Synthesis of Gold Nanoparticles Coated with Aspartic Acid and Their Conjugation with FVIII Protein and FVIII Antibody

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Abstract Carboxylate-modified gold nanoparticles (GNPs) were synthesized in a simple one-step process based on the reduction of tetrachloroauric acid by aspartic acid in water. GNPs were identified by UV–Vis spectroscopy, dynamic light scattering (DLS) and transmission electron microscopy. Conjugation of protein molecules with functionalized nanoparticles was performed through electrostatic interaction. The GNP–protein conjugates were characterized by gel electrophoresis. The interaction between functionalized GNPs and protein molecules lead to conformational transition of protein structure after conjugation of protein with GNPs. This process was investigated by fluorescence spectroscopy and circular dichroism spectroscopy.

Keywords Gold nanoparticles · Colloid · Aspartic acid · Bioconjugation · FVIII protein

Introduction

Unique properties of metal nanoparticles like optical, electronic and catalytic properties are presently under intensive study for many applications such as optoelectronic devices [1], ultrasensitive chemical and biological

sensors [2–4], and as catalysts in chemical and photochemical reactions [5, 6].

The GNPs are of prime importance in biochemical and biomedical applications [7]. These interests in GNPs are strongly dependent on optical resonance in the visible range and their sensitivity in environmental changes, the particle size, interparticle distance, and shape of the nanoparticles. Therefore, applications require synthesis protocols, which deliver well-defined shapes and sizes [8, 9].

One of the most common methods used for the synthesis of gold colloids, includes the reduction of an aqueous solution of tetrachloroauric acid (HAuCl_4) by trisodium citrate in water, suggested by Turkevich [10, 11]. These nanoparticles are known to be stabilized by a physical adsorption of excess citrate ions from the medium [11]. Other methods used for synthesis of GNPs involve the two-phase Brust method. GNPs produced by this method have superb stability. The gold particles coated and stabilized with thiol-derivative monolayers are dispersed in nonpolar and weakly polar organic solvents [10, 12]. But the byproduct of these reducing agents and the organic solvents used in these techniques may make them unsuitable for using in some bioanalytical applications. Therefore, it is preferable to use an in situ reduction technique so that the byproduct of the system remains compatible with biosystems.

GNPs synthesized with reduction technique compatible with biosystems in water and subsequently attached to biomolecules have contributed immensely in applications such as drug-delivery, gene transfer, bioprobes in cell and tissue analysis, and for observation of the biological processes at nanoscale, etc. [5, 13, 14]. The need of a bio-compatible synthesis of stable GNPs prompted us to explore an alternative technique for synthesizing GNPs in aqueous media by the use of an amino acid.

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In this study, synthesis and coating of stable GNPs by using an amino acid, was described. GNPs were prepared in a simple one-step process in which the amino acid acted as a reducing agent and then linked to GNPs surface. The prepared particles were characterized with a variety of methods such as X-ray diffraction, transmission electron microscopy (TEM), DLS, thermo gravimetric analysis and UV–Vis spectroscopy. Also, the binding of biomolecules, such as Factor VIII and anti-FVIII antibody (IgG), to the GNPs was studied qualitatively and quantitatively by various techniques.

Materials and Methods

Materials

HAuCl₄, aspartic acid, and sodium dodecyl sulfate (SDS) were purchased from Aldrich Chemicals. Factor VIII and anti-FVIII antibody were obtained from Pasteur Institute of Iran.

Synthesis of Gold Nanoparticles

1.5 mL of 25 mM aspartic acid solution was added to 25 mL of de-ionized water and the mixture was heated till boiling. Upon boiling, 5 ml of chloroauric acid solution (such that the molar ratio of aspartic acid to chloroauric acid was adjusted to 7.5), was added to this mixture under vigorous stirring and heating conditions till boiling. After a few minutes, the reduction of the gold salt (Au³⁺) to GNPs (Au⁰) was confirmed by the appearance of a dark-red colloidal solution. When the color of the colloid stabilized, the reaction was rapidly quenched in ice.

Instrumentation and Characterization of GNPs

UV–Vis Spectrophotometry

The optical properties of the gold colloidal solution were monitored on a Shimadzu dual beam spectrophotometer (Model 1601) in the range of 300–700 nm. Quartz cuvettes with 1 cm optical length were used for all measurements.

Transmission Electron Microscopy

Samples for TEM analysis were prepared by placing a drop of the gold colloidal solutions on carbon-coated copper TEM grids. The sample deposited on the grid was allowed to dry in air for a few minutes before analysis. The morphology and the size of the prepared gold nanoparticles were performed on a JEOL-JEN 2010 transmission

electron microscope operated at an accelerating voltage of 200 kV.

Dynamic Light Scattering (DLS)

Particle size distribution determination was carried out with Zetasizer (Malvern, UK) instrument by illuminating the gold colloidal solution with He–Ne laser (633 nm) in a sample cell.

Thermal Stability Measurements

TGA profiles of aspartic acid and aspartic acid-coated GNPs were monitored on a Seiko Instrument TG/DTA 32 at a heating rate of 20 °C/min in the temperature range of 24–700 °C under a nitrogen flow of 40 mL/min.

Bioconjugation of Protein to GNPs

By means of electrostatic and hydrophobic binding interactions between GNPs and protein, conjugation was happened. For preparation of GNPs–protein (GNPs–antibody) conjugates, GNPs were mixed (diluted) with phosphate buffer (10 mM, pH 7.4). The protein solution with different concentrations was added to the diluted solution containing GNPs to achieve different protein/gold nanoparticle mole ratios [(a)1:400, (b)1:600, (c)1:800, (d)1:1000, (e)1:1250, (f)1:1500], respectively. The mixture was stirred overnight at 4 °C.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Conjugation of GNPs–proteins (FVIII and IgG) was investigated by SDS-PAGE. Small aliquots (15 µL) of the conjugates were loaded on 10 % gel, and ran for 3 h at 80 V.

Circular Dichroism (CD) Spectroscopy

Determination of protein conformation and the study of protein interaction with other molecules were performed by CD spectroscopy. CD measurements were carried out with Aviv CD spectrometer in a wavelength range between 197 and 250 nm with a 0.1 cm path length quartz cell.

Fluorescence Spectroscopy

Fluorescence spectroscopy studies of GNPs–proteins solutions were performed using a Cary Eclipse Spectrofluorimeter between 300 and 450 nm

Results and Discussion

Generally, GNPs are produced by reduction of chloroauric acid with amino acid. The reduction of HAuCl_4 is carried out through electrons transition from amine group of the amino acid to Au^{3+} ions. This causes the formation of gold atoms. Various mechanisms are proposed about the amino acids oxidation by the gold salt, but the mechanism which has been presented in Fig. 1 is more reasonable [15]. The formed GNPs are then coated with amino acid molecules.

GNPs colloids are colorful. Colloidal GNPs are shown in Fig. 2. According to Mie theory some metals like gold and silver shows resonances known as Plasmon in UV–visible spectrum. These resonances are formed by interactions of electromagnetic waves and electron gas at the surface of nanoparticles. This resonance characteristic of the nanoparticles can be observed by spectroscopy. The characteristic is only clear when the particles reduce to certain scale of nanosize. Any change in the size and shape of the nanoparticles yields in the change and displacement of surface Plasmon resonance (SPR). As a result, the appearance, color and absorption properties of these

Fig. 1 Colloidal GNPs (bottle).UV–Vis spectra of aspartic acid-reduced GNPs (curve)

Fig. 2 **a** TEM image of aspartic acid reduced GNPs and **b** a histogram of particle size distribution determined by DLS

nanoparticles will change depending on the diameter of the molecule layers [16, 17]. Figure 2 depicts the UV–Vis spectrum of colloidal GNPs produced by aspartic acid. As can be inferred from the results, the nanoparticles have a broad absorption band in the visible region around 530 nm.

The TEM image of the nanoparticles is shown in Fig. 3a. Mono-dispersed spherical GNPs can be seen while their mean grain size is in the range of 10 ± 5 nm. The particle size distribution curve obtained by DLS is shown in Fig. 3b. In the DLS method, it is to be noted that the measured diameter is the hydrodynamic diameter of the particles. The comparison of the results achieved by TEM (10 ± 5 nm) and DLS (29.85 nm), on nanoparticles produced by aspartic acid, confirms the indicated information. In fact, the diameter measured in this technique is somehow larger than the real diameter of the particles achieved by TEM.

The conjugation of nanoparticles with drugs and proteins is widely used in biological processes. To apply nanoparticles in various processes such as diagnosis and therapeutics, they should be coated for more compatibility of the particles with biological systems. In the preparation method, amino acids initially act as reducing agent and then bind to GNPs surface. This is due to the fact that both the Au and N atoms of amine group in the amino acid are soft [5, 12–18]. Figure 4b schematically demonstrates the aspartic acid binding to GNPs surface.

TGA profiles of aspartic acid-coated GNPs and pure aspartic acid are shown in Fig. 4a. The aspartic acid-coated GNPs display three weight losses in the temperature intervals 180–240 °C (44 % weight loss), 320–355 °C (10 % weight loss) and 500–560 °C (2 % weight loss), while pure aspartic acid shows three sharp weight losses in the temperature intervals 235–270 °C (27 % weight loss), 395–430 °C (33 % weight loss) and 505–580 °C (21 % weight loss).

The monotonic weight loss (ca. 44 %) in the temperature interval 180–240 °C is attributed to desorption of trapped water and aspartic acid molecules which are bounded by

Fig. 3 **a** Thermogravimetric analysis profile of carefully weighed powders of purified aspartic acid-coated GNPs and pure aspartic acid. **b** The schematic representation of coated-GNPs with aspartic acid

non-covalent interactions such as hydrogen bonding to coated-GNPs. The complete decomposition of the amino acid molecules is bounded to the gold nanoparticle surface (ca. 12 %) in the temperature interval 320–560 °C. These results clearly indicate that aspartic acid molecules are bounded to the gold nanoparticle surface.

Due to presence of carboxylic acid groups, the nanoparticles formed by aspartic acid are proper to react with proteins. Lots of amine groups in proteins can react with carboxylic acids at the surface of GNPs and attach to them. The attachment of the functionalized nanoparticles to proteins creates stable gold–proteins complexes.

Fig. 4 UV-Vis spectra and size distribution of gold particles prepared by aspartic acid **a** GNPs conjugated with FVIII protein and **b** GNPs conjugated with IgG

Fig. 5 SDS-PAGE, stained by Coomassie Blue. **A** FVIII:GNPs and **B** IgG:GNP conjugates. Wells: (a) 1:400, (b) 1:600, (c) 1:800, (d) 1:1000, (e) 1:1250, (f) 1:1500, (O) control, (L) standard protein ladder. Molecular weights are 170, 130, 95, 72, 55, 43, 34, 26, 17, 10 kDa

The conjugation of GNPs to proteins was studied by UV–Vis spectrophotometry. Figure 5a shows UV–Vis spectrums of gold nanoparticles and GNPs conjugated with factor VIII protein (antibody). As can be seen, conjugation leads to red shift in the spectrum [19–21]. The particle size distribution curves made by DLS are demonstrated in Fig. 5b. Results show that conjugation increases the nanoparticles size and their distribution [21, 22].

A detecting method for conjugation stage is SDS-PAGE analysis. As can be seen in the Fig. 6a: FVIII, b: IgG the bands in conjugated sample are displaced from that of standard sample [23–26]. Increasing the protein concentration decreases the shift extent of conjugated protein bonds to the nanoparticles one. Once the molar ratio of colloidal gold solution to factor VIII and antibody reaches to 1:1,000, conjugation occurs.

Fluorescence spectroscopy is used for studying intrinsic fluorescence of tryptophan of antibody and protein molecules due to its high sensitivity. Figure 7 depicts the relative fluorescence intensity of tryptophan units of factor

VIII and antibody with GNPs. The conjugation of nanoparticles to protein (antibody) quenches the tryptophan fluorescence of the protein. It means that a change in conformation occurs due to binding of nanoparticle to tryptophan or to a group around tryptophan [27].

CD spectroscopy is an important method to study protein interaction with other molecules and also to determine protein structure. Far ultraviolet region (190–240 nm) is used to measure the secondary structure of proteins. A significant percentage of factor VIII has α -helical structure. α -helical structure has two negative bands in 208 and 222 nm. Any change in ellipticity of these regions refers to as distortion from standard situation [28, 29]. The decrease in ellipticity in 208 nm in CD spectrum of factor VIII indicates that the extent of α -helical structure of the protein is reduced as a result of conjugation with GNPs. In fact the CD spectrum of the conjugated nanoparticle-factor VIII protein displays a structural change from α -helix to β -sheets. In IgG molecules conjugated with GNPs a similar structural change occurs in secondary structure of IgG (Fig. 7).

Fig. 6 Fluorescence spectra conjugation of **a** GNPs–FVIII and **b** GNPs–antibody

Fig. 7 **a, b** show representative CD spectra of **a:** FVIII and FVIII conjugated with GNPs. **b** IgG and IgG conjugated with GNPs

Conclusions

In this synthetic method, aspartic acid plays an important role as a reducing and covering agent. Amino acids initially act as reducing agent and then bind to prepared GNPs. The particles are prepared roughly spherical with average particle size of 5–20 nm. The toxicity risk of the reducers is avoided through applying a non-toxic material in this synthesis method. Functionalized GNPs by amino acid are conjugated with protein and antibody.

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