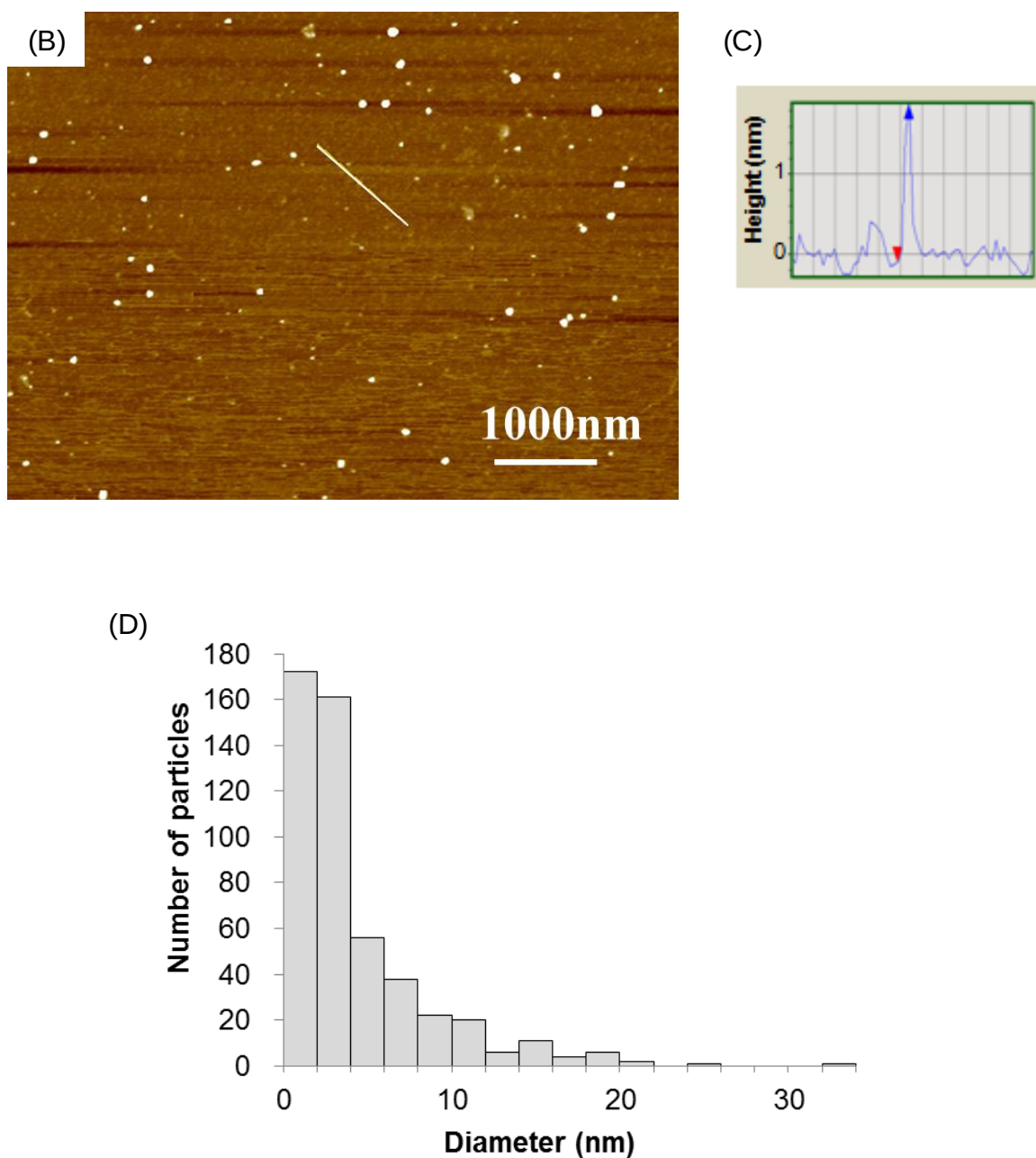
**Figure 3** (A) UV-Vis absorption spectra of the Ag nanoparticle dispersions, in terms of the same concentration of Ag. (B) TEM and STEM images of the samples. (C) Corresponding SEM images of Ag-DNA complexes after dialysis. The specified DNA solutions were: 1) LMw-DNA ( $10^{-3}$  mol/L), 2) LMw-DNA ( $10^{-2}$  mol/L), 3) IMw-DNA ( $10^{-3}$  mol/L), 4) IMw-DNA ( $10^{-2}$  mol/L), and 5) HMw-DNA ( $10^{-3}$  mol/L).

Figures 3B and 3C show typical microscopic images. The LMw-DNA at concentrations of  $10^{-3}$  and  $10^{-2}$  mol/L and the IMw-DNA at a concentration of  $10^{-3}$  mol/L produced fine Ag nanoparticles (Figure 3B and 3C, no. 1, 2, and 3), while the IMw-DNA at a concentration of  $10^{-2}$  mol/L and the HMw-DNA gave large Ag particles (Figure 3B and 3C, no. 4 and 5). The  $10^{-2}$  mol/L LMw-DNA and the  $10^{-1}$  mol/L aqueous  $\text{AgNO}_3$  solutions were selected for producing Ag nanoparticles in a high concentration, and then the characterization was carried out.

### Characterization of Ag nanoparticles

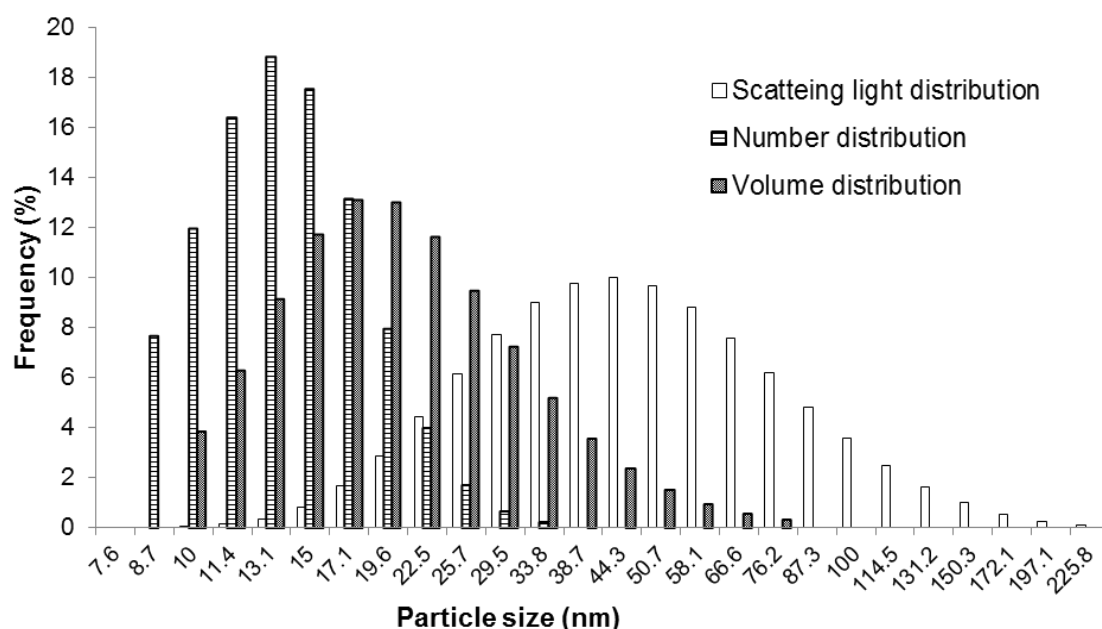
The concentrations of Ag, P, and Na in the Ag nanoparticle dispersions were measured by ICP-AES, and the DNA concentration was calculated from the amount of P. The Ag content in the nanoparticles was 85.2 wt%; the DNA content was calculated as 14.3 wt%, that is, the Ag nanoparticles consisted largely of Ag, with a low content of impurities such as Na.





**Figure 4** Particle images and size distribution of the Ag nanoparticles. (A) TEM and (B) AFM images of the Ag nanoparticles. (C) Height profile along the indicated line of the AFM image. (D) Histogram of the Ag nanoparticle size distribution as evaluated by AFM.

The TEM images of the Ag nanoparticles showed them to be spherical in shape with an average diameter of less than 10 nm (Figure 4A). The particle size distribution was determined by AFM (Figure 4B) using the height profile in the image (Figure 4C). The histogram of the size distribution is shown in Figure 4D; approximately 90% of the particles were less than 10 nm, with a median particle size of 2.6 nm.



**Figure 5** Particle size distributions of the Ag nanoparticles in aqueous solution by DLS.

On the other hand, the size distributions by DLS measurement of the Ag nanoparticle aqueous dispersion were shown larger than that by AFM images (Figure 5). The median particle sizes were 41.2, 15.1 and 14.7, for scattering light distribution, number distribution and volume distribution, respectively. The number distribution showed the smallest particle size distribution. However, the particles with a size of

under 8.7 nm were not detected in contrast to those observed in TEM and AFM images. The reason why the particle size by DLS was not in agreement with that by TEM and AFM was assumed as follows; i) it was difficult to measure accurately the size of nanoparticles in the range around 1-2 nm by DLS, ii) the nanoparticles with larger sizes affected the total size distribution toward the larger value, iii) DNA may affect the Brownian motion of Ag nanoparticles and lead to the overestimated value of particle size in water.

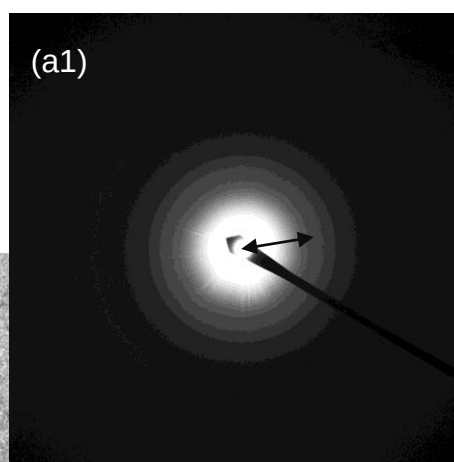
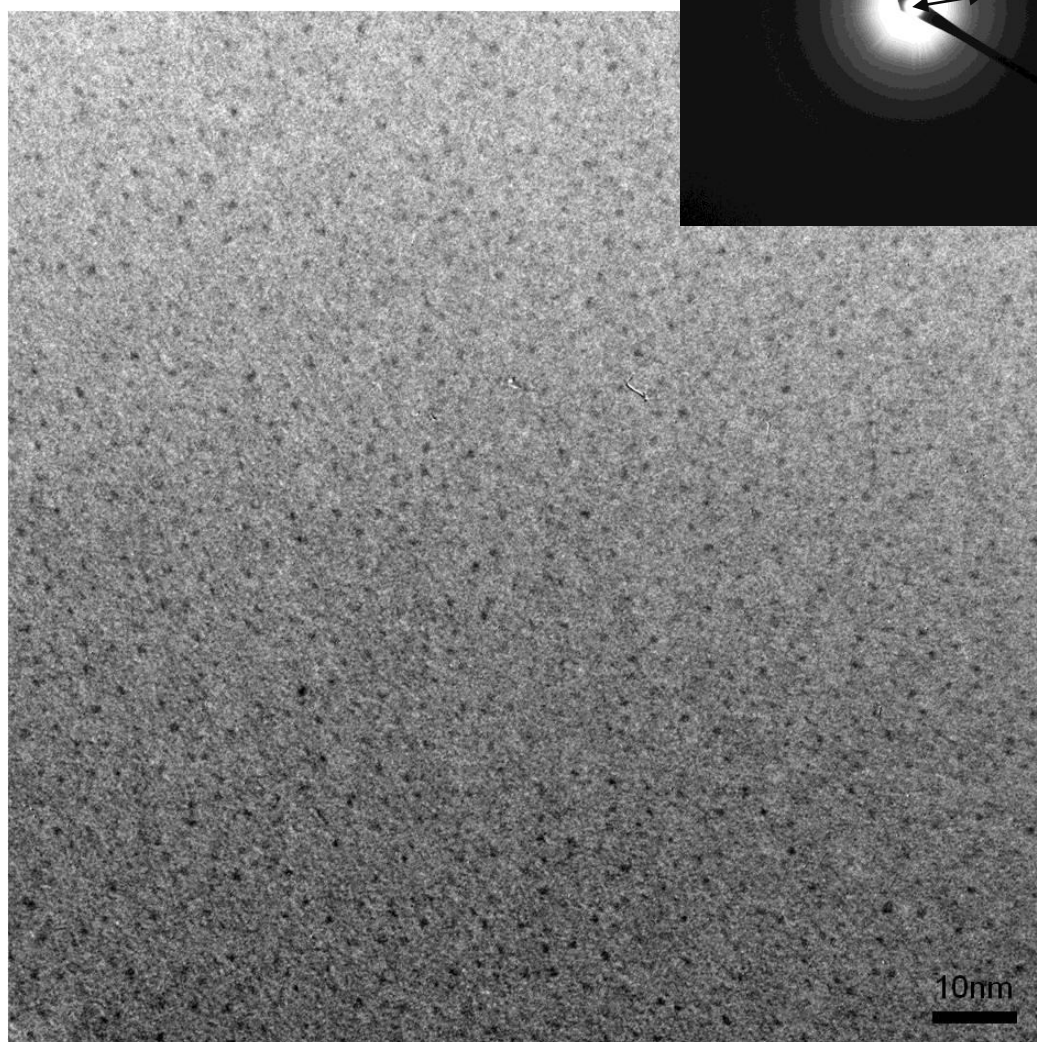
The uniform distribution of the dark spherical objects with the size of less than 2 nm was found by high resolution observation using FE-TEM (Figure 6A). The objects with the size of less than 2 nm were also found in large numbers as well as those around 10 and 20 nm in Figure 6B. The nano-beam electron diffraction pattern (inset b1 of Figure 6B) of the particle with the size of about 20 nm (b in Figure 6B) showed the single-crystal spot pattern with other faint patterns suggesting polycrystals including twins. The lattice spacing was calculated as follows,

$$L\lambda = dR$$

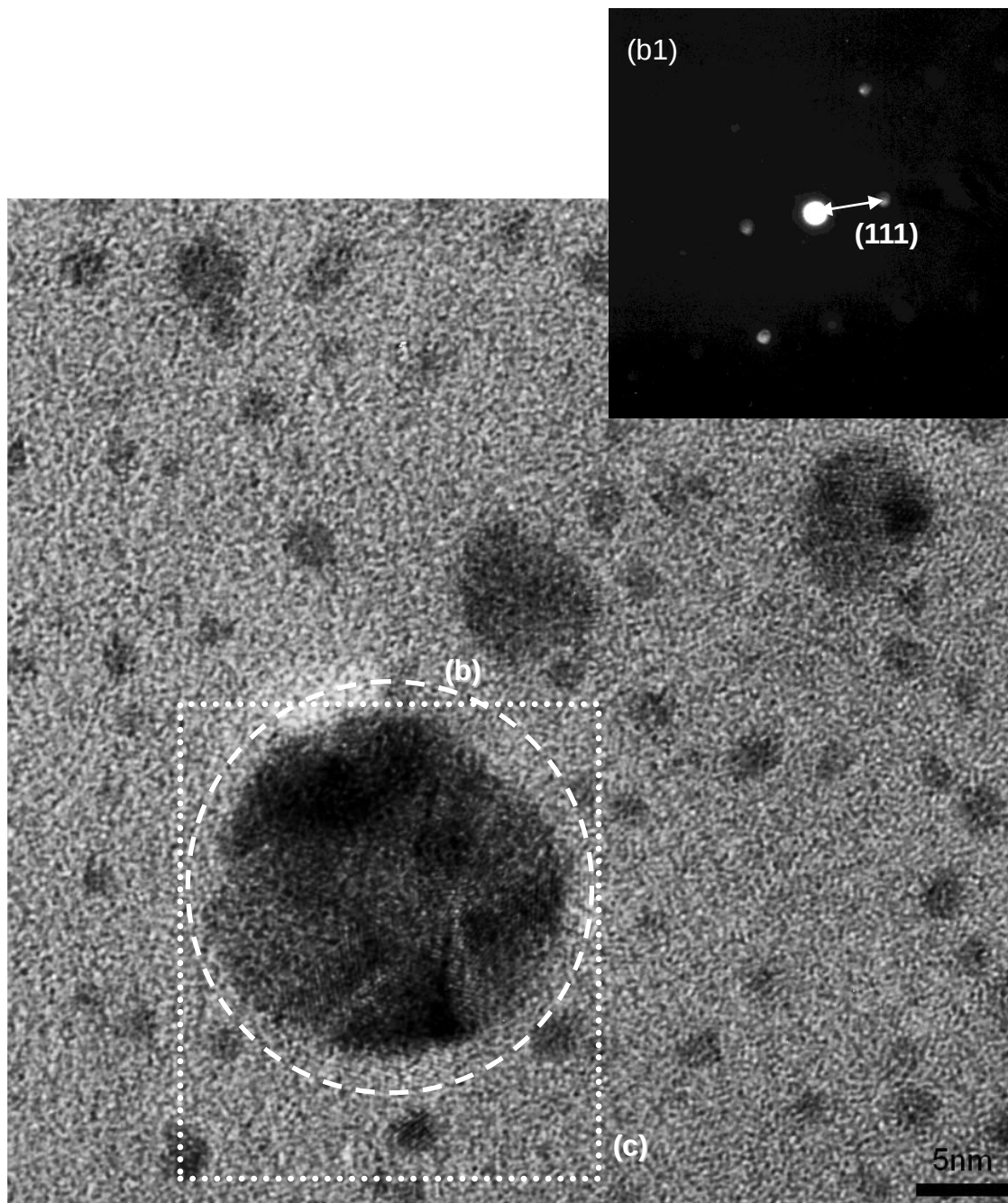
where L is camera length,  $\lambda$  is wavelength of the TEM, d is lattice spacing and R is measured diffraction ring radius or spot distance. The lattice spacing corresponding to the nearest spot was 0.243 nm, close to the (111) plane of face-centered cubic (FCC) Ag. A broad ring pattern (inset a1 of Figure 6A) with the same diffraction ring radius as the spot in Figure 6B could be observed in the selected area electron diffraction pattern from the area of the particles with the size less than 2 nm (Figure 6A), indicating that the particles consist of Ag crystal. The enlarged image of the Ag nanoparticle with the

size of around 20 nm showed the lattice fringes in various directions and Moire fringe, indicating that the particle consists of plural crystals (Figure 6C). The lattice fringe also could be observed in the nanoparticle with the size of around 2 nm (d in Figure 6C and 6D). The spacing of lattice fringes were 0.227 nm corresponding to the (111) plane of the FCC Ag, indicating that the finer nanoparticles also consist of Ag crystal. These results were well in agreement with the size distribution obtained by AFM.

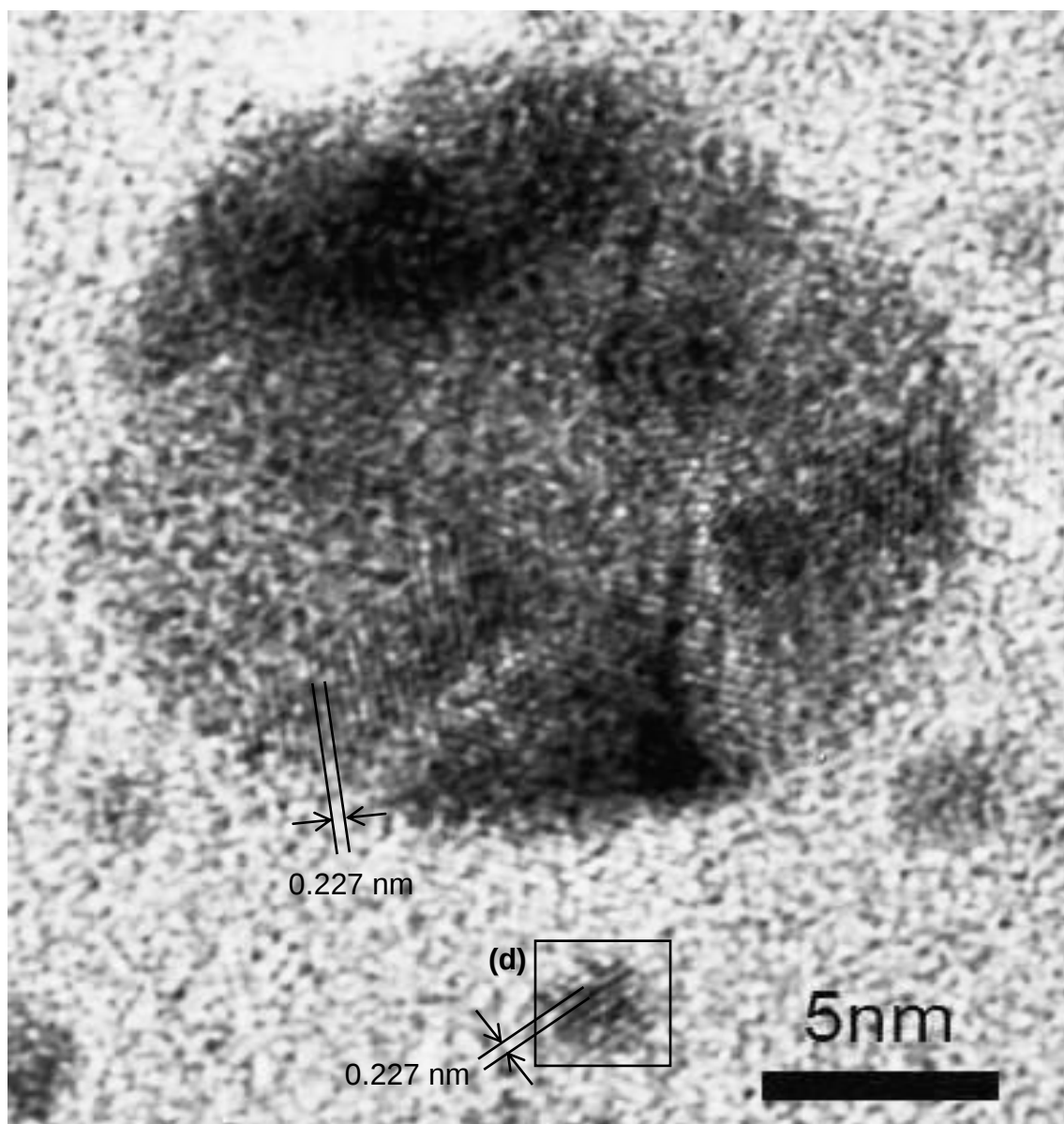
(A)



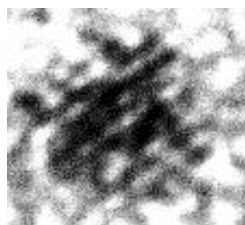
(B)



(C)

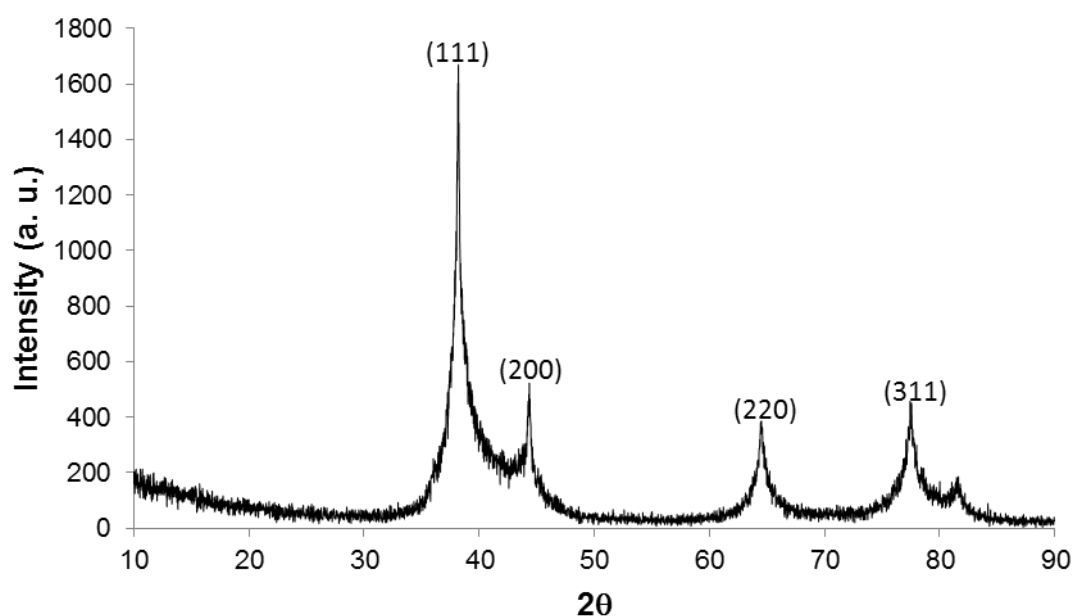


(D)



**Figure 6** High resolution FE-TEM images of the Ag nanoparticles. The insets (a1) and

(b1) in (A) and (B) show the corresponding selected area electron diffraction pattern recorded from the whole area and the nano-beam diffraction pattern from the area marked with the dashed line circle (b), respectively. (C) and (D) are the enlargements of the areas indicated with the dotted square (c) in (B) and the solid square (d) in (C), respectively.



**Figure 7** XRD pattern of the Ag nanoparticles.

XRD analysis was performed to identify the crystal phase of the Ag nanoparticles. The four peaks were attributed to the (111), (200), (220) and (311) planes of FCC Ag (Figure7). The peak shape looks to be a complex overlapped with sharp and much broader peak, which suggests the existence of very fine Ag nanoparticles. Assuming that the peak attributed to the (111) consisted of sharp and broader peaks, the

peak separation was done using the Lorentz function as peak shape and the crystal size was estimated from the half-peak width of the broader peak with the equation as follows,

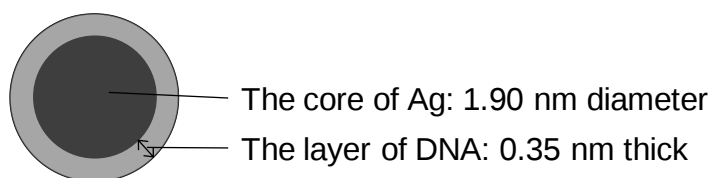
$$D = K\lambda/(\beta \cdot \cos\theta)$$

where D is crystal size, K is constant number,  $\lambda$  is X-ray wavelength from CuK $\alpha$ ,  $\beta$  is half-peak width after correction of the equipment error and  $\theta$  is Bragg angle of diffracted peak. The crystal size was estimated as less than 10 nm, which were consistent with the median value of the size distribution obtained by AFM images.

The zeta-potential is a measure of the degree of repulsion between similarly charged particles in a dispersion. A higher zeta-potential indicates a higher stability of the dispersion, which would be more resistant to aggregation. The zeta-potential of the Ag nanoparticles in water was -73.82 mV, which was negatively charged, similar to DNA. The absolute value was higher than that of DNA, -42.17 mV. This result suggested that the surfaces of the Ag nanoparticles were covered with DNA, which led to the high stabilization in aqueous media.

From the ratio of Ag to DNA, a schematic was drawn of the size of the Ag core and the thickness of the outer DNA layer wrapping the core. We applied the literature density value of  $1.05 \times 10^4 \text{ kg/m}^3$  for Ag,<sup>31</sup> and the measured density value of  $1.14 \times 10^3 \text{ kg/m}^3$  for DNA. For a particle of median size, 2.6 nm in diameter, the Ag core was calculated to be 1.90 nm in diameter and the DNA layer 0.35 nm thick (Figure 8). Adamcik and coworkers reported that the height of single-stranded DNA, dried in air, was  $0.35 \pm 0.05 \text{ nm}$ , according to their AFM observations.<sup>32</sup> Therefore, our results could

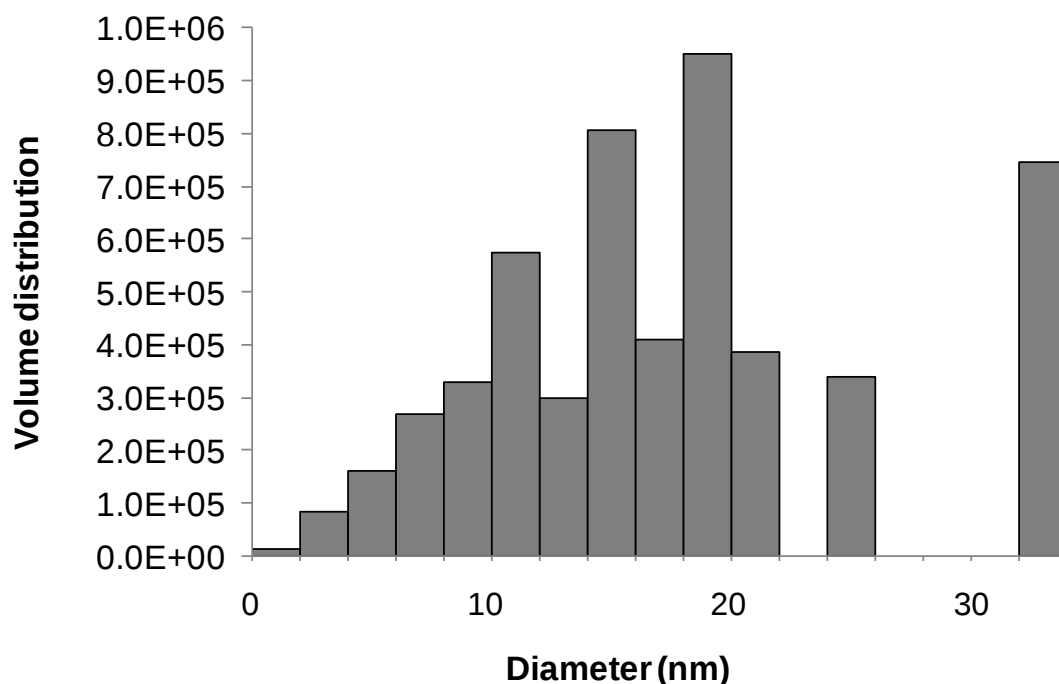
be explained by supposing that the Ag nanoparticles were coated with a single layer of single-stranded DNA.



**Figure 8** Proposed structure of the Ag nanoparticles.

Assuming that the Ag core was FCC crystal, the number of Ag atoms per Ag core or the size of Ag core was calculated from the lattice constant of bulk Ag,  $a_0 = 0.4086$  nm, though it was reported that the lattice constant of very small Ag particles (3.0-17.8 nm) was slightly decreased with the decrease of particle size.<sup>33</sup> Given the molar ratio of [nucleotide]:[Ag], which was calculated as 1:18, if the Ag nanoparticle was prepared from one molecule of DNA, the number of Ag atoms was around 1080 prepared from LMw-DNA with around 60 nucleotides. The size of Ag core consisted of the number of Ag atoms from one molecule of LMw-DNA was calculated to be 3.7 nm, which was larger than the Ag core size estimated from the median diameter. The number of Ag atoms of 1.9 nm of Ag core was 149, which was less than that corresponding to LMw-DNA. Since the larger particles were prepared as shown in the size distribution (Figure 4), the remained Ag atoms may prepare the large particles. Assuming that the particles consist of only silver, the volume distribution was calculated from the particle number distribution obtained by AFM (Figure 4) and the number of Ag atoms contained

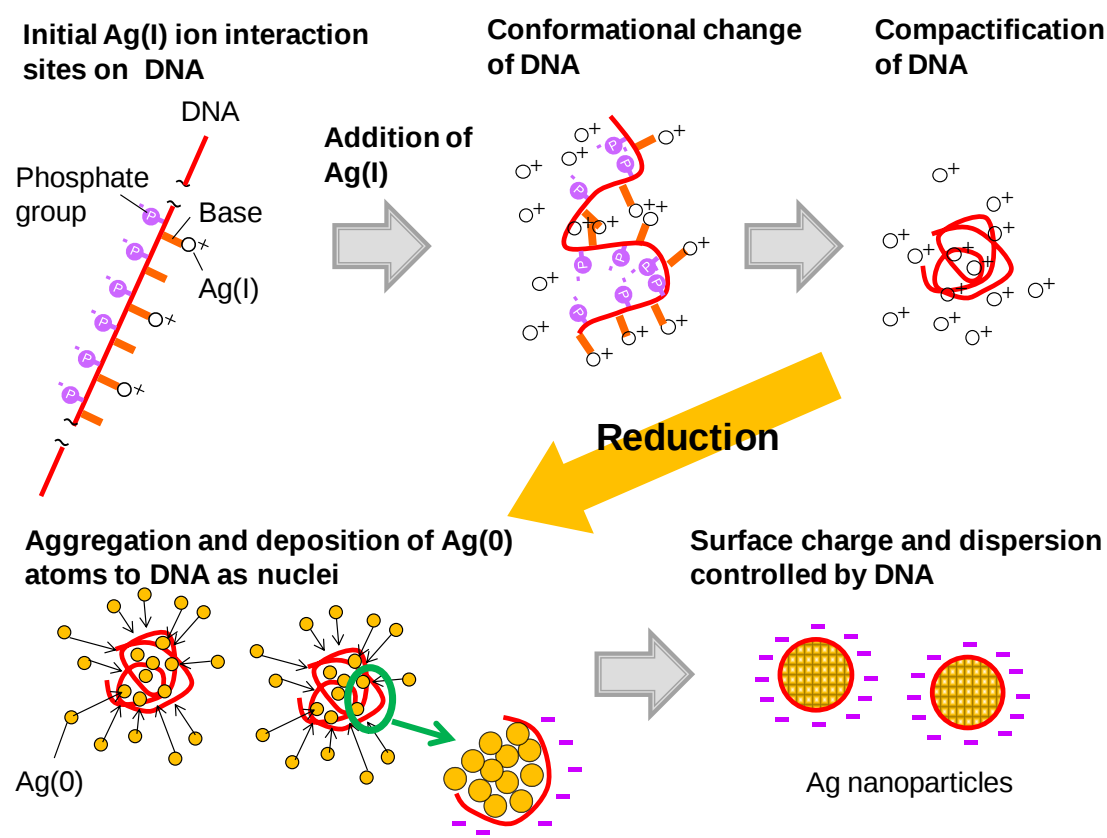
in each particle as shown in Figure 9. It shows that the considerable numbers of which is also consistent with the result of volume distribution obtained by DLS (Figure 5). Ag atoms are distributed in the Ag nanoparticles with the size of from 10 to 20 nm.



**Figure 9** Volume distribution of Ag nanoparticles calculated from the particle size distribution by AFM. Volume (total Ag atom numbers) was obtained as the product of the number of atoms/particle and the number of particles.

In the case of IMw-DNA with around 151-303 nucleotide, Ag nanoparticles prepared from one molecule was calculated to be 5.0-6.3 nm, though the size distribution was not calculated, the size appropriately agreed with the STEM images (Figure 3B, no. 3). On the other hand, Ag core prepared from one molecule of HMw-DNA, around 30 300 nucleotide, was calculated to be 29 nm or more, which was

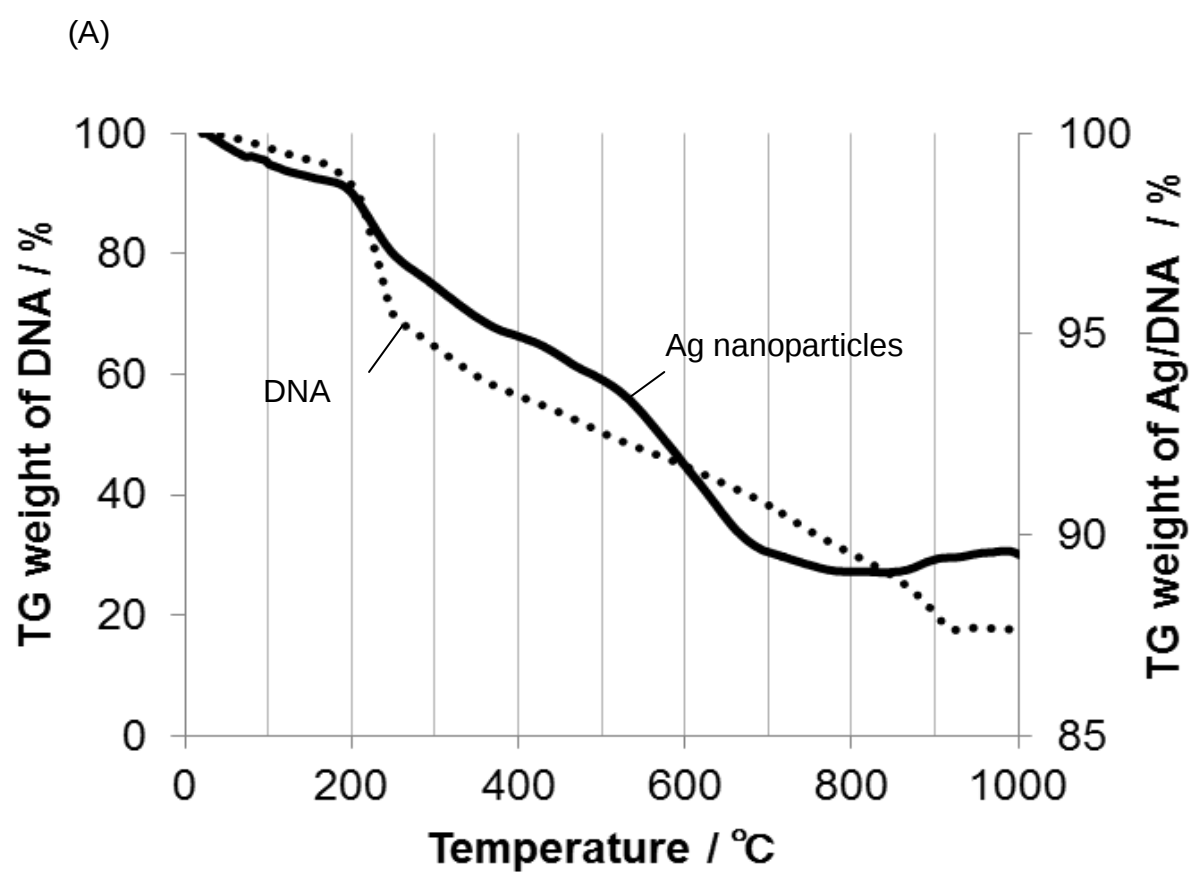
much smaller than the particle or agglomerate observed by STEM (Figure 3B and 3C, no. 5). These results suggest that the Ag nanoparticles from LMw-DNA and IMw-DNA were possible to be prepared from one molecule of DNA, meanwhile the particles from HMw-DNA were from some molecules of DNA. Assuming that one Ag nanoparticle was derived from one molecular of DNA, the formation mechanism of Ag nanoparticles using DNA as template was proposed as shown in Figure 10. At first, part of Ag(I) ions interacts with nucleotide base of DNA. However, most of other Ag(I) ions freely exist in the solution and do not interact with DNA since the numbers of Ag(I) ions added are for larger, about 20 times to nucleotide. The Ag(I) ions without interaction exists near DNA and induces the conformational change of DNA by neutralization of the charge on DNA, and then the DNA is finally compactified. By adding NaBH<sub>4</sub>, Ag(I) ions are reduced to Ag(0) atoms, and then the atoms aggregate together and deposit onto the DNA as nuclei. The aggregate of Ag atoms are crystallized and grown from cluster to nanoparticles. The size and dispersion are controlled by DNA, which covers and protect the surface of the nanoparticles and keeps the particle-particle separation by negative charge of the phosphate group. In this mechanism, DNA takes an important role as template and particle protection. The advantages of DNA as the template may be considered as follows; i) the conformation change of DNA is induced by adding cationic molecules, and then the compactificated DNA can be nuclei to aggregate the Ag atoms, ii) it is assumed that the interaction of DNA with Ag(I) ion is not so tight, therefore Ag(0) atoms can be aggregated and crystallized without involving of DNA.



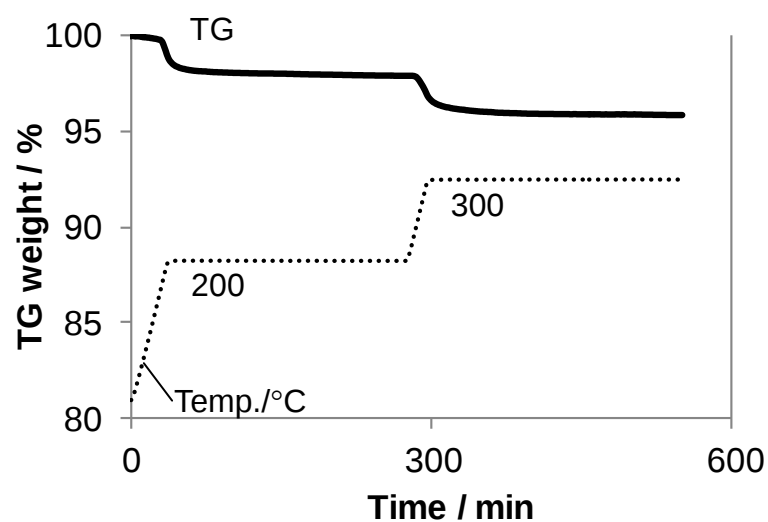
**Figure 10** Proposed formation mechanism of Ag nanoparticles using DNA as template.

The thermal behavior of the Ag nanoparticles was analyzed by TG. The weight loss of the nanoparticles was low from 250-500°C, followed by a remarkable change over 500°C. In contrast, the weight of DNA was continuously decreasing from 250-900°C (Figure 11A). In the stepwise heating from 200°C to 600°C, the weight changed little under 400°C, and a 15% decrease occurred at 500°C (Figures 11B and 11C). This value was identical to the analysis by ICP-AES. In other words, the Ag nanoparticles demonstrated good thermostability up to 400°C. Moreover, most DNA is expected to decompose by heating over 500°C, at which point the Ag nanoparticles may

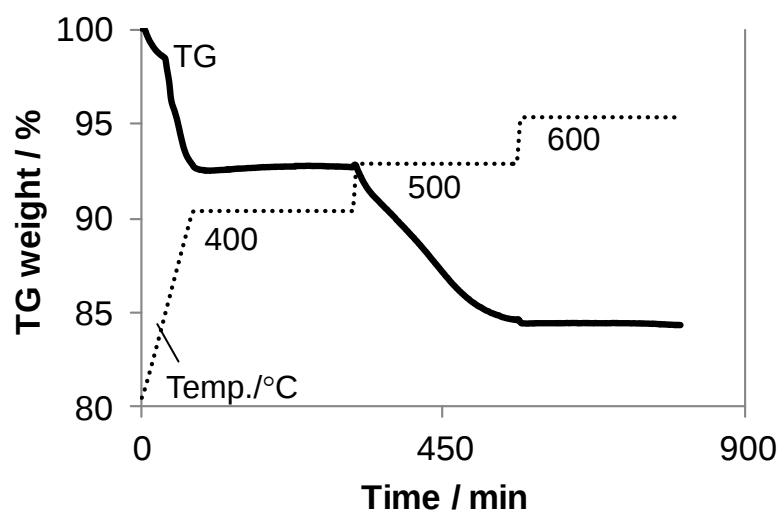
show potential as a conductive material.



(B)



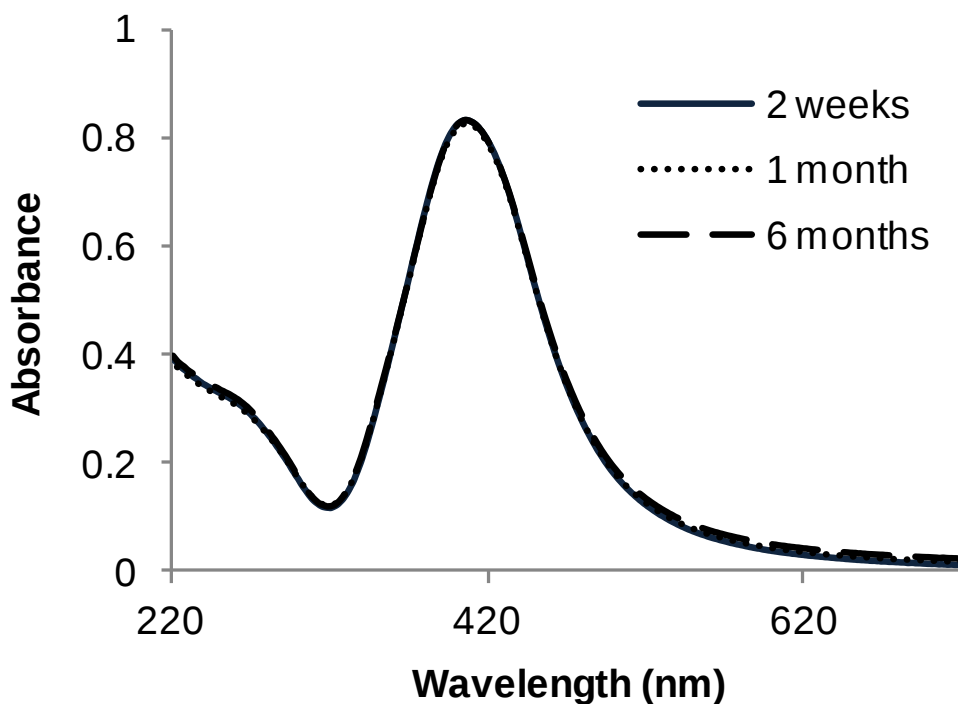
(C)



**Figure 11** TG curves of (A) DNA and the Ag nanoparticles at a heating rate of 5°C/min under ambient air, and TG curves with stepwise temperatures at (B) 200°C and 300°C, (C) 400°C, 500°C and 600°C, each for 4 h.

### Long-term stability of the Ag nanoparticles in water

Long term dispersion stability is necessary for Ag nanoparticle applications. Upon standing for over six months, the as-prepared dispersion did not change from a homogeneous dark brown based on visual inspection. The UV-Vis absorption spectrum indicated that the particle size and shape were remarkably stable in water (Figure 12). The change in the spectrum was the same or even smaller than that of previously reported highly stable Ag nanoparticles stored at lower concentrations.<sup>10,13,34</sup> These results demonstrate that the DNA played an important role as a protective agent in addition to serving as the template. This stabilization enabled the DNA-templated Ag nanoparticles to be stored on a large scale and used without re-dispersion.



**Figure 12** UV-Vis absorption spectra of the Ag nanoparticle dispersions stored for two weeks, one month and six months.

## 2.5 Conclusions

Cheaply available DNA from salmon milt was used as the bio-template to prepare Ag nanoparticles. Among the three selected templates, DNA with the lowest molecular weight (mainly molecular weight, ca. 20 000 Da, single-stranded) prepared the dispersion without Ag nanoparticle aggregation and containing the highest Ag concentration ( $5.3 \times 10^{-2}$  mol/L). Ag accounted for as high as over 85 wt% of such nanoparticles. These particles had spherical shape, and their diameters were mostly less than 10 nm. The templated DNA in the nanoparticles would decompose around 500 °C in air, and mostly be removed at higher temperature. Besides, highly negatively charged Ag nanoparticles demonstrated the long-term stability of the aqueous dispersion under ambient conditions. Thus DNA extracted from salmon milt was highly available for preparation of the Ag nanoparticle dispersion. The method possessed low cost and a simple process, highly suitable for mass production, and thus would benefit the Ag nanoparticle utilities in the fields of catalysis, optics, electronics and antimicrobial materials.

## 2.6 Acknowledgments

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## 2.7 References

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## Chapter 3

Antibacterial effect of Ag nanoparticles prepared using salmon milt DNA for practical application

### 3.1 Abstract

Antibacterial function of Ag nanoparticles prepared using salmon milt DNA was investigated against *S. aureus* as Gram-positive bacteria and *E. coli* as Gram-negative bacteria. The Ag nanoparticles had the growth inhibition effect against both kinds of bacteria with the concentration-dependent manner, and the effective concentration depended on the number of bacteria applied in the test. The Ag nanoparticles could be immobilized on a cationized cotton fabric via phosphate group of DNA. It showed the long time sustained release of Ag ions with the particles still fixed to the fabric. The fabric showed an enough inhibitory and killing effect against *E. coli* according to the antibacterial standard of Japanese Association for the Functional Evaluation of Textiles at a concentration of 10 ppm as Ag.

## 3.2 Introduction

Metal nanoparticles, whose structures display novel and improved physical, chemical, and biological properties and functions due to their nanoscale size, have attracted much interest.<sup>1-5</sup> Among the metal nanoparticles, Ag nanoparticles have been widely used as an antibacterial agent for various matters from consumer products, such as refrigerators, mobile phones, clothes, plasters, toothbrushes and cosmetics, to medical instruments and products, such as catheters, bandages, scalpels and needles.<sup>6</sup>

It has been known that Ag and its compounds have a broad spectrum of antimicrobial activities for bacteria, fungi and virus,<sup>6-12</sup> and they have been used as disinfection agents from the ancient time. Ag nanoparticles are expected to show more efficient antibacterial property due to their extremely large surface area, which can interact well with microorganisms. The antibacterial mechanisms of Ag nanoparticles have been extensively studied; however, they are not clearly understood. It has been assumed that Ag nanoparticles can release Ag ions, and then the Ag ions interact with the negatively charged bacterial cell wall, deactivating cellular enzymes, disrupting membrane permeability, and ultimately leading to cell lysis and death.<sup>13,14</sup> The antibacterial effect of Ag nanoparticles depended on the size<sup>15</sup> and shape,<sup>16</sup> where the antibacterial activity decreased with an increase of the particle size. Sondi *et al.* reported that the Ag nanoparticles with negative charge were incorporated into the membranes structure of *E. coli*, leading to cell damage.<sup>4</sup> Radzing *et al.*, reported that the Ag nanoparticles strongly inhibited biofilms formation of *E. coli*, *P. aeruginosa* and *S.*

*proteamaculans*.<sup>17</sup> Though Ag, its compound and nanoparticles have been reported to exhibit lower toxicity than other metals and some organic antibacterial agents,<sup>18-20</sup> potentiality of health and environmental risks of them have been discussed. It is assumed that the object, dose and processing method are important for actual use.

In this study, fine size and highly stable Ag nanoparticles were prepared using salmon milt DNA as the template described in chapter 2. The Ag nanoparticles were spherical in shape with a median particle size of 2.6 nm, and approximately 90% of the particles were less than 10 nm from the observation by transmission electron microscope and atomic force microscope. The particles had a high negative charge, suggesting that the surfaces were covered with DNA, and this leads to the aqueous dispersion very stable for long-term. The particles may have good antibacterial potential due to their fine particles size, meanwhile, the DNA layer may prevent microorganisms from contacting the Ag atoms/Ag<sup>+</sup> ions at the particle surface. In this study, the antibacterial property of the Ag nanoparticles based on DNA template was investigated against *S. aureus* as Gram-positive bacteria and *E. coli* as Gram-negative bacteria. The Ag nanoparticles were also immobilized on the fabric through electrostatic interaction between the phosphate group of DNA surface of the particles and the cationized surface of the fabric, and their antibacterial effect was confirmed.

### 3.3 Materials and Methods

#### Materials

The Ag nanoparticles were prepared as described in chapter 2. DNA (from salmon milt, mainly molecular weight, ca. 20 000 Da, single-stranded) was obtained from Nisseibio Co., Ltd., (Eniwa, Japan). Silver nitrate ( $\text{AgNO}_3$ , special grade), Potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ , special grade) and polyoxyethylene sorbitan monooleate (Tween 80®) were purchased from Kishida Chemical Co., Ltd. (Osaka, Japan). Ammonia solution (10%) and sodium tetrahydroborate ( $\text{NaBH}_4$ , chemical grade) were purchased from Wako Pure Chemicals Industries Ltd. (Osaka, Japan). Potassium peroxydisulfate (for N and P analysis grade), sodium chloride ( $\text{NaCl}$ , special grade) and sodium carbonate ( $\text{Na}_2\text{CO}_3$ , special grade) were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Ultra-pure water (supplied from Milli-Q water purification systems, Merck Millipore, Billerica, MA, USA,  $>18 \text{ M}\Omega$ ) was used in all experiments.

*S. aureus* (AHU1142) was obtained from Faculty of Agriculture, Hokkaido University (Sapporo, Japan). *E. coli* (NBRC3301) was purchased from the National Institute of Technology and Evaluation (Chiba, Japan). Peptone (Bacto Peotone) and beef extract (desiccated), which were purchased from BD (Franklin Lakes, NJ, USA), and Agar (BA-10, high quality), which was purchased from Ina Food Industry Co., Ltd., (Ina, Japan), were used to prepare culture medium.

Cotton broad fabric, fabric mass of  $122.5/\text{m}^2$ , was purchased from Shikisensya (Osaka, Japan). Cationic polymer solution (DANSHADE®) was provided from Nittobo

Medical (Tokyo, Japan) and used as cationization agent to cationize the cotton fabric.

### **Antibacterial evaluation of Ag nanoparticles**

Antibacterial effect of Ag nanoparticles was examined against *S. aureus*, AHU1142, as Gram-positive bacteria, and *E. coli*, NBRC3301, as Gram-negative bacteria. Ag nanoparticle aqueous dispersion was sterilized by filtration through 0.45  $\mu\text{m}$  syringe filter (Minisart®, Sartorius Stedim Biotech, Goettingen, Germany), and then diluted (2-200 ppm as Ag) with phosphate buffer solution (pH 7.2). The bacteria were pre-cultured in nutrient broth (5% peptone, 3% beef extract) for 16 h at 37 °C. The culture solutions were diluted with phosphate buffer solution (pH 7.2), and mixed with the Ag nanoparticle dispersion at a volume ratio of 1:1. The mixture (0.1 ml) was put into bacterial petri dish ( $\phi$  90 mm) with 20 ml of nutrient agar medium (5% peptone, 3% beef extract, 15% agar), and incubated for 24-48 h at 37 °C.

### **Preparation of Ag nanoparticles-immobilized fabric**

Cotton fabric was cationized in cationization agent diluted with water (1-10 000 ppm) for 20 min at 80 °C by using hot plate stirrer, and then sodium carbonate was added 1 wt% for the solution, which was kept for 30 min at 80 °C. Next, the fabric was washed enough with deionized water, and then soaked in Ag nanoparticles dispersion (5-5 900 ppm as Ag) for 30 min. After enough washing with deionized water, the fabric was dried in air. The amount of the immobilized Ag was measured with inductively coupled plasma atomic emission spectrometry (ICP-AES, ICPE-9000, Shimadzu, Kyoto,

Japan) after hydrolyzed in the presence of aqueous potassium peroxydisulfate (4.0%) at 120 °C.

#### **Ag release from the Ag nanoparticles-immobilized fabric**

Ag release behavior of the Ag nanoparticles-immobilized fabric was determined. A small piece (approximately 0.2 g) of the fabric with Ag nanoparticles (34 000 ppm as Ag) was immersed into water (20 ml) in 50 ml glass vial container with screw cap. The container was shaken at 37 °C in a swing shaker (around 110 rpm). The water (1 ml) was sampled at 2, 4, 8, 12, 24, and 48 h, and the Ag concentration was measured by ICP-AES.

#### **Antibacterial evaluation of Ag nanoparticles-immobilized fabric**

The antibacterial assay of Ag nanoparticles-immobilized fabric (with 10 and 30 ppm as Ag) was performed according to the Japanese industrial standard (JIS L 1902: 2008- Adsorption test method) using *E. coli*. as follows: The bacteria were pre-cultured in nutrient broth for 16 h at 37 °C, and then diluted with 20-fold diluted nutrient broth (pH 6.8) as bacteria suspension containing  $1-3 \times 10^5$  colony-forming units (CFU)/ml. The bacteria suspension (0.2 ml) was inoculated to each specimen (0.4 g). Three untreated specimens and test specimens were washed immediately with 0.2% tween 80-containing saline (20 ml), and the washed bacterial suspension (1 ml) was taken into bacterial petri dish ( $\phi$  90 mm) with 20 ml of nutrient agar medium, and then incubated at 37 °C for 24-48 h to determine the number of bacteria. The other three untreated

specimens and test specimens were incubated at 37 °C for 18 h, and the number of bacteria was determined in the same way as at the beginning of contact time. The bacteria growth (BG) value, bacteriostatic (BS) value, and bactericidal (BC) value of the Ag nanoparticles-immobilized fabric were then calculated according to JIS L 1902: 2008.

### 3.4 Results and Discussion

#### Antibacterial evaluation of Ag nanoparticles

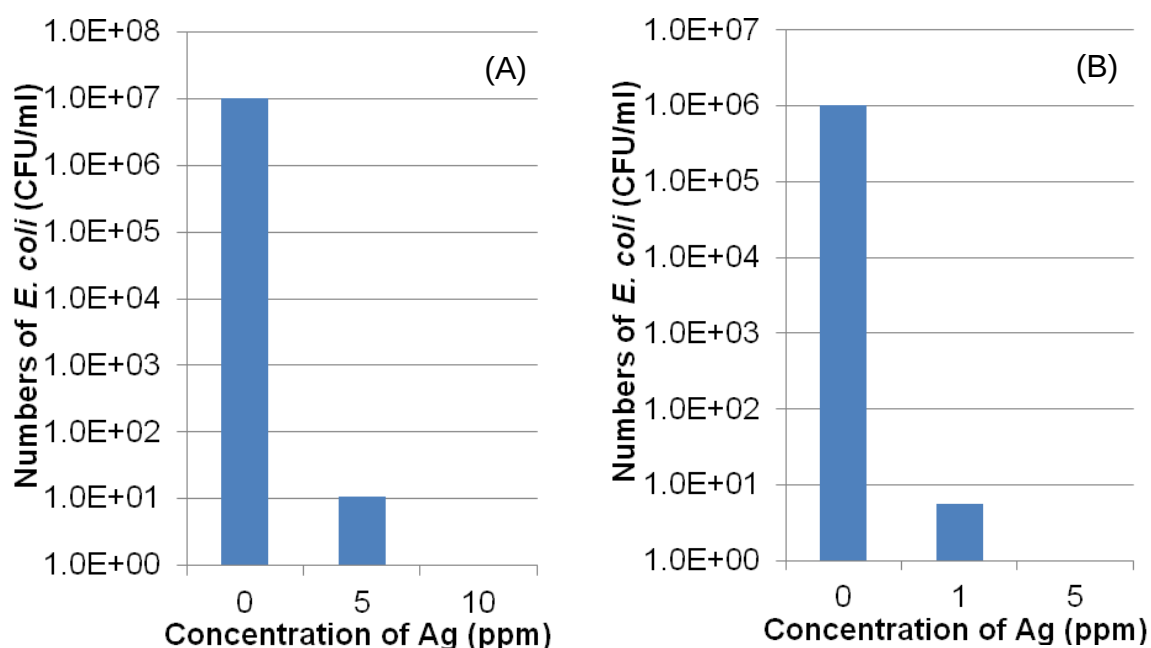
Preparation of the Ag nanoparticles was described in chapter 2. Antibacterial effect of the Ag nanoparticles was tested on both Gram-positive *S. aureus* and Gram-negative *E. coli* mixed with different concentrations of Ag nanoparticles. Figure 1 and Figure 2 show the number of bacterial colonies on nutrient agar plates after incubation with and without Ag nanoparticles. These results revealed that the Ag nanoparticles had the growth inhibition effect against both kinds of bacteria. The growth inhibition effect was observed in a concentration-dependent manner, and the effective concentration depended on the number of bacteria applied in the test. The inhibitory concentrations of several Ag nanoparticles concluded in a review article were in the range of 0.5 to 20 ppm against *E. coli* and 7.5 to 33.71 ppm against *S. aureus* for  $10^3$ - $10^5$  CFU/ml,<sup>1</sup> though the results are not strictly comparable owing to different experimental conditions, such as test method, strain and number of the bacteria, etc. In this study, if around  $10^6$  CFU/ml of bacteria were applied, the concentrations of Ag nanoparticles to completely prevent bacterial growth were 5 ppm against *E. coli*, and 50 ppm against *S. aureus* (Figure 1B and 2B). The effect of the Ag nanoparticles is considered to favorably compare with other Ag nanoparticles in previous reports.<sup>6</sup>

The Ag nanoparticles were more effective against *E. coli* than *S. aureus*. Gram-positive and Gram-negative bacteria have differences in the membrane structure. The most distinctive feature is the thickness of the peptidoglycan layer. The cell wall of

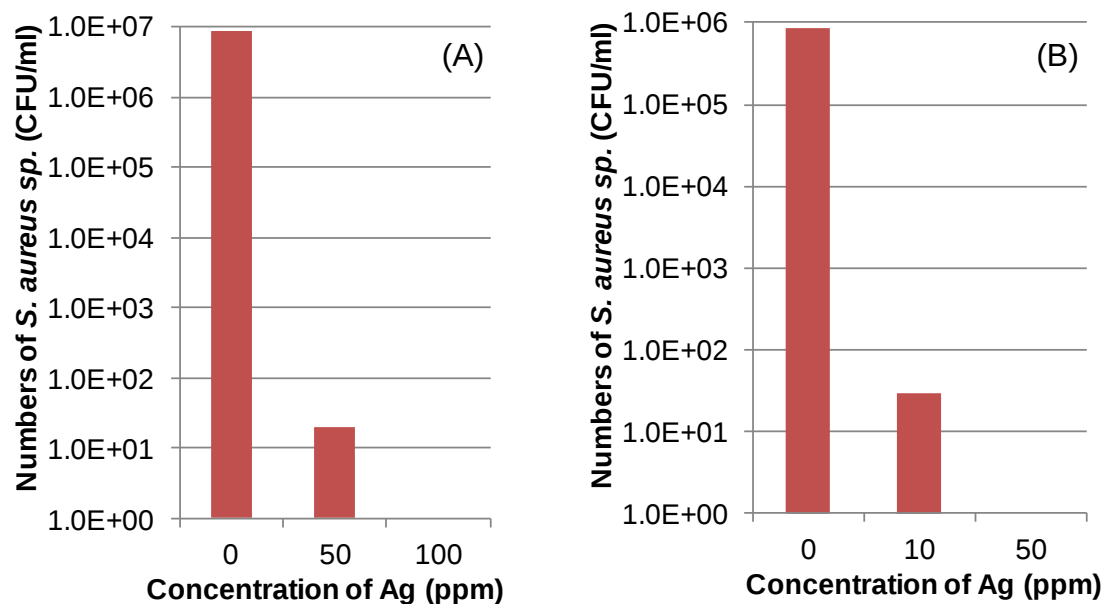
Gram-positive bacteria have the 30 nm thick peptidoglycan layers, while Gram-negative bacteria have the 2 to 3 nm peptidoglycan layer, which is covered with an outer membrane composed of phospholipids and lipopolysaccharides, facing towards the external environment.<sup>8</sup> The difference of the antibacterial effect of the Ag nanoparticles against *E. coli* and *S. aureus* may derive from the difference of membrane structure. Feng *et al* reported that Ag ions make DNA lost its replication ability and the protein became inactivated, and the morphological changes of *S. aureus* after treated with Ag ions was slighter than that of *E. coli*.<sup>13</sup> Sondi *et al* reported that during treatment with Ag nanoparticles, *E. coli* cells were damaged, showing formation of “pits” in the cell wall of the bacteria, while the Ag nanoparticles were found to accumulate in the bacterial membrane. A membrane with such a morphology exhibits a significant increase in permeability, resulting in death of the cell.<sup>4</sup> Kim *et al* reported that *E. coli* was inhibited at the low concentration of Ag nanoparticles, whereas the growth inhibitory effects on *S. aureus* were mild, and the free-radical generation effect of Ag nanoparticles was investigated by electron spin resonance spectroscopy as the mechanism of the growth-inhibitory effects.<sup>5</sup> Although it is necessary to further comparative study between various Gram-negative and Gram-positive bacteria to confirm the mechanisms of antibacterial effect and their differences of the species, the Ag nanoparticles showed the same tendency of the previous reports in this study.

The complete coverage of the Ag nanoparticles by DNA layer may lead to prevent microorganisms from contacting the particle surface and to show no antibacterial effect. However, our results confirmed the high antibacterial effect of the

Ag nanoparticles. It was reported that the Au nanoparticles prepared using DNA as a template showed the higher catalytic activity than those using polyvinyl pyrrolidone (PVP), Au nanoparticles was examined to have stronger binding with PVP than DNA by X-ray photoelectron spectroscopy.<sup>21</sup> Too strong binding of PVP probably hindered the direct osculation of the reaction substrate with the Au nanoparticles, thereby resulting in a lower catalytic activity of the Au catalyst in the report. In this study also the interaction of the Ag nanoparticles with DNA may be not so strong, therefore the Ag nanoparticles was not prevented from contacting microorganisms and antibacterial activity is kept at high level.



**Figure 1** Number of *E. coli* colonies after incubation with Ag nanoparticles in different concentrations. (A)  $1.04 \times 10^7$  CFU/ml , (B)  $1.04 \times 10^6$  CFU/ml, of bacteria were applied.

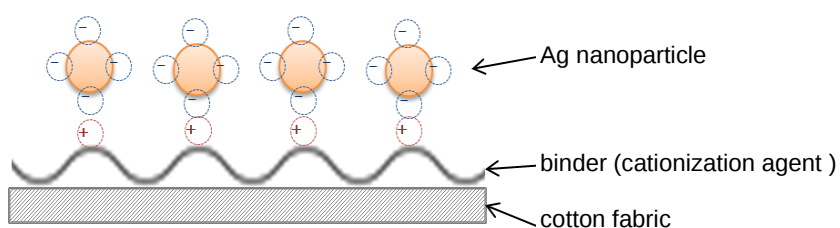


**Figure 2** Number of *S. aureus* colonies after incubation with Ag nanoparticles in different concentrations. (A)  $8.65 \times 10^6$  CFU/ml, (B)  $8.65 \times 10^5$  CFU/ml, of bacteria were applied.

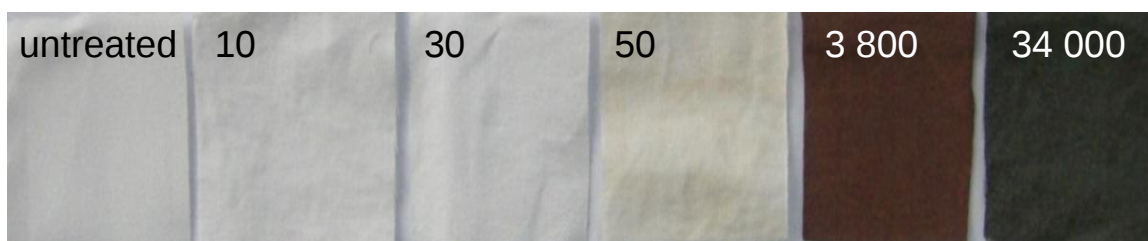
### Preparation of Ag nanoparticles-immobilized fabric

Since Ag nanoparticles prepared using DNA had the negative charge, the particles were immobilized on cationized cotton fabric by electrostatic interaction (Figure 3). The immobilized amount as estimated by ICP-AES was from 10 ppm as Ag content for the fabric treated with 1 ppm of cationization agent and 5 ppm of Ag nanoparticles aqueous dispersion, to 34 000 ppm as Ag content for the fabric treated with 10 000 ppm of cationization agent and 5 900 ppm of Ag nanoparticles aqueous dispersion. The amount depended on the concentrations of the cationization agent and Ag nanoparticles aqueous dispersion. The appearances of the fabrics were almost

unchanged for the low amount, 10 and 30 ppm of Ag, whereas it turned to dark brown, original color of the Ag nanoparticles, for the high amount, 3 800 and 34 000 ppm (Figure 4).



**Figure 3** Schematic of Ag nanoparticles immobilized to cotton fabric.

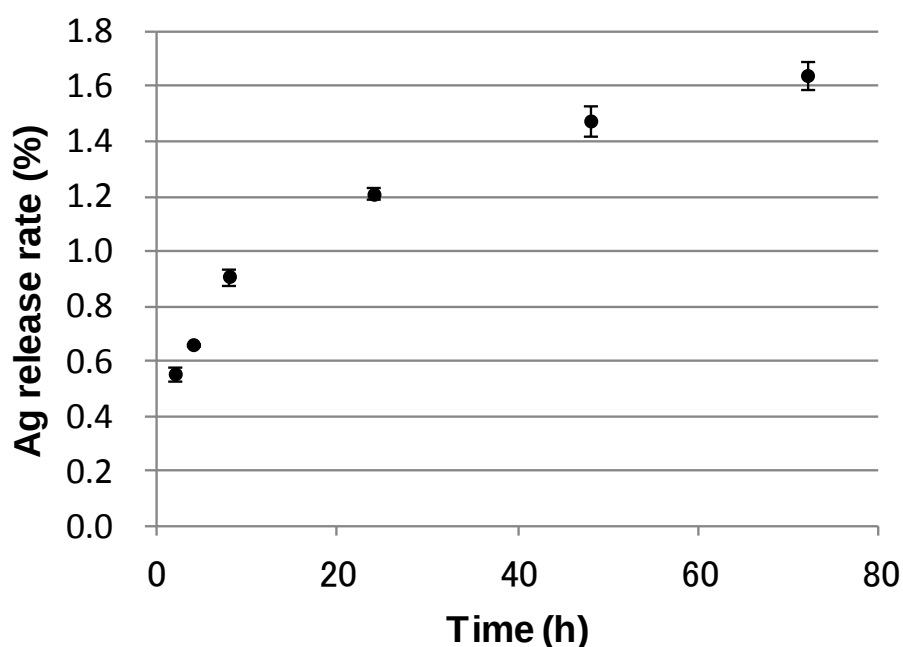


**Figure 4** Photographs of Ag nanoparticles-immobilized fabric. The amounts of immobilized Ag nanoparticles were shown as Ag (ppm).

#### **Ag release from the Ag nanoparticles-immobilized fabric**

The Ag ion release behavior was tested for the fabric with the immobilized Ag nanoparticles by 34 000 ppm as Ag content. The fabric sample (approximately 200 mg)

was immersed into the water of about 100 times the weight, and then it was shaken at 37 °C. Since the typical peak of the Ag nanoparticles at around 410 nm was not observed in UV-Vis absorption spectrum of the water, it was assumed that the most Ag was not in the state of Ag nanoparticles but Ag ion. The Ag concentrations of the water were about 1.9 ppm and 6.4 ppm after 1 h and 72 h, respectively. The Ag release rate was calculated based on the Ag concentration and the amount of immobilized Ag to the fabric (approximately 6.8 mg as Ag) as shown in Figure 5. The release rate is relatively high in the first day and decreases with time. The result indicates that the immobilized Ag nanoparticles released Ag ions gently, and most of the particles were still fixed to the fabric. Thus the Ag nanoparticles-immobilized fabric would show sustainably the antibacterial effect in water.

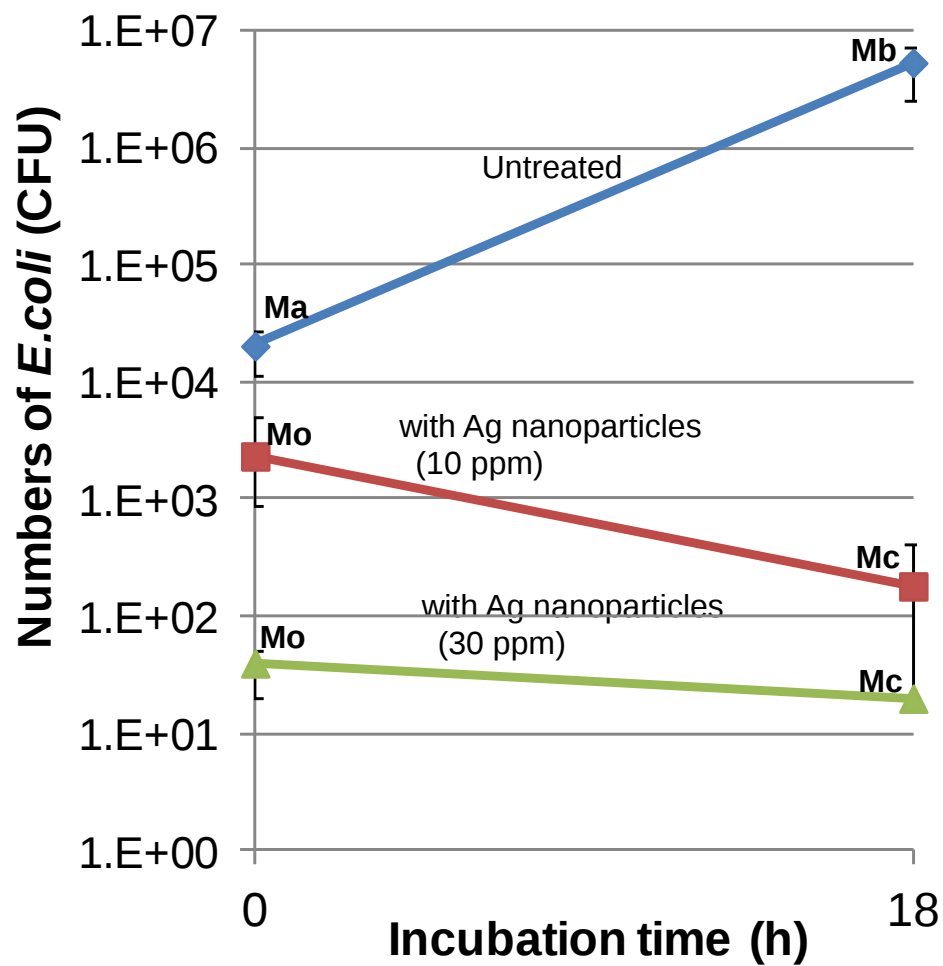


**Figure 5** Ag release profiles of Ag nanoparticles-immobilized cotton fabric (34 000 ppm as Ag). Error bars represent maximum and minimum value (n = 2).

### **Antibacterial evaluation of Ag nanoparticles-immobilized fabric**

Antimicrobial effect of the Ag nanoparticles-immobilized fabric was tested according to JIS L 1902: 2008 using *E. coli.*, whose concentration of seeding was  $2.6 \times 10^5$  CFU/ml. The numbers of the bacteria at the beginning of contact time and after incubation for 18 h were shown in Figure 6 and Table 1. The number of the bacteria inoculated to the untreated specimen increased after incubation. At the starting point of measurement at 0 h, the numbers of bacteria inoculated to both fabrics with 10 and 30 ppm Ag nanoparticles were already fewer than the untreated specimens, indicating the fabrics showed the antibacterial effect instantly after inoculation. The numbers of the bacteria inoculated to the Ag nanoparticles-immobilized fabric decreased after incubation 18 h.

The value of bacteria growth activity (BG), bacteriostatic value (BS) and bactericidal value (BC) were calculated as shown in Table 1. According to the antibacterial standard of Japanese Association for the Functional Evaluation of Textiles (JAFET), when BS is greater than 2.2, the test indicated that the test specimen may have inhibitory bacteria effect, and when BC is greater than 0, the test indicated that the test specimen may have bacteria-killing effect. In this study, both fabrics with the 10 and 30 ppm Ag nanoparticles show the inhibitory bacteria effect and bacteria-killing effect, because both BS and BC values are higher than 2.2 and 0. Since the colors of both fabrics were very little different from untreated fabric as shown in Figure 4, the Ag nanoparticles can add the enough antibacterial effect without change in appearance of the base materials.



**Figure 6** The numbers of the bacteria at the starting point of measurement and after incubation for 18 h. Error bars represent maximum and minimum value (n = 3). Ma, Mb, Mo and Mc correspond with Table 1.

**Table 1** Antibacterial test of Ag nanoparticles-immobilized fabric (JIS L 1902: 2008)

| Testing Item | Test results       |                                     |        |
|--------------|--------------------|-------------------------------------|--------|
|              | Untreated          | Ag nanoparticles-immobilized fabric |        |
|              |                    | 10 ppm                              | 30 ppm |
| Ma           | $2.05 \times 10^4$ | -                                   | -      |
| Mb           | $5.40 \times 10^6$ | -                                   | -      |
| Mo           | -                  | $2.35 \times 10^3$                  | <40    |
| Mc           | -                  | $1.82 \times 10^2$                  | <20    |
| BG           | 2.4                | -                                   | -      |
| BS           |                    | >3.6                                | >2.7   |
| BC           |                    | >2.3                                | >3.0   |

Notes: Ma: the number of the bacteria recovered from the inoculated untreated specimen at the starting point of measurement. Mb: the number of the bacteria after 18 h inoculated untreated specimen. Mo: the number of the bacteria recovered from the inoculated test specimen at the starting point of measurement. Mc: the number of the bacteria after 18 h inoculated test specimen. BG: the value of bacteria growth activity =  $\log(\text{Mb}/\text{Ma})$ . BS: bacteriostatic value =  $\log(\text{Mb}/\text{Ma}) - \log(\text{Mc}/\text{Mo})$ . BC: bactericidal value =  $\log(\text{Ma}/\text{Mc})$ . The mean value was used as the number of the bacteria (n=3).

### 3.5 Conclusions

Ag nanoparticles prepared using DNA described here showed the high antibacterial effect against both Gram-positive bacteria, *S. aureus* and Gram-negative bacteria, *E. coli*. The growth inhibition effect was observed in a concentration-dependent manner, and the effective concentration depended on the number of bacteria applied in the test. The effect of the Ag nanoparticles is favorably comparable with other Ag nanoparticles in previous reports. The Ag nanoparticles were more effective against *E. coli* than *S. aureus*, which is the same tendency as other Ag nanoparticles in previous reports. The Ag nanoparticles were negatively charged on the surface due to the phosphate group of DNA layer, and then the particles could be successfully immobilized to the cationized cotton fabric in the concentration range from 10 to 34 000 ppm as Ag content with almost no change in the appearance of the fabrics for the low amount, 10 and 30 ppm. The immobilized Ag nanoparticles released Ag ions gently, however, most of the particles still remained to be fixed to the fabric. The fabric showed an enough inhibitory effect and killing effect against *E. coli* according to the antibacterial standard of JAFET at a concentration of 10 ppm as Ag content. In this case, the appearance of the fabric was pretty much unchanged from the fabric without Ag nanoparticles, therefore the Ag nanoparticles may have the potential to add the antibacterial function to various textile products.

### 3.6 Acknowledgments

Nittobo Medical for providing DANSHADE® is gratefully acknowledged.

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## CONCLUSIONS

In this study, DNA extracted from salmon milt, which is discarded as industrial waste, was investigated to use as a template to prepare Ag nanoparticles for practical use. Three types of salmon milt DNA with different molecular weights were examined and comparisons of the obtained nanoparticles under different conditions were executed. As a result, DNA with the lowest molecular weight led to the Ag nanoparticles with the finest particle size and the highest total Ag content. The particle properties such as the morphology, composition, thermal behavior, aqueous dispersity and stability were also examined with the expectation as the industrial materials. Moreover, the antibacterial property of particles was evaluated for industrial use. The conclusions of this work were summarized as following.

1. Widely available DNA from salmon milt was used as a bio-template to prepare Ag nanoparticles. Among the three selected templates, DNA with the lowest molecular weight (mainly molecular weight, ca. 20 000 Da, single-stranded) could prepare the dispersion of Ag nanoparticles without aggregation and containing the highest Ag concentration ( $5.3 \times 10^{-2}$  mol/L).
2. Under this condition, the Ag nanoparticles largely consisted of Ag, over 85 wt%. The nanoparticles were spherical in shape, and their mean diameter was less than 10 nm. The templated DNA associated with Ag nanoparticles decomposed at approximately 500°C in air and was mostly removed at higher temperatures. The highly negatively charged Ag nanoparticles demonstrated the long-term stability as aqueous dispersion

under ambient conditions.

3. The Ag nanoparticles displayed antibacterial activity against both of Gram-positive bacteria, *S. aureus* (AHU1142) and Gram-negative bacteria, *E. coli* (NBRC3301). The Ag nanoparticles were immobilized electrostatically on cationized cotton fabric, and the resultant fabric also showed an antibacterial effect.

Thus, DNA extracted from salmon milt is a viable template for the preparation of stable Ag nanoparticle dispersion. The method presented is inexpensive and simple, highly suitable for mass production, and thus would benefit the utility of Ag nanoparticles in various fields, such as catalysis, optics, electronics, and antimicrobial materials. In particular, the Ag nanoparticles may have potential to add the antibacterial function to various textile products, which is expected as one of the most important and widely applicable industrial products.

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## Publications in International Journals:

1. Tomomi Takeshima, Ling Sun, Yanqing Wang, Yoshihisa Yamada, Norio Nishi, Tetsu Yonezawa and Bunshi Fugetsu, “Salmon milt DNA as a template for the mass production of Ag nanoparticles” *Polymer Journal*, In press (advance online publication, 26 June 2013; doi:10.1038/pj.2013.58).

## Presentations in Domestic Conferences:

1. Tomomi Takeshima, Yoshihisa Yamada, Masao Nishihara, Masahito Sugi, Norio Nishi, Tetsu Yonezawa and Bunshi Fugetsu. Preparation of Ag Nanoparticles Using Salmon Milt DNA. *The 77th the Society of Chemical Engineers Japan Annual Meeting*, March 15-17, 2012, Tokyo, Japan
2. Tomomi Takeshima, Yoshihisa Yamada, Yuya Tada, Masao Nishihara, Masahito Sugi, Norio Nishi, Tetsu Yonezawa and Bunshi Fugetsu. Salmon Milt DNA as the Potential Template for the Massive Production of Ag Nano-particles. *62nd the society of Polymer Science Japan Annual Meeting*, May 29-31, 2012, Yokohama, Japan
3. Tomomi Takeshima, Yoshihisa Yamada, Yuya Tada, Yanqing Wang, Masao Nishihara, Masahito Sugi, Norio Nishi, Tetsu Yonezawa and Bunshi Fugetsu. Characterization of Ag Nanoparticles Using Salmon Milt DNA as the Template. *61st Symposium on Macromolecules*, September 19-21, 2012, Nagoya, Japan
4. Tomomi Takeshima, Yuya Tada, Yoshihisa Yamada, Yanqing Wang, Masao Nishihara,

Masahito Sugi, Norio Nishi, Tetsu Yonezawa and Bunshi Fugetsu. Studies on the Antibacterial Efficiency of the Salmon Milt DNA Template Based Ag Nanoparticles. *61st Symposium on Macromolecules*, September 19-21, 2012, Nagoya, Japan

### **Presentations in International Conferences:**

1. Tomomi Takeshima, Yoshihisa Yamada, Yuya Tada, Yanqing Wang, Masao Nishihara, Masahito Sugi, Norio Nishi, Tetsu Yonezawa and Bunshi Fugetsu. Preparation and Characterization of Novel Ag Nanoparticles Using Salmon Milt DNA as the Template. *The 9th the society of Polymer Science Japan International Polymer Conference*, December 11-14, 2012, Kobe, Japan

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