Synthesis, capping and binding of colloidal gold nanoparticles to proteins

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Abstract

Bovine serum albumin (BSA) was used as a stabilizing agent and biofunctionalized layer for water-dispersed gold nanoparticles (NPs) synthesized from metal precursor HAuCl₄. The BSA binding to gold NPs was characterized qualitatively and quantitatively by transmission electron microscopy, UV-VIS and FTIR spectrophotometers. HER2 (human epidermal growth factor receptor 2) specific phage antibodies were attached to BSA stabilized gold NPs to form a gold–antibody complex. An ELISA (enzyme-linked immunosorbent assay) test was done to confirm the bioactivity of antibodies attached to gold NPs.

Keywords: colloidal gold nanoparticles, protein, nanostructure complex

Classification numbers: 2.04, 4.02, 5.08

1. Introduction

Gold nanoparticles (NPs) exhibit a unique phenomenon, known as surface plasmon resonance, which is responsible for their large absorption and scattering cross-sections of four to five orders of magnitude larger than those of conventional dyes. In addition, their optical properties can be controlled by varying their size, shape and composition. Gold NPs synthesized in water and subsequently linked to biomolecules have many applications in the life sciences, such as drug-delivery, gene transfer, bioprobes in cell and tissue analysis and studies of biological processes at the nanoscale [1–5]. The conjugation of proteins with NPs not only affords stabilization of the system but, more importantly, also introduces biocompatible functionalities into these NPs for further biological interactions. The surface modifications of gold NPs to provide stable bimolecular functionalized NPs in biomedical applications have been reported by various groups [2, 6–8]. On the other hand, antibody–antigen binding is a fundamental phenomenon in the fields of biochemistry and biology. Most bioassays are based on an antibody–antigen binding pair [9]. Serum albumin is the major protein component of blood plasma but is distributed to the interstitial fluid of the body tissues [10]. Serum albumin is capable of binding to a wide variety of drugs, and there is strong interest in this abundant protein because of its effects on drug delivery [10, 11]. Therefore, immobilization and immunoreaction of a fundamental protein, such as serum albumin on gold NPs, is very important for bioapplications.

HER2 (human epidermal growth factor receptor 2) is a protein that is highly aggressive in breast cancers. Overexpression of the receptor in breast cancer is associated with increased disease recurrence and worse prognosis, so breast tumors are routinely checked for overexpression of HER2 [12]. In order to detect a tumor before it turns into late stage cancer, virus based NPs are more and more being used for diagnostic imaging. The use of ‘smart’ NPs, which combine multiple functions of targeting, imaging and drug delivery, have great potential in increasing the sensitivity and specificity of therapies [13]. The bacteriophage M13 presents many attractive features, such as having a high surface density (300–400 m² g⁻¹), so it can stick to whatever
it has been designed to target. Furthermore, a phage has 2700 copies of the major coat protein P8 and 5 copies of two different minor coat proteins (P9, P6 and P3), which can be genetically engineered to express peptides that have a high affinity for cancer markers, other proteins and inorganic materials [14, 15].

In this paper, we report the synthesis of gold NPs from metal precursor HAuCl\(_4\) using trisodium citrate dihydrate as a reducing agent. Bovine serum albumin (BSA) was used as a stabilizing agent and a biofunctionalized layer for the gold NPs. Phage HER2 specific antibodies were attached to the synthesized NPs.

2. Experimental

2.1. Materials

Tetrachloroauric acid trihydrate 99.5% (HAuCl\(_4\)·3H\(_2\)O), trisodium citrate dehydrate (Na\(_3\)C\(_6\)H\(_5\)O\(_7\)·2H\(_2\)O) and N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (EDC), horseradish peroxidase (HRP) and 3, 3’, 5, 5’-tetramethylbenzidine dihydrochloride were purchased from Merck. Bovine serum albumin (BSA) was purchased form Biochem. Polyethylene glycol (PEG) 4000 was obtained from Sigma-Aldrich. Double distilled water was used throughout the course of this investigation.

2.2. Preparation of gold NPs

Gold colloids were prepared by sodium citrate reduction of HAuCl\(_4\) following our previous work [16]. 90 ml of 3 \times 10^{-2} M aqueous solution of chlorauric acid (HAuCl\(_4\)) was allowed to boil, at which point 3.6 ml of 6.8 \times 10^{-2} M sodium citrate was added dropwise with stirring. Following the addition of sodium citrate, the solution began to darken and turn bluish-gray or purple. After approximately 10 min, the reaction was completed and the final color of solution was a deep wine red. The solution was cooled to room temperature with continued stirring. The suspension was stored in the refrigerator at 4°C until needed. The average diameter of the citrate capped gold NPs (AuNPs) was determined from a high resolution scanning electron microscope (HSEM, Hitachi-S480) image, measuring 15.8 \pm 3.8 nm.

2.3. Biofunctionalization by bovine serum albumin (BSA)

Some BSA was added into AuNPs solution, synthesized by the method as described above, under stirring at room temperature till optically clear. The minimum amount of protein BSA required to stabilize AuNPs was determined by employing the surface plasmon absorption resonance assay. In this assay, serial dilutions of the BSA–AuNPs at pH 4 and pH 7.4 were prepared, varying the quantities of BSA from 0 to 300 \(\mu\)g in 1 ml of AuNPs solution (\(\mu\)g ml\(^{-1}\)), followed by UV-VIS spectrophotometric analysis. The minimum amount of protein BSA necessary to cap AuNPs was deduced graphically from the concentration at which the absorbance at pH 4 became nearly constant.

The AuNPs and BSA–AuNPs solutions were characterized using a transmission electron microscope (TEM, JEM 1011) and a UV-VIS spectrophotometer (JASCO-V570-UV-VIS).

2.4. Attaching BSA–AuNPs to anti HER2 phage display antibodies (HER2 phage)

(a) HER2 specific phage display antibodies (provided by Institute of Biotechnology, VAST) are M13 phage displayed HER2 specific antibodies (HER2 Abs). Anti HER2 phage single-chain Fv variable (scFv) fragment antibodies were panned and selected from Griffin1 highly diverse human scFv library, based on its affinity with HER2 target antigens. Griffin1 library includes 10\(^6\) different antibody cloned fragments with heavy chain (V\(_H\)) and light chain (V\(_L\)) variable regions. These scFv fragments were fused with shell protein P3 of phage M13 to create the anti HER2 fragments displayed on the phage. HER2- phages were dispersed in PBS buffer (phosphate buffered saline) of pH 7.4.

(b) Attaching BSA–AuNPs to HER2-phage antibodies:
- Mix BSA–AuNPs (150 \(\mu\)g ml\(^{-1}\)) with EDC activated antibodies (1 ml, 10\(^3\) CFU in PBS) and react for 1 h at 4°C temperature; then shake the mixture at 200 rpm for 1 h keeping at 4°C temperature.
- Separate AuNP–Ab conjugates from excess free antibodies and AuNPs via centrifugation of 12,000 rpm at 4°C temperature in 400 \(\mu\)l solution containing 20% PEG for 15 min (twice). The concentrated Au–Ab complex was rediluted in 100 \(\mu\)l PBS buffer.

(c) ELISA (enzyme-linked immunosorbent assay) test was done to confirm the bioactivity of the Au–Ab complex. The ELISA test was performed with a flat bottomed ELISA plate coated with HER2 antigen (100 \(\mu\)l, 10 \(\mu\)g ml\(^{-1}\)) overnight at 4°C. The wells were washed three times with PBS, blocked with skimmed milk (2% in PBS). After washing as described above, with PBS-T1 (0.05% (v/v) Tween 20 in PBS), 100 \(\mu\)l neat culture medium containing HER2 or Au–HER2 antibodies was added. The wells were incubated for 90 min at 37°C and washed three times with PBS-T2 (0.1% (v/v) Tween 20 in PBS pH 7.2). Then, the wells were incubated for 1 h at room temperature with 100 \(\mu\)l of the secondary antibody conjugated with HRP diluted in PBS. Wells containing PBS alone were included as assay blanks. The substrate used for color development was 3, 3’, 5, 5’ tetramethylbenzidine dihydrochloride. The wells were decanted and washed thoroughly. Absorbance at 450 nm of samples was measured in a plate reader.

3. Results and discussion

3.1. Biofunctionalization of gold NPs by BSA protein

Figure 1 (black line) shows the UV-VIS absorption spectra of AuNPs at pH 7.4. A strong plasmon absorption peak at 519 nm attributed to AuNP collective electron oscillations or localized surface plasma resonance (SPR) is presented and is a feature of 16 nm AuNPs. The red line in figure 1 shows the UV-VIS absorption spectra of BSA protein stabilized AuNPs. It is clear that the plasmon absorption peak of AuNPs shifts from 519 nm to 526 nm after the modification of BSA. The resonance wavelength and bandwidth of AuNPs are dependent on the particle size and shape, the refractive index of the surrounding medium and the temperature. This shift after conjugation of AuNPs with BSA is attributed to
the changes in the dielectric nature surrounding the AuNPs due to BSA presence \((n = 1.9)\). The inset in figure 1 shows the TEM image of the BSA stabilized AuNPs. Compared to citrate capped AuNPs, a shadow around the AuNPs is clearly observed in the BSA stabilized NPs and represents the BSA protein layer on the surface of gold NPs. The thickness of shadow surrounding the gold NPs is about 4 nm and is quite close in size to that of the BSA, which has a molecular weight of 69 kDa with dimensions \(4 \times 4 \times 14\) nm\(^3\).

The FTIR spectra of gold NPs without (lower line) and with (upper line) BSA conjugate molecules are shown in figure 2. The main peaks at 1589 cm\(^{-1}\) and 1403 cm\(^{-1}\) due to CH\(_2\)-O deformation and C=O stretching of citrate ligand in AuNPs spectra, respectively, disappeared after BSA conjugation. Several other peaks appeared at 727, 1629 and 3255 cm\(^{-1}\), which are associated with the N–H deformation and stretching vibration, respectively. These results verify the adsorption of BSA onto gold NPs via a dative bond between gold and sulfur to form a stable protein-gold conjugate \([2]\).

At pH 4, the AuNPs are not stable due to charge neutralization following the aggregation of gold particles \([10]\), and their absorption spectra are large and low in intensity compared with the situation at pH 7.4 (figure 3(a)). As shown, addition of protein BSA forms new capping by replacing the citrate ions on the gold surface. If the added amount of BSA protein is not enough to replace all the citrate
capping molecules, the residue citrate ions continue to react with hydrons in solution to form citrics, leaving gold to tend to aggregate following the dependence of its absorption spectra on BSA added amounts (figure 3(a)). When the BSA molecules are enough to cap all the surface of gold to form a stable protein–gold conjugate, its absorption spectra become constant. The minimum amount of protein BSA necessary to prevent aggregation of AuNPs was deduced graphically from the concentration at which the absorbance at pH 4 became nearly constant. This value must be determined for every gold solution. In this case, the minimum BSA amount necessary for capping was about 100 µg ml\(^{-1}\) (figure 3(b)).

### 3.2. Attaching BSA–AuNPs to anti HER2 antibodies

Figure 4 shows the UV-VIS spectra of the BSA conjugated AuNPs with (red line) and without (black line) addition of HER2 antibodies. As shown, the SPR peak has a red shift from 519 nm to 529 nm and is much broader after the addition of HER2 antibodies. Particle aggregation typically results in a red shift of the peak position as well as a peak broadening, caused by the near-field coupling between adjacent particles. Moreover, the magnitude of the red shift is highly dependent on the interparticle distance and the size of the aggregations.

Figure 5 shows the TEM images of BSA: (a) stabilized AuNPs only, (b) HER2 phage antibodies only and (c) with the addition of BSA–AuNPs (c), respectively. As is well known, the size of the phage is about 6–8 nm wide and 1000 nm long. In this study, the concentration of phages is high, so that they wind and join together to create bigger filaments and heads. The gold NPs are well dispersed before adding antibodies. After the addition of HER2 Abs, the AuNPs bind along the phage body and become concentrated on the phage head. The gold binds phages form networks at the micrometer scale with large islands—phage heads like aggregations consisting of hundreds of gold NPs. Consequently, when HER2 antibody was added into the solution of BSA stabilized AuNPs, aggregation occurred.

Figure 6 presents the absorbance at 450 nm of samples in the ELISA test. The absorbance of wells containing Au–HER2 Abs is shown in columns 1, 2, 3 and 5, while that of bare HER2 Abs and PBS are shown in columns 4 and 6, respectively. These results demonstrate that the Au–HER2 Abs had the activity of HRP as well as bare HER2.

### 4. Conclusion

The BSA protein functionalized stabilized gold NPs were prepared. The minimum amount of protein BSA necessary to stabilize AuNPs of 16 nm was determined as about 100 µg ml\(^{-1}\). The BSA stabilized gold NPs were successfully attached to phage HER2 antibodies by a simple route, forming a complex Au–HER2 antibody. The bioactivity of the complex Au–HER2 was proved by the ELISA test. These results show the quality of BSA conjugated gold NPs as bioprobes, and the ability of the Au–HER2 antibody complex as cancer marker. Other studies must be done in order to introduce gold NPs into biomedical applications.
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