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RESEARCH PAPER

UV–Visible intensity ratio (aggregates/single particles) as a measure to obtain stability of gold nanoparticles conjugated with protein A

M. A. Ríos-Corripio · B. E. García-Pérez · M. E. Jaramillo-Flores · V. L. Gayou · M. Rojas-López

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Abstract We have analyzed the titration process of gold nanoparticles with several amounts of protein A (0.3, 0.5, 1, 3, 6, and 9 μg/ml) in the presence of NaCl, which induces aggregation if the surface of particles is not fully covered with protein A. The colloidal solutions with different particle size (16, 18, 20, 33 nm) were synthesized by citrate reduction to be conjugated with protein A. UV–Visible spectroscopy was used to measure the absorption of the surface plasmon resonance of gold nanoparticles as a function of the concentration of protein A. Such dependence shows an aggregation region ($0 < x < 6$ μg/ml), where the amount of protein A was insufficient to cover the surface of particles, obtaining aggregation caused by NaCl. The next part is the stability region ($x \geq 6$ μg/ml), where the amount of protein used covers the surface of particles and protects it from the aggregation. In addition to that the ratio between the intensities of both: the aggregates and of the gold nanoparticle bands was plotted as a function of the concentration of protein A. It was determined that 6 μg/ml is a sufficient value of protein A to stabilize the gold nanoparticle–protein A system. This method provides a simple way to stabilize gold nanoparticles obtained by citrate reduction, with protein A.

Keywords Gold nanoparticles · UV–Visible · Protein A · Titration

M. A. Ríos-Corripio · V. L. Gayou · M. Rojas-López (✉)
CIBA-Tlaxcala, Instituto Politécnico Nacional, Tepetitla, Tlaxcala, Mexico
e-mail: marlonrl@yahoo.com.mx

B. E. García-Pérez
Departamento de Inmunología, ENCB, Instituto Politécnico Nacional, Mexico, DF, Mexico

M. E. Jaramillo-Flores
Departamento de Ingeniería Bioquímica, ENCB, Instituto Politécnico Nacional, Mexico, DF, Mexico

Introduction

Colloidal metallic particles, in particular gold, have been found to possess biological applications as affinity labels in the diagnostic field and also as probes in light and electron microscopy (Horisberger 1992; Sonvico et al. 2005; Jennings and Strouse 2007). Gold nanoparticles 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 (Chen et al. 2008).

Conjugation of gold nanoparticles with proteins not only affords stabilization of the system but, more importantly, also introduces biocompatible functionalities into these nanoparticles for further biological interactions (Wangoo et al. 2008). In particular, protein A (cell wall protein) produced by most strains of *Staphylococcus aureus* (Forsgren 1970), has been employed to be conjugated to the surface of gold

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nanoparticles (Dilan et al. 2007). Protein A is functionally bivalent (Langone 1982) and it results to be very useful into the sensing field because it reacts with the Fc region of immunoglobulin G from various mammals (Forsgren and Sjöquist 1966; Patrick 2009) and in some species with IgA and IgM as well (Langone 1978; Goudswaard et al. 1978).

Conjugated gold nanoparticles, are obtained from the negative charge on the gold colloids, (synthesized by citrate reduction) having an affinity for proteins that are positively charged at neutral or physiological pH (Norde 1986; Hayat 1989; Hermanson 2008). Some proteins, in particular antibodies, can adsorb strongly to colloidal gold to form stable conjugates with the protein retaining its biological property. The adsorption of proteins to gold nanoparticles is non-covalent process based on three separate but dependent phenomena: (a) ionic interaction between the negatively charged nanoparticle and the positively charged sites on the protein; (b) hydrophobic attraction between the protein and the metal surface; (c) dative binding between the metal and the conducting electrons of nitrogen and sulfur atoms of the protein (Smita et al. 2010).

On the other hand, antibody–antigen binding is a fundamental phenomenon in the fields of biochemistry and biology that may be mostly used with gold nanoparticles adequately conjugated with protein A. The success of the performance of nanoparticle-based immunoassays could depend on the chemical properties of the gold nanoparticles, as well as the bioactivity and adequate conjugation of the protein A.

In this article, we report the obtaining of a stability condition of gold nanoparticles conjugated with protein A through the estimation of the UV–Visible intensity ratio (aggregate/individual particles). This method provides a simple way to stabilize gold nanoparticles obtained by citrate reduction, with protein A.

Materials and methods

Materials

Tetrachloroauric acid trihydrate 99.5 % ($HAuCl_4 \cdot 3H_2O$) as precursor purchased from Sigma-Aldrich, sodium citrate dehydrate ($Na_3C_6O_7 \cdot 2H_2O$) as a reducing agent was from JT Baker, and Protein A was obtained from Sigma-Aldrich. This was stored in phosphate buffer saline (PBS) buffer pH 7.4, 0.01 M. Sodium chloride NaCl was purchased from Sigma-Aldrich. Transmission electron microscope was used to analyze the particle size, and UV–Visible spectrophotometer Thermo Scientific Evolution 606 was used to measure the surface plasmon resonance of both single and protein A conjugate–gold nanoparticles.

Preparation of gold nanoparticles

Citrate-reduction method to produce gold nanoparticles was used according to Hermanson (2008). It is based on the reduction of tetrachloroauric acid ($HAuCl_4$) by sodium citrate in presence of heat. The gold nanoparticles are synthesized because the citrate ions act as both a reducing agent, and a capping agent. This method involved the preparation of 1 ml of $HAuCl_4$ at 4 % in deionized water, then 0.5 ml was added to this solution to 200 ml of deionized water and brought to boiling, the solution was kept under constant stirring. Once the sample reaches the range of temperature between 97 and 100 °C, 3 ml of 1 % sodium citrate were added. As sodium citrate was added, the solution began to darken and turn bluish-gray or purple. After 30 min, the reaction was completed and the final color of solution was a deep wine red indicating that the colloidal solution of gold nanoparticles was obtained. Four colloidal solutions were prepared at several temperatures to obtain gold nanoparticles with different size. After the solutions were cooled, the gold nanoparticles were centrifuged at 3,500 rpm for 40 min, the supernatant was removed and the nanoparticles were re-suspended to 4 ml with deionized water. The obtained suspension was stored in refrigeration at 4 °C until needed.

Titration of gold nanoparticles with protein A

The titration procedure of gold nanoparticles with protein A was carried out following the protocol of Slot and Geuze (1984), to obtain a correct approximation of the amount of protein A which must be added to maintain the stability of the gold nanoparticles. This protocol was to perform various protein A solutions at different concentrations ranging from 0.3 to 9 μg/ml. Was added one volume of 3–90 μl of protein A (100 μg/ml) in 1 ml of gold nanoparticles to

reach these concentrations. After 5 min, were added 1 ml of sodium chloride at 10 % with the aim of increasing the rate of precipitation of the nanoparticles. The minimum amount of protein A required to stabilize gold nanoparticles was determined using the ratio of UV–Visible intensities of aggregate/single particles as a function of the concentration of protein A. The stabilization of the nanoparticles in the presence of NaCl was also monitored by a color change of the colloids from red to dark blue. When the concentration of protein A added is sufficient to stabilize the gold nanoparticles, the colloidal solution do not suffers color changes and the intensity of the absorbance is maintained, although a displacement of about 4 nm can be observed.

Results and discussion

Size and concentration of gold nanoparticles

Gold nanoparticles were synthesized according to the method mentioned in this study, thus several colloidal solutions were obtained by repetition. Figure 1 shows the UV–Visible spectra of the colloidal solutions analyzed in this study. All of them were prepared by following the same procedure varying lightly the temperature of the synthesis from 97 to 100 °C. UV–Visible spectra of these solutions allow us to observe differences in intensity between them which are produced by changes on particle size and concentration. The typical appearance of the nanoparticles is observed in the TEM image shown inset of the Fig. 1, showing the spherical nature and an average size of 15.4 nm determined statistically.

We have used a simple and fast method to determine the size and concentration of gold nanoparticles, proposed by Haiss et al. (2007). This model is based on multipole scattering theory with correction of the complex refraction index of the gold nanoparticles. This correction was realized because for small particles the mean free path is reduced due to collisions of electrons with the particle surface, compared with the mean free path of the conduction electrons in the bulk (~42 nm for gold). This correction affects the dielectric constant of the particles, and affords a better quantitative description of their UV–Visible absorbance and also to the determination of particle size.

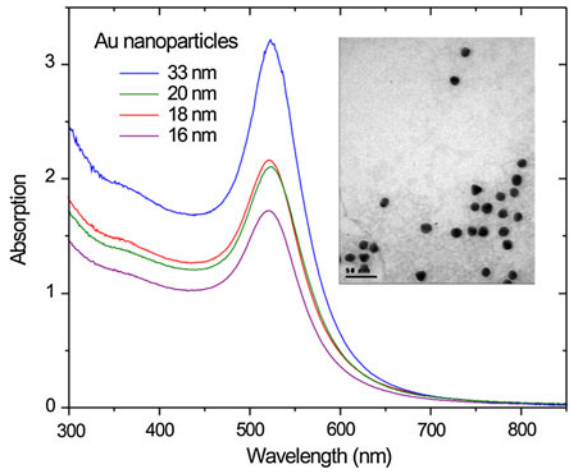

Fig. 1 UV–Visible spectra of the group of colloidal solutions with different concentrations and particle size used in this study. *Inset* TEM image of gold nanoparticles analyzed in this study. For this case $d = 15.4$ (TEM) and $d = 16$ nm (theoretical model)

This method uses the information of the UV–Visible spectra obtained experimentally.

The particle size was calculated by:

$$d = \exp\left(B_1 \frac{A_{spr}}{A_{450}} - B_2\right) \quad (1)$$

where (A_{spr}) is the absorbance of the surface plasma resonance, (A_{450}) is the absorbance at 450 nm. B_1 is the inverse of the slope of the linear fit between (A_{spr}/A_{450}) and ln(d), whereas B_2 is the interception. Using the experimental values of the parameters B_1 and B_2 reported in Haiss et al. (2007), $B_1 = 3.0$ and $B_2 = 2.2$. The calculations of particle diameters using Eq. (1) allow to determine the size of gold nanoparticles. These calculations are in good agreement with the experimental measurements of several authors (Alekseeva et al. 2006; Slouf et al. 2006; Njoki et al. 2007), and with the theoretical simulations reported by Khlebtsov (2008), Kimling et al. (2006) and by Haiss et al. (2007). Estimations of size made in this study using Eq. (1) and spectral UV–vis parameters are valid from 5 to 80 nm (Haiss et al. 2007), however they also show good agreement with other theoretical models too (Khlebtsov 2008; Kimling et al. 2006).

Also, concentration of the nanoparticles can be estimated using the expression:

$$N = \frac{A_{450} \times 10^{14}}{d^2\left(-0.295 - 1.36\exp\left(-\left(\frac{d-96.8}{78.2}\right)^2\right)\right)} \quad (2)$$

Using both equations and the information from the UV–Visible spectra, we have calculated size and concentration of the gold nanoparticles analyzed in this study. The results are summarized in Table 1.

The relation between concentration and particle size can be observed in Fig. 2. Experimental data were obtained from Eqs. (1) and (2), and the line is a fit of these data. This relation arises from the dependence of N and d on the quotient (A_{spr}/A_{450}) of the UV–Visible spectra. The use of the ratio of the absorbance at the surface plasma resonance peak (A_{spr}) to the absorbance at 450 nm (A_{450}), which is near to the local minima has shown be in good agreement with experimental determination of particle size from other authors (Khlebtsov 2008; Haiss et al. 2007).

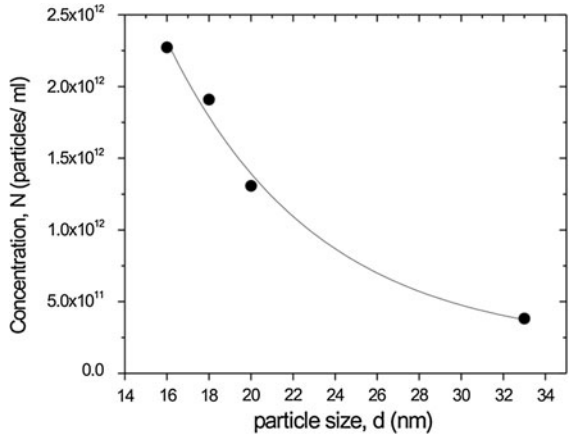

Fig. 2 Relation between concentration and particle size for the colloidal solutions analyzed in this study

Titration of gold nanoparticles with protein A, for size and particle concentration constants

Colloidal solutions were conjugated with different concentrations of protein A, from 0.3 to 9 μg/ml. After 10 min of incubation, the conjugated solutions were mixed with NaCl (10 %) to promote aggregation of the gold nanoparticles in case of the surface is not fully covered with protein A. The UV–Visible spectra of this reaction can be observed in Fig. 3 for one of the colloidal solutions analyzed in this study, which has a particle size of 18 nm, calculated using Eq. (1).

For low concentrations of protein A we can observe two absorption bands, one of these near 525 nm and the other near 686 nm. The first band is associated to the gold nanoparticles absorption, whereas the second band is associated to the absorption of the aggregates, which is typical when gold nanoparticles are immersed in NaCl solution. Protein A has an absorption band at 276 nm that fortunately is situated far from the surface plasmon resonance (near 520 nm). As the protein A concentration increases, the wavelength of the absorption band of the aggregates diminishes toward the absorption band of the gold nanoparticles. When an optimum value of protein A has been reached, the spectrum of the conjugated gold nanoparticle–protein A is very similar to the gold nanoparticle spectrum, except by a shift of about 4 nm, in good agreement with the reported by other authors (Losin et al. 2009). In this case, the titration procedure for one of the colloidal solutions (d and N constants) is shown in Fig. 3, where the concentration of protein A was varied. The typical dependence of the absorption profile with protein A concentration during the titration process with NaCl is shown in Fig. 4. This graph suggests a concentration of 6 μg/ml (protein A) as an adequate value to avoid nanoparticles aggregation.

Titration of gold nanoparticles with protein A, with variation of size and particle concentration

The same procedure as in the previous section was applied for all the colloidal solutions studied in this study (Table 1). According to the Fig. 5, three concentration values of protein A were selected (0.5, 3, and 6 μg/ml) corresponding to the initial, exponential, and

Table 1 Size and concentration of gold nanoparticles obtained using the Eqs. (1) and (2) and also from the UV–Visible spectra of the colloidal solutions

No. Exp.	λ_{spr} (nm)	A_{spr}	A_{450}	d (nm)	N (part/ml)
7	523	3.21	1.69	33	3.82E11
9	521	2.16	1.27	18	1.90E12
6	522	2.10	1.21	20	1.30E12
8	520	1.71	1.03	16	2.27E12

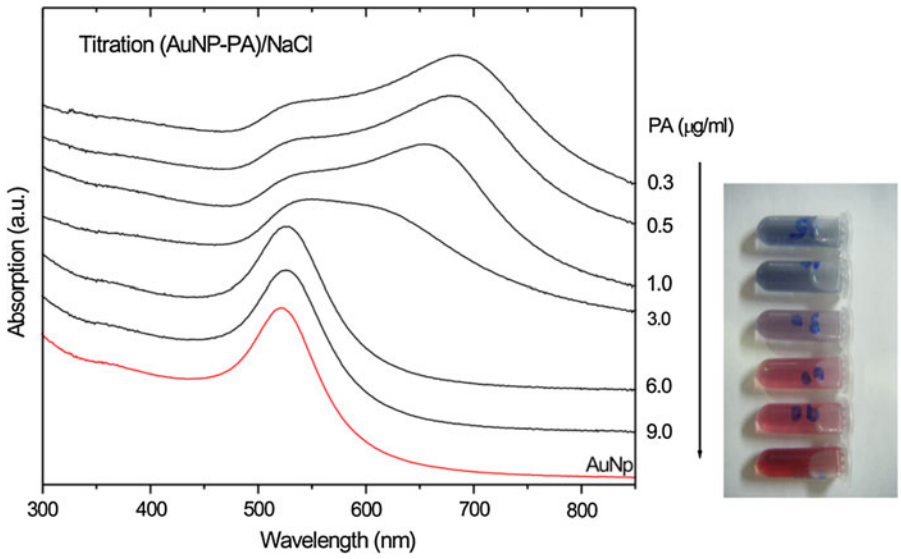

Fig. 3 UV–Visible spectra of gold nanoparticles solutions during the titration process with NaCl for several protein A concentrations. In this case the particle size is 18 nm and particle concentration is 1.9E12 part/ml (using Eqs. 1, 2)

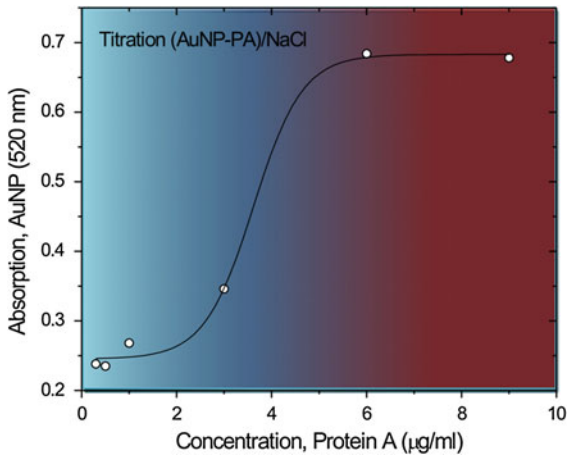

Fig. 4 Typical absorption profile of gold nanoparticles as a function of concentration of protein A, in the presence of NaCl. In this case the particle size is 18 nm and particle concentration is 1.9E12 part/ml (using Eqs. 1, 2)

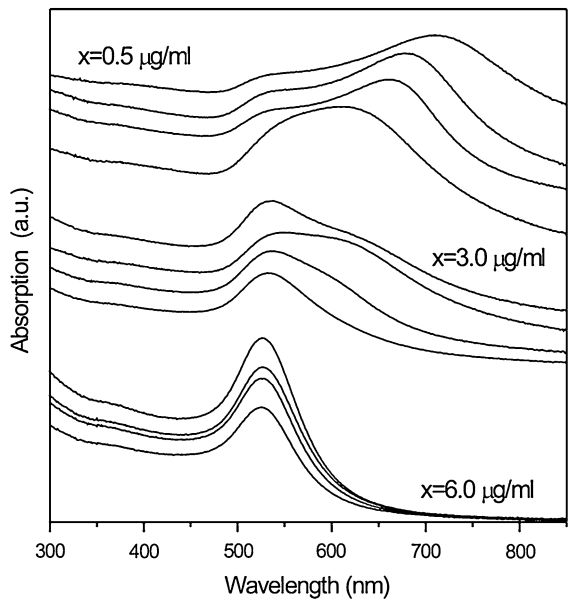

Fig. 5 UV–Visible spectra of the gold nanoparticles conjugated with protein A (0.5, 3.0, and 6.0 μg/ml) for four colloidal solutions (with 16, 18, 20, and 33 nm of particle size) in the presence of NaCl

stable phase of the gold nanoparticles absorption band, respectively. The last was done to observe the line shape variations of the UV–Visible absorption for all the particle concentrations, being the concentration of protein A constant.

In the first case, for low protein A concentration (0.5 μg/ml) we observe both absorption bands (single and aggregate gold), Fig. 5. A very intense absorption of the aggregates suffers a shift to low wavelengths, whereas the absorption of gold remains unaltered. There is a systematic relation between the shift to low wavelengths of the absorption of aggregates with particle size and also with particle concentration (20, 18, and 16 nm), except for the colloidal solution with 33 nm, being not clear the reason of the last. However, in most of cases a common behavior indicates that bigger particles react more quickly with NaCl to form aggregates than short particles.

In the second case (3 μg/ml), Fig. 5, the intensity of the absorption of aggregates starts to diminish with respect to the previous case (0.5 μg/ml), because now more protein A covers the surface of gold nanoparticles, keeping their intensities very similar. As in the previous case, there is a systematic relation between the shift to low wavelengths of the absorption of aggregates with particle size and particle concentration (20, 18, and 16 nm). Colloidal solution with 33 nm which has the lowest particle concentration presents mostly the absorption of the gold nanoparticles and a very low intensity associated to aggregates.

In the last case (6 μg/ml) we have no absorption bands associated to aggregation, only bands from gold nanoparticles are present, Fig. 5. This fact indicates that the particles are covered by protein A and aggregation is not present using NaCl, having a stable condition. Finally we have compared the relative intensities between the absorption of the aggregate particles (I_2) and the absorption of the individual gold nanoparticles (I_1), to obtain the dependence of the intensity ratio I_2/I_1 on the concentration of protein A, Fig. 6. This method allows to obtain a critical concentration value of protein A from which gold nanoparticles conjugated with this protein can stabilize the system, which is independent from size–concentration of the colloidal solutions. This is because the particle size and the particle concentration are not independent, but they are related by the Eqs. (1) and (2) and also by the continuous line shown in Fig. 2.

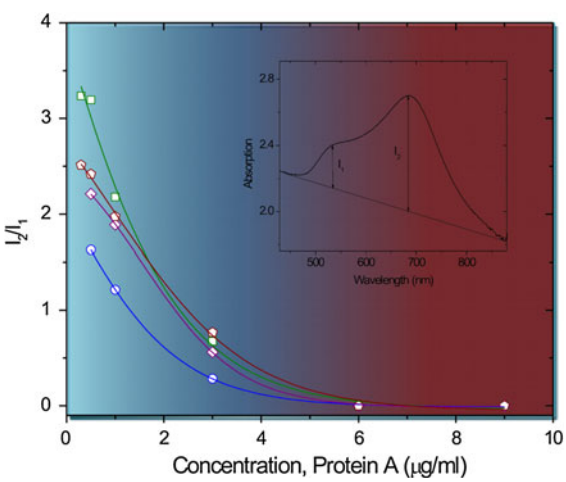

Fig. 6 Dependence of the ratio I_2/I_1 on the concentration of protein A. The particle sizes of the colloidal solutions used were 16, 18, 20, and 33 nm, according to the order cited in Fig. 1

Conclusions

An easy way to estimate the concentration value of protein A that stabilize gold nanoparticles synthesized by citrate reduction at neutral pH was obtained. Such method involves the performance of the titration procedure of gold nanoparticles conjugated with protein A in the presence of NaCl, for several colloidal solutions having different particle size and particle concentration. In a first stage the size and concentration are constant, varying only the protein concentration, whereas in a second stage, several values of protein A are kept constant and the variation of particle size and particle concentration were analyzed. From these results we analyzed the ratio between the intensities of the aggregates and individual gold nanoparticles bands as a function of the concentration of protein A, to obtain 6 μg/ml as the sufficient amount of protein A to stabilize the gold nanoparticle–protein A system, which is independent of the particle size and particle concentration of the colloidal solutions prepared by citrate reduction.

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