Chapter 5
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In recent years, the bottom up approach, which mimics inherent construction principles of biological systems, is gaining importance for fabrication of ordered nanostructures as compared to top down techniques which are technically complicated, expensive and inefficient. Although the bottom up approach also has its share of difficulties pertaining to perfection and detail, with a rapidly growing understanding of the corresponding principles of nanofabrication, this approach may be technologically viable in certain application domains and advantageous over the lithographic techniques. Techniques such as electron beam lithography, ion beam lithography, X-ray lithography suffer from several shortcomings such as complexity and cost intensiveness of the equipments, slow performance rate and requirement of reliable, specific sources of electron, ion and X-ray beams, respectively. Recent advances in such techniques have made them efficient but expensive too. Currently used state-of-the-art physical and chemical methods also suffer from their inability to efficiently, precisely and reliably adjust interparticles distances and lattice symmetries of the super lattices formed, and are confined to closed packed type array features (Alivassatos et al., 1996; Mirkin et al., 1996; Braun et al., 1998).

Attempts have been made to assemble nanomaterials on various templates such as diblock co-polymers (Sohn et al., 2003), surfactants (Chen et al., 2003), carbon nanotubes, fullerenes (Lim et al., 2005), DNA (Aldaye and Sleiman, 2007) etc. In these examples, interactions at molecular level or intrinsic material properties of a template are used as driving force to assemble nanomaterials. Liquid-liquid interface (Kumar et al., 2002) and air-water interface (Selvakannan et al., 2005) have also been used to assemble nanoparticles. These approaches use interfacial properties, which limit the degree of freedom of nanoparticles present at the interface to coordinate them.
into assemblies. However, such methods are time consuming and employ laborious synthesis protocols. Non-biological techniques for the formation of self-assembled monolayers of pre-synthesized nanoparticles have some attractive features but these also suffer from certain drawbacks pertaining to the system and surface specificity. This scenario has made scientists look for biosystem-based alternatives for the realization of patterned assemblies of nanoparticles. Indeed, exploiting the natural tendency of biomolecules such as proteins, DNA to yield perfect self-assemblies through molecular specificities, thereby creating interactive surface templates for inorganic/organic molecular functionalizations clearly offers novel alternatives for nanofabrication.

Bacterial S-layer lattices exhibit pores of identical size (2-8 nm), morphology, and uniform thickness (5-35 nm). The functional groups on the surface and pores are aligned in well-defined position and orientation, and are accessible for binding to functional molecules in a very precise fashion. Most importantly, the isolated S-layer subunits can self-assemble and are capable of recrystallizing into monolayers onto solid supports, at the air/water interface, on Langmuir lipid films and on liposomes. These unique properties displayed on a nanometer scale make S-layer lattices ideal structures for functionalization of surfaces and interfaces for fabricating nanostructures required for the development of new generation optical and electronic devices, and for the formation of metal nanocluster arrays such as gold for nanoelectronic digital circuits.

Recent research has established that nanoparticles of gold are most stable metal nanoparticles. They exhibit interesting properties like assembly at multiple level, size related electronic, magnetic and optical properties due to quantum size effect, which makes them ideal for applications in the field of electronics, material science, catalysis and biology. Due to such wide spectrum applications gold nanoparticles will be key materials and building blocks of nanofabrication in 21st century. In order to fabricate new generation of functional devices one of the major step is synthesis and patterning of nanoparticles into controlled architecture.
In the present investigation an attempt is made to use the remarkable supramolecular features of S-layer protein of *Bacillus subtilis* for the synthesis and patterning of gold nanoparticles. In contrast to some studies performed earlier, our method relies on a simple chemical protocol, which embodies concurrent *in situ* reductive synthesis and assembly.

### 5.1. Synthesis of Gold Nanoparticles using S-layer Protein

The S-layer protein was isolated and purified from *Bacillus subtilis*, *Bacillus fusiformis*, and *Brevibacillus parabrevis*, according to the protocol mentioned in Chapter 2, Section 2.2. The purified S-layer protein (1 mg/ mL) was mixed with chloroauric acid (HAuCl₄·3H₂O, 1 mM) and allowed to react for 24 h at 37 °C. The absorbance spectrum of the reaction solution was recorded after 24 h using a UV-Visible spectrometer (Nano Drop, USA).

Fig. 5.1 shows the UV–Vis spectra of the solution containing the purified S-layer protein and chloroauric acid after 24 h. No specific absorption features in the wavelength range 500-560 nm, characteristic of gold nanoparticles, were observed.

![Fig. 5.1. UV-Vis spectrum of S-layer protein samples incubated with chloroauric acid.](image)
Visually, the color of the solution remained unaltered after the incubation period (24 h).

Thus, the S-layer protein of B. subtilis, B. fusiformis, and Brevibacillus parabrevis does not possess the ability to reduce ionic gold to gold nanoparticles.

5.2. Patterning of gold nanoparticles using S-layer (B. subtilis) as template

Since the purified S-layer protein showed an inability to synthesize gold nanoparticles, the subsequent in-situ patterning of these nanoparticles using the array-like features of the S-layer was not possible. However, the synthesis and patterning of gold nanoparticles on an S-layer array may be facile if an external reducing agent is used. This reductant would reduce chloroauric acid to gold nanoparticles, which subsequently would be arranged in a definite pattern on the S-layer template. However, recrystallization of the S-layer on a solid support prior to the synthesis of gold nanoparticles is required for the patterning of synthesized gold nanoparticles.

Since, the S-layer of B. subtilis showed an excellent recrystallization property (Chapter 2, Section 2.6) compared to the S-layers of B. fusiformis and Brevibacillus parabrevis, the S-layer of B. subtilis was chosen for the patterning of gold nanoparticles. Two different approaches were followed:

1) In the first approach, synthesis and patterning of gold nanoparticles was attempted on glass coverslips. The structural analysis was performed using AFM and SEM and elemental analysis was performed using Energy Dispersive X-ray Analysis (EDAX).

2) In the second approach, synthesis and patterning of gold nanoparticles was performed on carbon-coated copper grids and analyzed by Transmission Electron Microscopy (TEM).
5.2.1. Synthesis, patterning and imaging of gold nanoparticles on S-layer array recrystallized on glass cover slip

5.2.1.1. Isolation and purification of S-layer protein

The isolation and purification of S-layer protein from *B. subtilis* was performed as per the standardized protocol described in Chapter 2, Section 2.2. A total protein concentration of 1.0 mg/mL was used for recrystallization on glass coverslip as a solid support.

5.2.1.2. Recrystallization of S-layer on glass coverslip

The S-layer protein of *B. subtilis* was recrystallized from 50 mM Tris buffer solution containing 10 mM CaCl₂ onto piranha treated glass coverslips. The recrystallization process was carried out in Petri plates, wherein the protein solution (concentration 1 mg/mL) was incubated on the glass coverslip for 60 min. After completion of the incubation period, the excess protein solution was blotted and the coverslip was washed with sterile double distilled water to remove the unattached protein.

5.2.1.3. Synthesis of gold nanoparticles on recrystallized S-layer array

The glass coverslip coated with S-layer protein array was flooded with chlorauric acid solution (0.01 %, 100 μL) and incubated at room temperature for 1 h. The excess solution was blotted and the coverslip was allowed to dry in air. Synthesis of gold nanoparticles on S-layer array was achieved by addition of 1 % tri-sodium citrate solution (Frens, 1978). To increase the rate of nanoparticle synthesis, the coverslip was incubated at 37 °C for 1 h. The time of incubation (1 h) was fixed based on the results of the control sample (without the S-layer protein), wherein 1 % tri-sodium citrate completely reduced 1 mM chlorauric acid to gold nanoparticles (appearance of surface plasmon peak at 530 nm). The excess solution was blotted and the coverslip was washed with sterile double distilled water to remove impurities and
excess sodium citrate. An S-layer coated glass coverslip without exposure to chloroauroic acid served as control.

5.2.1.4. AFM imaging of gold nanoparticles on S-layer array

To detect the presence and arrangement of gold nanoparticles on the S-layer array, AFM studies of the glass coverslips were carried out. AFM scanning was performed in non-contact mode to ensure minimal sample damage. All measurements were performed at ambient temperature (20 °C) in air.

Fig. 5.2. AFM images of S-layer array of B. subtilis without treatment of chloroauroic acid

A- Cross sectional analysis of 2-D AFM image and
B- Three dimensional image of S-layer array
Fig. 5.3. AFM images of S-layer array of *B. subtilis* after treatment of chloroauric acid and tri sodium citrate
A- Cross sectional analysis of 2-D AFM image and
B- Three dimensional image of S-layer array

Figs 5.2A and 5.2B show the AFM images of the control glass coverslip, i.e., recrystallized S-layer without exposure to chloroauric acid. The presence of
an S-layer array with pore size 28-35 nm was observed, similar to the AFM images of the S-layer protein shown in Chapter 2, Section 2.4.4.

Figs 5.3A and 5.3B depict the AFM images of the glass coverslip containing gold nanoparticles on the S-layer array. 2-D cross sectional analysis and 3-D surface topology showed an increase in sample height. The increase in sample height indicated uneven deposits on the outer surface of the S-layer protein. When compared with AFM images of untreated S-layer array (control coverslip), accompanied by ~ 2-3 times increase in the height of the S-layer array was observed.

However, AFM analysis did not indicate the presence of gold nanoparticles and their distribution inside the pores of the S-layer, i.e., patterning of gold nanoparticles. The latter was expected since AFM is a surface-scanning technique and the AFM probe cannot image the gold species present inside the pores. Moreover, AFM analysis did not provide any chemical information on the micron-sized deposits on the S-layer surface. These structures could be aggregates of gold nanoparticles or aggregates of S-layer protein subunits.

5.2.1.5. SEM imaging of gold nanoparticles on S-layer array

To confirm the chemical nature of the surface deposits, Scanning Electron Microscopy and Energy Dispersive X-ray Analysis (SEM-EDAX, JEOL) was performed. SEM analysis confirmed the presence of sub-micron-sized (~400-600 nm in diameter) particles (Fig. 5.4). EDAX analysis confirmed the presence of gold in these particles (Fig. 5.5). Thus, the uneven deposits observed on the S-layer protein array are aggregates of gold nanoparticles. The presence of gold aggregates indicated that a major fraction of the gold nanoparticles, formed by the reduction of chloroauric acid, did not penetrate the pores of the S-layer as anticipated.
Fig. 5.4. Scanning electron micrograph of S-layer of *B. subtilis* after deposition and reduction of gold.
Scale bar: 5 μm

Fig. 5.5. Energy dispersive X-ray scattering spectrum of gold particles formed on S-layer array of *B. subtilis*.

The exact reason behind this phenomenon is not clear at the moment. One possible explanation is the incompatible electrostatic nature of the surface functional groups on the S-layer and the surface of the gold nanoparticles. The outer surface of S-layers from most members of the family *Bacillaceae*...
contain amine (-NH2) and carboxyl (-COOH) groups in equimolar amounts (Sleytr et al., 1994). On the other hand, gold nanoparticles formed by the citrate-reduction method are electrostatically stabilized due to presence of negative charge on its outer surface (Hayat, 1989, Dieuluweit et al., 1998). These electrostatically stabilized gold nanoparticles may attach to the free amino/carboxyl groups on the outer surface of S-layer due to its strong affinity (Marie-Christine and Didier, 2004). This process may act as a site of nucleation and further attachment of gold particles on the surface only. This would eventually lead to aggregation of the gold nanoparticles on the surface, as observed by AFM and confirmed by SEM analysis.

Although a fraction of the gold nanoparticles may have bypassed the surface functional groups and penetrated into the S-layer pores, it was difficult to confirm this using AFM. Transmission electron microscopy, on the other hand, has the ability to image both the surface and depth of the pores, due to the penetration of the electron beam through the S-layer sample. Therefore, in the second approach, transmission electron microscopy was used for detection of gold nanoparticles and their distribution on the S-layer template.

5.2.2. In situ synthesis of nanoparticles on S-layer array and TEM imaging

5.2.2.1. Recrystallization of S-layer on carbon-coated copper grids

The recrystallization of S-layer protein was carried out on carbon coated copper grids, which are used as sample holders for transmission electron microscopy (as mentioned in section 2.4, Chapter 2). Recrystallization was carried out in 30 mm plastic petri plates (Tarsons Ltd., India) filled with 3 mL of 50 mM Tris buffer solution (pH 7.5) containing 10 mM CaCl2. The S-layer protein (1 mg/mL) was added in the recrystallization buffer and the grids were placed horizontally at the liquid-air interface. The plate was incubated at 4 °C for 24 h. During the incubation period, the isolated S-layer subunits reasssembled in the recrystallization buffer to form self-assembly products, which subsequently were adsorbed onto the grids (Pum et al., 1993). After
incubation, the grids were carefully removed using tweezers and washed with Tris buffer solution (50 mM, pH 7.5) to remove the excess, unattached protein. The recrystallized S-layer was cross-linked with 2.5 % glutaraldehyde solution (2.5% v/v in 50 mM Tris buffer) for 5 min at room temperature.

5.2.2.2. Exploring assembly of preformed gold nanoparticles on S-layer array

In one set of experiments assembly of preformed gold nanoparticles on S-layer array was examined. In this experiment, gold nanoparticles of mean diameter (5±1 nm) were synthesized according to method described by Hermanson (1996). To arrange gold nanoparticles on the template of S-layer, copper grid coated with S-layer was exposed to 20 µL solution of gold nanoparticles. Excess solution was blotted dry and sample was investigated using transmission electron microscope.

![Fig. 5.6. Assembly of gold nanoparticles on the S-layer template of B. subtilis. Scale bar- 100 nm.](image)

When used for site specific immobilization of gold nanoparticles (diameter 5±2nm) the TEM analysis showed randomly positioned aggregates of gold nanoparticles on the S-layer template (Fig. 5.6). This implies that the
sedimentation and landing kinetics of preformed gold nanoparticles in liquid medium does not offer favorable conditions for realization of ordered nanoparticle assembly. The pores being extremely tiny (nanoscale), they apparently do not offer strong enough anchoring modulation for liquid borne nanoparticles to trap individually in separate pores in an ordered fashion. Other dominant force fields seem to cause coagulation. Hence another approach of concurrent in situ synthesis and assembly of gold nanoparticles inside the pores of S-layer array was examined.

5.2.2.3. Synthesis of gold nanoparticles on recrystallized S-layer array

In another set of experiments concurrent in situ synthesis and assembly of gold nanoparticles on the S-layer was explored. The copper grid coated with the monolayer of unstained S-layer protein was exposed to 1 mM chloroauric acid solution (50 µL) and incubated for 5 min. The copper grids exposed to gold were blotted and allowed to dry in air. Further, synthesis of gold nanoparticles was achieved by exposing the coated grid to hydrazine solution (6 mM, 50 µL) for 15 min for complete reduction of Au^{3+} to Au^{0}. The S-layer sample stained with 2 % ammonium molybdate and unexposed to chloroauric acid served as control.

5.2.2.3.1. TEM imaging of gold nanoparticles on S-layer array

The carbon-coated copper grids containing gold nanoparticles coated on the recrystallized S-layer protein were analyzed by TEM on a JEOL model 1200EX transmission electron microscopy instrument operated at an accelerating voltage at 80 kV. Fig. 4.6 denotes the TEM micrograph of the control sample (stained S-layer unexposed to gold). The native architecture of the protein containing uniform sized pores (~ 2 nm) was observed (Fig. 5.7).
Fig. 5.7. Transmission electron micrograph showing S-layer array of *B. subtilis* stained with 2% ammonium molybdate. Scale bar- 50 nm

Fig. 5.8. Transmission electron micrograph showing patterned and symmetrically arranged gold nanoparticles synthesized in the pores of S-layer array of *B. subtilis*. Scale bar-100 nm.
Fig. 5.9. Magnified view of transmission electron micrograph showing arrangements of gold nanoparticles on S-layer template

Figs 5.8-5.10 shows the presence of spherical gold nanoparticles (2 ± 1 nm), formed by the hydrazine reduction method, arranged in a periodic assembly according to the native geometry of S-layer protein. Two types of periodic arrangements of the nanoparticles were observed: circular (highlighted in Fig. 5.9) and linear (highlighted in Fig. 5.9 and 5.10). However, the linear arrangement was found to be more dominant than the circular geometry. Thus, the regular arrangement of gold nanoparticles confirmed the penetration of the nanoparticles within the pores of the S-layer protein, which provided a template for the patterning of the nanoparticles.

Additionally, the presence of nanoparticles aggregates was also observed as large black spots (indicated by square boxes), which indicate the aggregation of nanoparticles on the protein surface. This is in corroboration with the AFM and SEM analysis of the gold deposits on the S-layer surface.
Fig. 5.10. Transmission electron micrograph of gold nanoparticles formed in pores of S-layer array of B. subtilis. Scale bar- 50 nm.

Fig. 5.11. Electron diffraction pattern obtained from the gold nanoparticles shown in Fig. 5.10.

The electron diffraction pattern of the patterned gold nanoparticles is shown in Fig. 5.11. The presence of discrete spots and rings indicated that the gold nanoparticles had a crystalline nature.
Fig. 5.15. Particle size distribution histogram of gold nanoparticles synthesized on S-layer template of *B. subtilis*.

Fig. 5.15 shows the particle size distribution of gold nanoparticles synthesized using hydrazine on S-layer template. The particle size distribution suggests that the average particle size is 6-7 nm in the case of gold nanoparticles formed on S-layer template. The quantity of the salt incorporated in a pore should depend on the interface governed shape and size of the droplet incorporated within or on the pore and the same would govern the size of the nanoparticles formed.

Apart from the successful synthesis and periodic patterning of the gold nanoparticles on the S-layer template, a major highlight of the TEM studies was the establishment of a protocol for rapid sample preparation for electron microscopy.

Dieлуweit and coworkers (2005) reported the synthesis of gold nanoclusters of ~4 nm diameter using S-layer of *B. sphaericus* CCM 2177, wherein the time required for the formation of the nanoparticles ranged from hours to several days. The reduction of gold was achieved by treating the gold-exposed S-
layer array to H2S for 2 days. Additionally, physical methods, e.g., electron beam have been used for reduction of metal deposited on S-layer (Dieluweit et al., 1998, Mertig et al., 1999, 2001). Merroun et al. (2006) used H2 gas as the reducing agent to synthesize gold nanoparticles in presence of S-layer protein of B. sphaericus JG-A 12. Compared to these reported processes, the protocol developed in the present study was fast and required no specialized equipment.

Synthesis of gold nanoparticles anchoring on the pores of S-layer is achieved by chemical reduction of respective metal salt laden protein template. Transmission electron microscopy reveals formation of well ordered and separated gold nanoparticles with an average diameter of 6±1 nm. The periodic arrangement of nanoparticles is dictated by the native structure of S-layer protein array as the nanoparticle locations are found to be correlated to the nanosized pores of the crystalline S-layer array. Electron diffraction studies revealed a crystalline nature of the patterned gold nanoparticles.

Thus in the present study we have successfully used recrystallized S-layers of B. subtilis for synthesis and patterning of uniform-sized gold nanoparticles. Moreover, a simple and rapid process of synthesis and subsequent patterning of gold nanoparticles on the S-layer array was established. This novel process can be a new generic method for patterning of nanoparticles.