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Instability of Cationic Gold Nanoparticle Bioconjugates: The Role of Citrate Ions

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Abstract: Gold nanoparticles of 6, 8, and 16 nm, synthesized with $HAuCl_4$ and sodium citrate, were derived with biomolecules based on the peptide CIPGNVG and possessing different terminal charges. We have studied the stability of these conjugates as a function of ionic strength, pH, and the presence of other species in solution. It was observed that multiple electrostatic interactions between the conjugates mediated by cross-linking species led to an effective strong bond and consequently to irreversible aggregation and precipitation. In the presence of citrate or diamine ions, nanoparticles precipitated when two-headed ions had charges opposite (and therefore attractive) to the conjugate, thus acting as bridging molecules. This effect depends on the pH, the concentration of particles, and their size, and it is relevant to designing bioconjugates for biomedical applications.

Introduction

The ability to synthesize aqueous-stabilized nanoparticles of controlled size and shape that can easily be functionalized with biomolecules (i.e., peptides, enzymes, antibodies, DNA) is highly desirable.[1,2] In this context, citrate-synthesized gold nanoparticles (AuNPs) derived with peptides have promising biomedical applications for diagnosis and therapy, such as for detection of homocysteine in blood,[3] as an indicator of cardiovascular disease, or for the local and remote manipulation of β-amyloid aggregation,[4] related to Alzheimer's disease, among many others. However, to perform these kinds of sophisticated experiments to which nanoparticle conjugates are specially suited, high stability in electrolytic environments at concentrations as high as possible is required, which is not always easily achievable. Conjugate instability has been observed even when conjugating soluble NPs with soluble molecules,[5,6] since colloids are out-of-equilibrium systems despite their apparent stability. According to this, solid particles suspended in a liquid medium in a non-interacting regime (at concentrations below 10^{10} NPs/mL for 10 nm AuNPs), whether conjugated or not, sediment following Stokes's law.[7,8] At such concentrations, as long as the AuNPs do not experience spontaneous aggregation, a 10 nm AuNP will take about 3 years to sediment from a solvent height of 1 cm.[9] Unfortunately, in biomedical applications, concentrations of 10^{13} NPs/mL are desirable for diagnosis and therapy.[10]

The general mechanism for stabilization of colloidal materials in water has been described in the Derjaguin−Landau−Verwey−Overbeek (DLVO) theory, which combines the effects of van der Waals attraction and electrostatic repulsion due to the so-called double layer of counterions.[11,12] This theory proposes that the NPs are in an interactive regime where strong attractive forces between NPs can be prevented by an energy barrier resulting from electrostatic and/or steric repulsive forces. The generation of electrostatic repulsion between charged conjugates is the most common strategy to keep NPs separated in aqueous medium and, subsequently, stable under physiological conditions.[13] The theoretical implications of the DLVO theory in the case of gold conjugates have been extensively studied by Whitesides et al.,[14] who measured the stability of colloidal dispersions at different pH values and ionic strengths in the presence of a series of alkanethiols differing in the terminal

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(1) Alivisatos, A. P. *Sci. Am.* **2001**, *285*, 66–73.
(2) Niemeyer, C. M. *Angew. Chem., Int. Ed.* **2001**, *40*, 4128–4158.
(3) Gates, A. T.; Fakayode, S. O.; Lowry, M.; Ganea, G. M.; Murugeshu, A.; Robinson, J. W.; Strongin, R. M.; Warner, I. M. *Langmuir* **2008**, *24*, 4107–4113.
(4) Kogan, M. J.; Bastus, N. G.; Amigo, R.; Grillo-Bosch, D.; Araya, E.; Turiel, A.; Labarta, A.; Giralt, E.; Puntes, V. F. *Nano Lett.* **2006**, *6*, 110–115.
(5) Doty, R. C.; Tshikhudo, T. R.; Brust, M.; Fernig, D. G. *Chem. Mater.* **2005**, *17*, 4630–4635.
(6) Pengo, P.; Broxterman, Q. B.; Kaptein, B.; Pasquato, L.; Scrimin, P. *Langmuir* **2003**, *19*, 2521–2524.
(7) Pusey, P. N.; van Megen, W.; Bartlett, P.; Ackerson, B. J.; Rarity, J. G.; Underwood, S. M. *Phys. Rev. Lett.* **1989**, *63*, 2753–2756.
(8) van Blaaderen, A.; Wiltzius, P. *Science* **1995**, *270*, 1177–1179.
(9) Calculated using the Stokes' law: $v = a^2(\rho_s - \rho_l)g/18\eta$, where v is the particle settling velocity, g is the gravitational acceleration, ρ_s is the mass density of the particles, ρ_l is the mass density of the fluid, η is the dynamic viscosity of the fluid, and a is the diameter of the NPs.
(10) Batarseh, K. I. *Leukemia* **2007**, *22*, 448–450.
(11) Derjaguin, B.; Landau, L. *Acta Phys. Chem. URSS* **1941**, *14*, 633–662.
(12) Verwey, E. J. W.; Overbeek, J. T. G. *Theory of the stability of lyophobic colloids;* Elsevier: Amsterdam, 1948.
(13) Bastús, N. G.; Casals, E.; Vázquez-Campos, S.; Puntes, V. *Nanotoxicology* **2008**, *2*, 99–112.
(14) Weisbecker, C. S.; Merritt, M. V.; Whitesides, G. M. *Langmuir* **1996**, *12*, 3763–3772.

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functionality and showed an enhanced stability for the ionizable surfaces. In principle, by ensuring that the particles have a positive or negative net charge in aqueous media, one may recall overall repulsive interactions. However, a detailed literature search for thiol-mediated conjugation of charged species to citrate-prepared AuNPs reveals that, overall, negatively capped AuNPs possess an enhanced stability, such as in the case of mercaptopropanesulfonate,[15] amino acids,[16−18] and peptidic sequences.[19,20] This significantly contrasts with the highly aggregating character observed in similar situations regarding mercaptoethylamine[15] or positively charged peptides.[19] Of significant importance is the case of the conjugation of AgNPs to differently charged biomolecules.[5] While the conjugation of the negatively charged biomolecule could successfully be performed with both citrate-stabilized AgNPs and AgNPs prepared by two-phase organic synthesis, conjugation of the positively charged biomolecule was only reported for the second case where the citrate is absent.

It would therefore be useful to elucidate the factors responsible for AuNP conjugate stability in order to predict the behavior of these systems, where nanoparticles, conjugated species, additives, residues from the AuNP synthesis, and the nature of the solvent may play important roles in the interaction between NPs. In order to perform these studies, biomolecules based on a peptide sequence (CIPGNVG) conjugated to AuNPs have been chosen as a case model. Monitoring of the aggregation and/or precipitation of these species has been carried out by UV−vis spectroscopy, which has been considered to be the most appropriate technique due to the surface plasmon resonance (SPR) of both single AuNPs (520 nm) and their aggregates (broad band above 600 nm), and which has successfully been employed to evaluate stability in similar studies.[17,19,21] In this paper, we observe that the presence of charged molecules in solution may induce NP aggregation by bridging particles together.

Experimental Section

Chemicals. The peptides **1**−**4** were synthesized following a Fmoc strategy and solid-phase synthesis. A 10 mM stock solution of peptide was prepared by dissolving it in phosphate buffer (160 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1 mM KH_2PO_4). The pH was adjusted to 7 employing 0.1 M NaOH, and the resulting solution was filtered using 0.2 μm filters and kept in a freezer (at −20 °C). Sodium citrate, ethylenediamine, and ethanolamine were purchased from Sigma-Aldrich.

Instrumentation and Measurements. UV−vis absorption spectra were recorded with a Shimadzu UV-2401PC spectrophotometer at room temperature. The samples were allowed to settle for 30 min before measurements, unless otherwise stated. AuNPs were visualized using transmission electron microscopy (TEM; JEOL 1010, Japan) at an accelerating voltage of 80 kV. The sample (10 μL) was drop-cast onto ultrathin Formvar-coated 200-mesh copper grids (MonoComp) and left to dry in air. For each sample, the size of 200 particles was measured to obtain the average and the size distribution. Zeta-potential and dynamic light scattering (DLS) measurements were carried out on a Zetasizer Nano ZS (Malvern Instruments). Standard purification by dialysis, unless otherwise stated, was performed with samples of 2 mL using 16 mm membranes (MWCO = 8 kDa) against 5000 mL aqueous solution of sodium citrate, 2.3 mM. Characterization by 500 MHz ^{1}H NMR was carried out on a Bruker DMX-500 spectrometer.

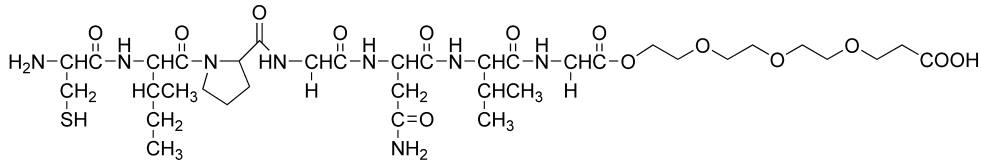
Synthesis of AuNPs. Citrate-stabilized monodispersed AuNPs (10^{12}−10^{13} NPs/mL) were prepared on the basis of the standard sodium citrate reducing methodology described in the literature for obtaining 6, 8, and 16 nm AuNPs.[22,23] In brief, sodium citrate (2.2 mM) was dissolved in H_2O (150 mL) in a three-neck round-bottom flask and heated to 100 °C. A solution of $HAuCl_4 \cdot 3H_2O$ (1 mL, 25 mM) in H_2O was injected, and the reaction mixture was maintained at the boiling temperature for a further 3.5 min before being allowed to cool to room temperature. The resulting AuNPs were characterized by UV−vis, TEM, DLS, and zeta-potential measurements (Supporting Information).

Conjugation of Peptidic Biomolecules. In order to ensure complete encapsulation of the AuNPs, conjugation was done in the presence of a 10-fold excess of biomolecule (calculated by the number of Au atoms at the surface of the NP and assuming that each thiolated molecule occupies 2.14 Å^2 of the AuNP surface).[24,25] Samples were prepared mixing 100 μL of a 10 mM stock solution of biomolecule **1**, **2**, **3**, or **4** with 5 mL of the appropriate AuNP solution at room temperature under stirring for 1 h. The rapidness of these conjugations was measured by challenging the stability of these systems (Supporting Information). The resulting conjugates were exhaustively purified by dialysis during 3 days, and the solution was changed 3 times in order to eliminate the excess of peptidic biomolecules.

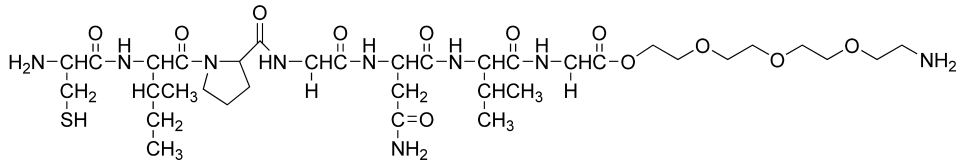
Results and Discussion

Peptidic Biomolecule Design. The peptidic chains selected to carry out these studies were based on the heptapeptide sequence CIPGNVG, where the SH group of the cysteine in the *N*-terminus provides a strong affinity for gold. The nonpolar side chains of the amino acid sequence give to the structure the ability to self-assemble into a dense layer that excludes water due to the hydrophobic core. These hydrophobic interactions, together with the hydrogen bonding between the backbone amides, result in a very efficient packing, optimal for high conjugate molecule density, which decreases molecule mobility, avoiding an effective steric repulsion between AuNPs and at the same time yielding large surface charge densities.[26] Due to the poor solubility of some of the peptides themselves, pegylation (poly(ethylene glycol), PEG, MW = 220) was carried out at the *C*-terminus with the aim to provide the peptidic biomolecules with both a hydrophilic terminus and an enhanced stability in water.[5,27] Since the main aim of these studies was the effect of the terminal charge in the final nanoparticle−biomolecule conjugate, two different PEG termini were designed, ending in either acidic (−COOH) or amino ($-NH_2$) functionalities, which depending upon the pH would result in

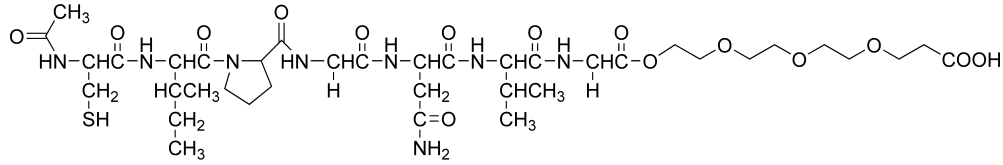
(15) Bellino, M. G.; Calvo, E. J.; Gordillo, G. *Phys. Chem. Chem. Phys.* **2004**, *6*, 424–428.
(16) Aryal, S.; K.C, R. B.; Bhattarai, N.; Kim, C. K.; Kim, H. Y. *J. Colloid Interface Sci.* **2006**, *299*, 191–197.
(17) Lim, I. I. S.; Ip, W.; Crew, E.; Njoki, P. N.; Mott, D.; Zhong, C. J.; Pan, Y.; Zhou, S. *Langmuir* **2007**, *23*, 826–833.
(18) Zhang, F. X.; Han, L.; Israel, L. B.; Daras, J. G.; Maye, M. M.; Ly, N. K.; Zhong, C.-J. *The Analyst* **2002**, *127*, 462–465.
(19) Levy, R.; Thanh, N. T. K.; Doty, R. C.; Hussain, I.; Nichols, R. J.; Schiffrin, D. J.; Brust, M.; Fernig, D. G. *J. Am. Chem. Soc.* **2004**, *126*, 10076–10084.
(20) Xiao, M.; Yoojin, A.; Chang-Soo, Y.; Seungju, M. Y. *Angew. Chem., Int. Ed.* **2006**, *118*, 2325–2328.
(21) Wang, G.; Sun, W. *J. Phys. Chem. B* **2006**, *110*, 20901–20905.
(22) Frens, G. *Nature−Phys. Sci.* **1973**, *241*, 20–22.
(23) Turkevich, J.; Stevenson, P. C.; Hillier, J. *J. Phys. Chem. B* **1953**, *57*, 670–673.
(24) Bishop, K. J. M.; Grzybowski, B. A. *Chem. Phys. Chem* **2007**, *8*, 2171–2176.
(25) Sellers, H.; Ulman, A.; Shnidman, Y.; Eilers, J. E. *J. Am. Chem. Soc.* **1993**, *115*, 9389–9401.
(26) Pale-Grosdemange, C.; Simon, E. S.; Prime, K. L.; Whitesides, G. M. *J. Am. Chem. Soc.* **1991**, *113*, 12–20.
(27) Kanaras, A. G.; Kamounah, F. S.; Schaumburg, K.; Kiely, C. J.; Brust, M. *Chem. Commun.* **2002**, *20*, 2294–2295.



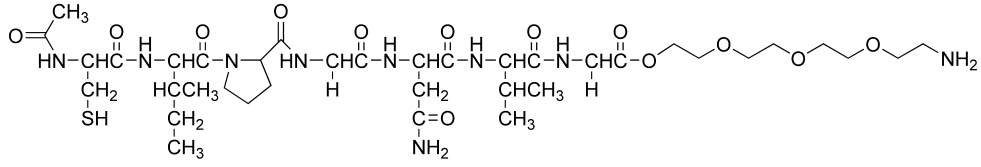
CIPGNVG-PEG$_a$ (1)



CIPGNVG-PEG$_b$ (2)



Ac-CIPGNVG-PEG$_a$ (3)



Ac-CIPGNVG-PEG$_b$ (4)

Figure 1. Structures of peptidic biomolecules **1**−**4**.

final negative or positive net charges, respectively. In addition, as stated in the literature, amino groups also have a strong interaction with gold surfaces, which may exert a complementary effect with the thiol that strongly chemisorbs onto the negatively charged nanoparticle surface.[5,15,19] Therefore, not only the NH_2-terminal biomolecules **1** and **2** but also their *N*-acetylated analogues **3** and **4**, possessing a bulkier terminal cysteine, were designed to perform these studies with the main purpose of changing the surface density of the conjugates (Figure 1).

Conjugation of Peptidic Biomolecules 1 and 2. Unexpectedly, the conjugation of peptidic biomolecules CIPGNVG-PEG$_a$ (**1**) and CIPGNVG-PEG$_b$ (**2**) with 8 nm AuNPs gave very disparate results. In the case of the COOH-terminated sequence **1**, the addition to a solution of AuNPs of 8 nm showed a red-shift in the SPR band from 518.5 to 523.5 nm (Figure 2A), together with an increase of the hydrodynamic diameter from 15.4 to 24.5 nm and a drop in the negative surface charge from −43.0 to −54.4 mV (Supporting Information). These results were indicative of a dense coverage of the biomolecule onto the surface of the AuNP. By contrast, the addition of the NH_2-terminated peptide **2** induced a decrease of the absorbance at 518.5 nm while a broad wavelength band above 600 nm evolved, which was accompanied by a color change of the solution from red to purple. The appearance of a new absorption band at longer wavelengths could be explained due to the dipole coupling between the plasmons of neighboring particles forming the aggregates.[28] Biomolecule conjugation was confirmed by the presence of a positive charge of 11.8 mV in the zeta-potential measurements, and the aggregation was confirmed by the increase of the hydrodynamic effective diameter up to 110.9 nm (Supporting Information). Examples of aggregation of NH_2-terminated, peptide-capped AuNPs have been previously reported,[15,19,29] but it was not clear why aggregation occurred. A possible explanation could rely on the electrostatic interactions between the citrate ions and the conjugates. The AuNPs are synthesized in the presence of an excess of citrate, which remains in the solution fully deprotonated at pH 7 and could interact with the cationic species. Given neutral conditions of pH, the negatively charged carboxyl group ($pK_a \approx 5$) of the CIPGNVG-PEG$_a$-AuNP conjugates would experience electrostatic repulsion toward the also negative carboxylic acids of the citrate molecules (Figure 2B). However, for the CIPGNVG-PEG$_b$-AuNP system, the terminal amino groups of the peptidic biomolecule **2** ($pK_{aH} \approx 9$) would be positively charged at the given pH value, and they would therefore interact electrostatically with the negative charges of the citrate molecules ($pK_a =$ 3.2, 4.8, and 6.4), thus forcing the aggregation of the conjugated AuNPs. Since aggregates are complex systems that are difficult to study due to their inherent nature, we decided to explore the instability of CIPGNVG-PEG$_b$-AuNP conjugates by employing

(28) Storhoff, J. J.; Lazarides, A. A.; Mucic, R. C.; Mirkin, C. A.; Letsinger, R. L.; Schatz, G. C. *J. Am. Chem. Soc.* **2000**, *122*, 4640–4650.
(29) Slocik, J. M.; Stone, M. O.; Naik, R. R. *Small* **2005**, *1*, 1048–1052.

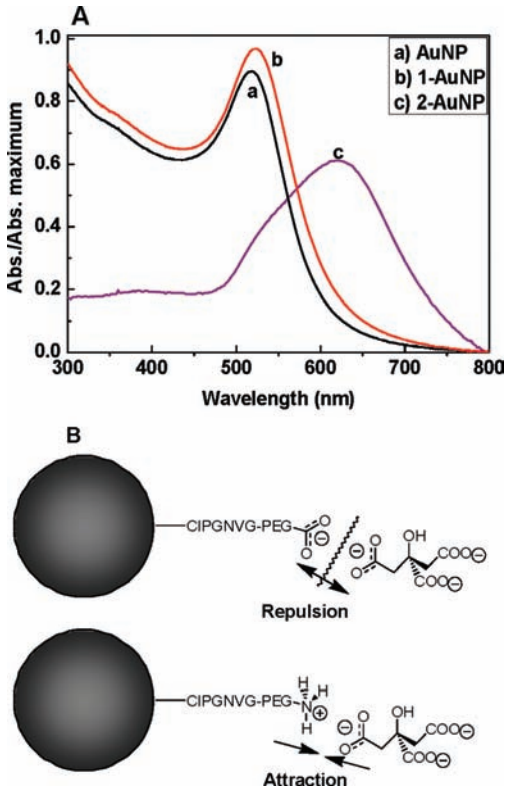


Figure 2. (A) UV−vis absorption studies of the conjugation of peptidic biomolecules **1** and **2** to AuNPs of 8 nm. (B) Representative model for electrostatic repulsion vs attraction between peptidic biomolecule-AuNP conjugates and citrate ions.

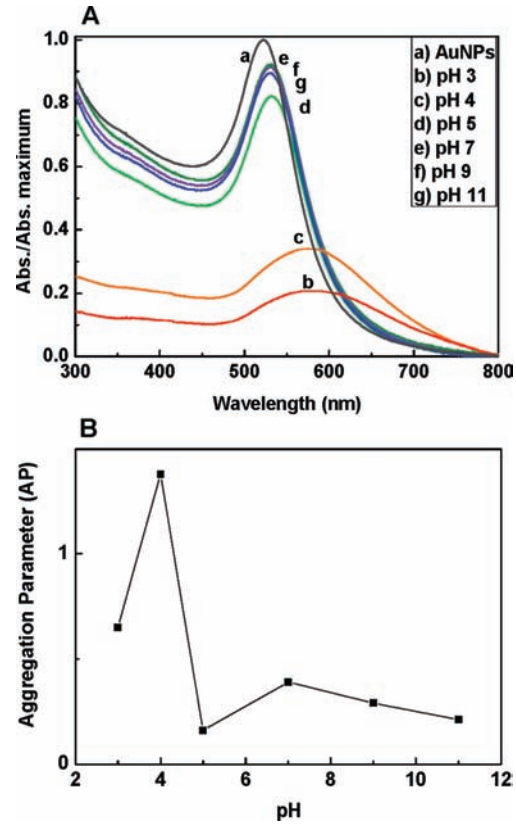


Figure 3. (A) UV−vis absorption spectra of CIPGNVG-PEG$_a$-AuNPs at different pH values. (B) Aggregation parameters as a function of pH.

their carboxylic acid analogues, CIPGNVG-PEG$_a$-AuNP, and destabilizing them as a function of pH, ionic strength, and other cross-linking species present in solution.

Stability of CIPGNVG-PEG$_a$-AuNPs as a Function of pH. Assuming the key importance of the charge of the biomolecule, we proceeded to study the behavior of the conjugated system by modifying the pH. The pH of an aqueous solution of CIPGNVG-PEG$_a$-AuNPs was varied by direct addition of either 1 M HCl or 1 M NaOH (at nonaggregating ionic concentrations, *vide infra*) to the solution and monitored by UV−vis spectroscopy. The biomolecule-capped AuNPs were stable at basic, neutral, and slightly acidic conditions (Figure 3A). However, when the pH of the solution was below the pK_a (∼5) of the terminal carboxylic acid group, AuNP conjugates agglomerated due to neutralization of the negative charge of the acidic groups and the subsequent decrease of the electrostatic repulsion between the particles. These results demonstrate that, despite the PEG moiety, the main mechanism for the stabilization of these biomolecule-AuNP conjugates is electrostatic. In addition, the formation of intermolecular H-bonding between protonated carboxylic acid functionalities of surface-bound peptidic biomolecules between different particles could also contribute to the observed aggregation.[30] The aggregation parameter (AP) was also calculated by measuring the variation of the integrated absorbance between 600 and 700 nm, and it was defined as AP $= (A - A_0)/A_0$, where A is the integrated absorbance of the sample at a given moment and A_0 is the integrated absorbance of the initial solution of AuNPs (Figure 3B).[19]

Stability of CIPGNVG-PEG$_a$-AuNPs as a Function of Ionic Strength. The electrostatic stability mechanism of these systems was further evaluated by studies of aggregation of CIPGNVG-PEG$_a$-AuNP (8 nm) conjugates in aqueous solution over a range of ionic strengths in the absence of free biomolecules. The addition of NaCl at a constant value of pH 7 was performed by adding a solid amount of the salt, which induced aggregation at 0.1 M NaCl (Figure 4). The decrease of the absorbance at 523.5 nm and the shift to longer wavelengths could be explained by aggregate formation due to the screening of the electrostatic repulsion between the negatively charged acidic terminus of the peptide chains.[21] Other peptidic conjugated AuNP solutions with significant steric repulsion withstand much higher concentrations of NaCl without compromising stability.[4,19] Electrostatic repulsion is mediated by the electrical double layer, which is a structure that describes the variation of electric potential near the surface of the NP and is formed by fixed surface charges (nanocrystal + biomolecules) and a layer of counterions (diffuse part). The thickness of the diffuse layer is inversely proportional to the square root of the electrolyte concentration in the solution.[31] This means that the layer is more compressed when there is more electrolyte concentration in the solution, and this would confirm that the stability factor in the case of CIPGNVG-PEG$_a$ is predominantly due to electrostatic instead of steric interactions.

Influence of the Addition of a Diamine to CIPGNVG-PEG$_a$-AuNPs Conjugates. In order to support the cross-linking role of the citrate molecules, further experiments were carried out by mixing the CIPGNVG-PEG$_a$-AuNP (8 nm) conjugate and a

(30) Si, S.; Mandal, T. K. *Langmuir* **2007**, *23*, 190–195.

(31) Bard, A. J.; Faulkner, L. R. *Electrochemical Methods*, 2nd ed.; John Wiley & Sons, Inc.: New York, 2001.

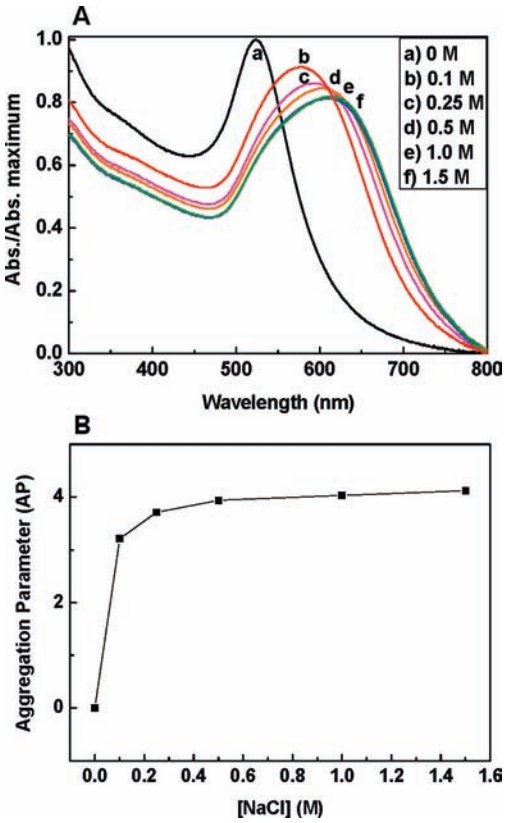


Figure 4. (A) UV−vis absorption spectra of CIPGNVG-PEG$_a$-AuNPs at pH 7 in the presence of an added amount of NaCl. (B) Aggregation parameters as a function of [NaCl]. The quantities refer to concentrations in the final solution.

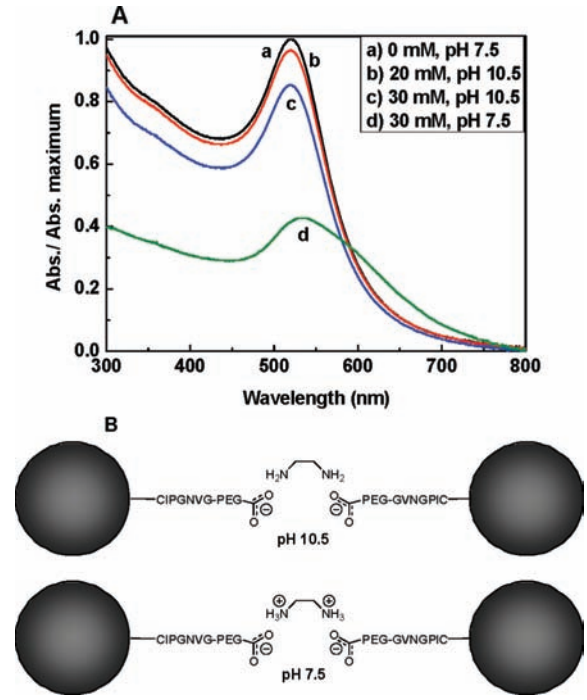


Figure 5. (A) UV−vis absorption spectra of CIPGNVG-PEG$_a$-AuNP after the addition of an amount of ethylenediamine (a−c) and after addition of ethylenediamine followed by adjustment of the pH to 7.5 (d). Quantities refer to concentrations in the final solution. (B) Representative model for the cross-linking effect of diamine molecules.

diamine molecule with the aim to induce electrostatic interactions but with opposite charges than before. Initially, the addition of solid ethylenediamine to obtain concentrations in the conjugate solution of 20 and 30 mM did not cause significant changes in the UV−vis absorption spectrum compared to the initial situation (Figure 5A), which was justified by an increase of the pH value in the final solution toward basic conditions caused by the excess of amine molecules. Under these conditions, the carboxylic acid functionalities of the peptidic chains are negatively charged and the amine groups of the ethylenediamine molecule are in neutral form, thus preventing electrostatic interaction between the diamine molecule and the conjugates (Figure 5B). However, neutralization of the solution to pH 7.5 (with 1 M HCl in order to avoid excessive dilution of samples) caused immediate aggregation of the CIPGNVG-PEG$_a$-AuNPs conjugates as a result of the appearance of a positive charge in the amine moieties of the ethylenediamine molecules, which are able to act as cross-linking elements between the AuNP conjugates (Figure 5).

Similarly, a monoamine molecule, ethanolamine, was used as a control experiment under reaction conditions identical to those used before in order to prove that the aggregation was solely due to the bridging character of the diamine functionality. As expected, addition of ethanolamine did not cause significant changes in the UV−vis absorption spectrum, neither under basic (pH 10.5) nor under neutral conditions (pH 7.5), as long as the carboxylic acid of the conjugate was not protonated at pH below 5 (Supporting Information).

As expected, the electrostatic interaction between the free biomolecules and either the citrate or diamine ions did not cause any observable aggregation after long periods of time at the working concentrations, which was indicative of a complete solubility and stability of the dispersants. Therefore, the aggregation resulting from the biomolecule-AuNP conjugates could be explained by an increase of the local concentration of the peptidic chains. In principle, the energy of a single electrostatic interaction is insignificant compared to the thermal agitation, and this would therefore be reversible at room-temperature conditions. However, the cooperative effect of multiple electrostatic bonds acting simultaneously would lead to high effective affinities (Figure 6B). Electrostatically induced aggregations observed in similar studies of AuNPs conjugated to oligocationic species acting as cross-linkers,[32] and the electrostatic interactions of amine-functionalized AuNPs with negatively charged siRNA[33] are examples that support these hypotheses.

Influence of the Size of the AuNPs. The assumption of cooperativity would suggest that the observed precipitation is strongly dependent on the size of the conjugates, since larger AuNPs would have the possibility to form more bonds. In order to further confirm such a hypothesis, conjugation of the peptidic biomolecule CIPGNVG-PEG$_b$ (**2**) was performed with different sizes (6, 8, and 16 nm) under the same conditions. As can be observed from the images in Figure 6A, whereas no significant aggregation is observed for 6 nm AuNPs, larger diameters kinetically favored aggregation. As shown in the TEM images (Figure 7), samples taken out of the aggregating solution less than 20 s after addition of the biomolecule show extensive clustering features of the 8 and 16 nm AuNPs, which differs

(32) Shipway, A. N.; Lahav, M.; Gabai, R.; Willner, I. *Langmuir* **2000**, *16*, 8789–8795.
(33) Lee, S. H.; Bae, K. H.; Kim, S. H.; Lee, K. R.; Park, T. G. *Int. J. Pharm.* **2008**, *364*, 94–101.

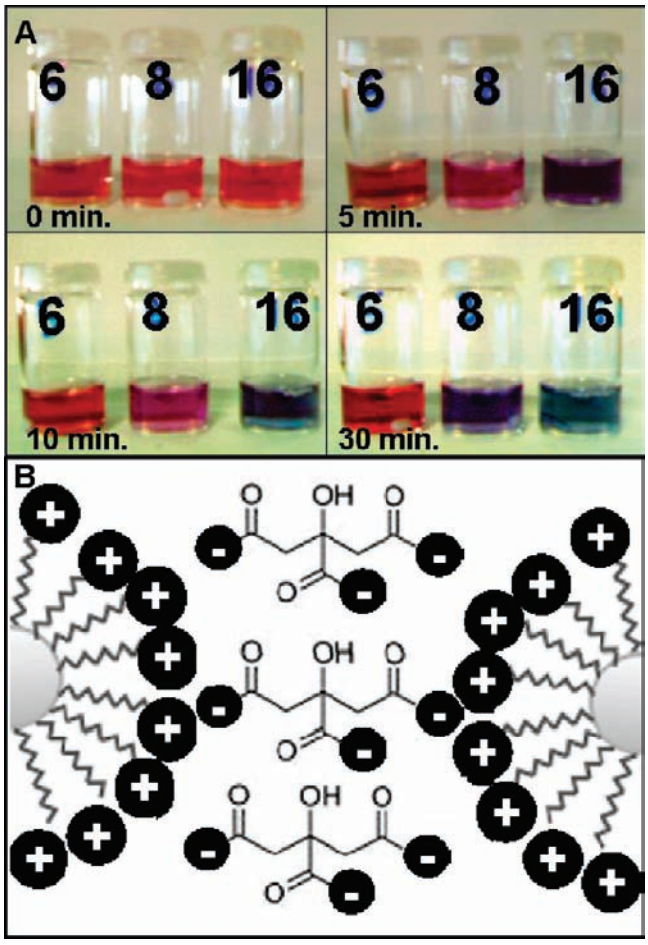


Figure 6. (A) Images corresponding to the conjugation of biomolecule CIPGNVG-PEG$_b$ (**2**) to AuNPs of different sizes at pH 7 for different periods of time. (B) Schematic view of the aggregation mechanism (drawn not to scale).

from the less-extensive clustering observed for 6 nm AuNPs. This is in agreement with what would be predicted from the multivalence effect model (Figure 6B). Therefore, the larger the diameter of the AuNPs, the more surface would be available to accommodate positively charged biomolecules, which leads to an increased number of binding sites that can readily interact with citrate molecules and results in a greater degree of aggregation between the AuNPs. Closer monitoring of the conjugation of the biomolecule CIPGNVG-PEG$_b$ (**2**) upon the addition to AuNPs of different sizes was carried out every 1 min up to 30 min using the UV−vis spectrophotometer (Figure 8). Comparison of the UV−vis spectra further confirmed the

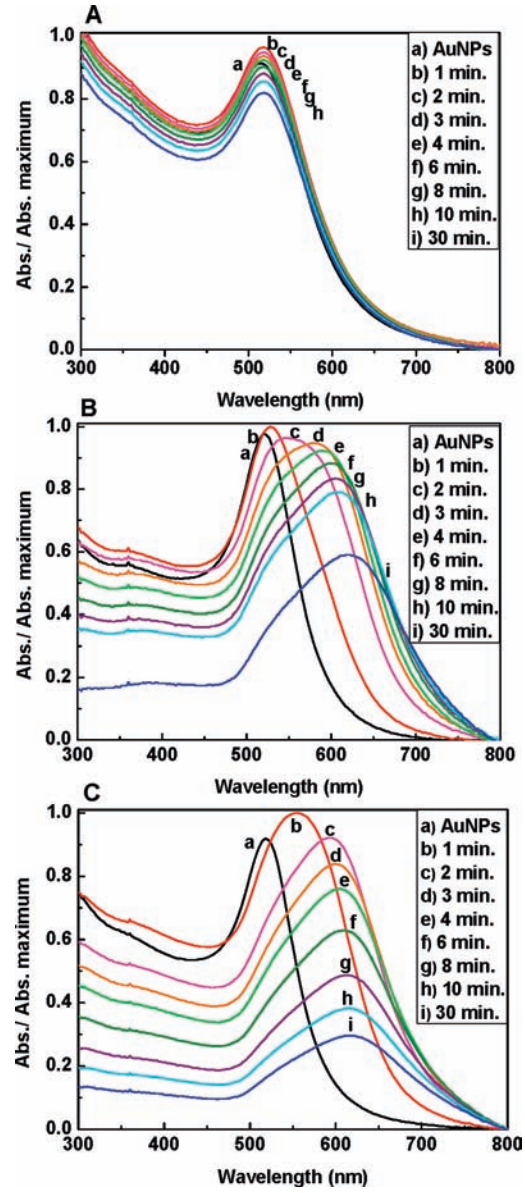


Figure 8. UV−vis absorption spectra of the conjugates resulting after the addition at pH 7 of CIPGNVG-PEG$_b$ to AuNPs of different sizes: (A) 6, (B) 8, and (C) 16 nm.

aggregating tendency as a function of the size of the AuNPs in the order 6, 8, and 16 nm. It must be noted that, for all sizes, the unconjugated AuNPs were stable for long periods of time and that the observed behavior is not due to a similar degree of

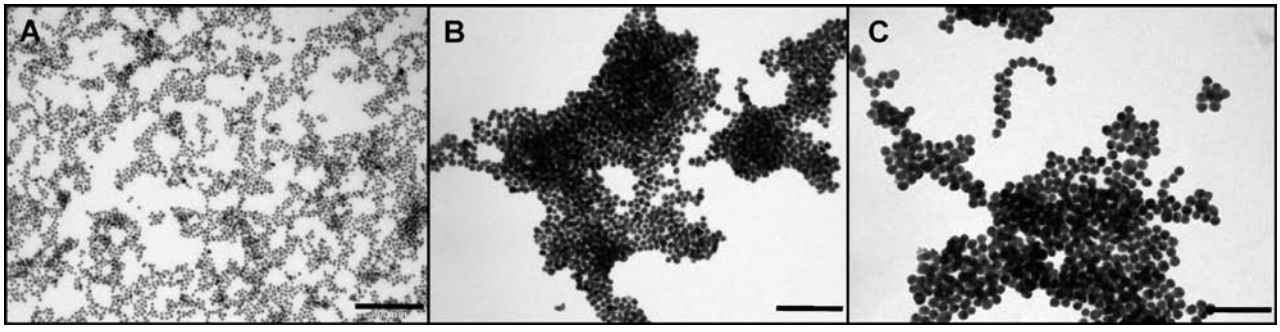


Figure 7. TEM images taken immediately (20 s) after the addition of biomolecule CIPGNVG-PEG$_b$ (**2**) to AuNPs of different sizes: (A) 6, (B) 8, and (C) 16 nm. Scale bars are 100 nm.

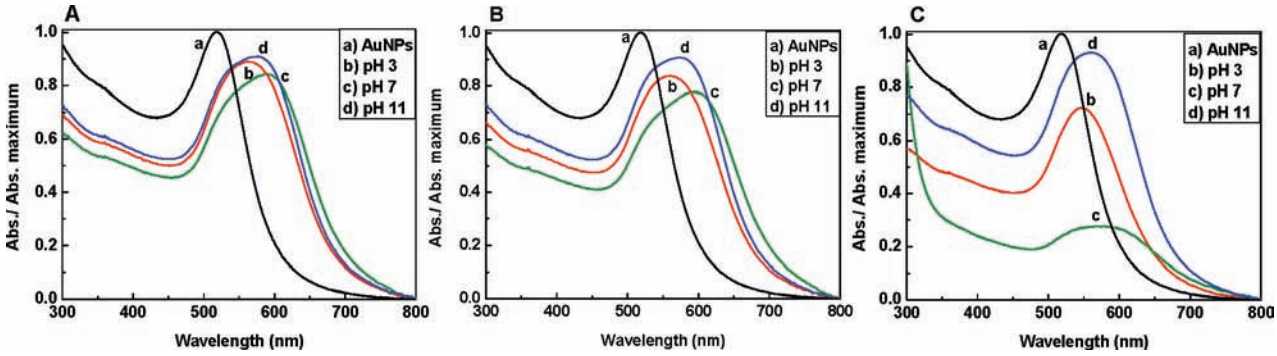


Figure 9. UV−vis absorption spectra of CIPGNVG-PEG$_b$-AuNPs at different pH values, taken after addition of the biomolecule **2** to the AuNPs solution: (A) 10 min, (B) 30 min, and (C) 24 h.

aggregation for the different NPs, but to an increased aggregation potential as the particles are larger.

Conjugation of the *N*-Acetylated Biomolecules 3 and 4. An alternative way of modifying the number of available positively charged biomolecules readily for cross-linking with citrate ions would be to decrease the Au surface coverage of the biomolecules *via* introduction of a bulkier group at the cysteine anchorage. For this reason, *N*-acetylated biomolecules **3** and **4** were conjugated with 8 nm AuNPs under the same conditions and compared to their free amine group analogues **1** and **2**, respectively. While in the case of the biomolecule **3** no significant difference in the UV−vis absorption spectrum could be observed, the conjugation of biomolecule **4** showed a clearly lower aggregating character of the system, thus maintaining an acceptable stability after 2 h of reaction but resulting in aggregates after 24 h (Supporting Information). The slightly improved stability of the *N*-acetylated biomolecule **4** could be a consequence of both the reduced biomolecule loading and the density of hydrophobic packing. This would imply the introduction of molecule mobility and therefore of an enhanced steric repulsion, together with a lower surface density of positive charges to which citrate molecules could attach. Proof of this lower thickness could be obtained from the reduced hydrodynamic effective diameter and the smaller negative surface charge in the Ac-CIPGNVG-PEG$_a$-AuNPs compared to CIPGNVG-PEG$_a$-AuNP conjugates for 8 nm AuNPs (Supporting Information). These results further confirm the cross-linking role of the citrate molecules.

Reversibility of the Aggregation of CIPGNVG-PEG$_b$-AuNPs. The aggregation of CIPGNVG-PEG$_b$-AuNP conjugates could be reversed upon modification of the pH of the solution by direct addition of either 1 M HCl or 1 M NaOH, which determines whether the amino group on the biomolecule and the carboxylic acids on the citrate present in the solution are charged or neutral. Unfortunately, although the induced changes in the pH prevented the immediate aggregation of CIPGNVG-PEG$_b$-AuNPs, it was not possible to fully recover the characteristic absorbance band around 520 nm in the UV−vis spectrum nor the typical reddish color of the gold colloidal suspension once the particles started to aggregate (Figure 9). If the negative charges of the citrate ions were neutralized before addition of the conjugating biomolecules, the absence of charge at the Au surface induced the AuNPs to aggregate. When the pH was decreased immediately after the addition of the conjugating molecule, a certain degree of irreversible aggregation occurred. In addition, when variation of pH was carried out for longer than 30 min, it was not possible to redissolve the aggregates, thus giving an idea of the stability of the forming bonds.

Similarly, if citrate is responsible for the aggregation, removal of citrate ions from solution should result in more stable systems. However, citrate proved to be difficult to completely eliminate from the sample of CIPGNVG-PEG$_b$-AuNP (8 nm) conjugates. In order to prevent immediate aggregation, samples were first acidified with 1 M HCl. Repeated (three times) centrifugation and redissolving in aqueous media resulted in irreversible aggregation of the CIPGNVG-PEG$_b$-AuNPs, as was already shown in similar studies. Exhaustive dialysis against 1 M HCl (10 days, changing the dialysis medium daily) was then carried out with the aim to remove the excess citrate. However, a ^{1}H NMR spectrum of the remaining sample revealed still the presence of citrate molecules, as indicated by the peak appearing at 2.6 ppm (Supporting Information). Further work to obtain stable CIPGNVG-PEG$_b$-AuNP conjugates is underway.

Conclusions

The overwhelming majority of biocompatible AuNPs are made in the presence of citrate. Citrate is a well-known biocompatible reducer, which is in fact involved in the citrate−oxalate reaction used by mitochondria. It also has the ability to bind onto the gold surface strongly enough to prevent aggregation but weakly enough to be exchanged with any other desired surfactant bearing N, P, or S atoms. However, in the case of positively charged molecules conjugated to AuNPs, citrate anion may act as a cross-linker by means of electrostatic interactions between the positively terminated biomolecule-AuNP conjugates and the negatively charged citrate. Although such an interaction is, in principle, reversible and insignificant, the cooperative effect of large amounts of biomolecules attached on the particle surface causes dramatic aggregation due to the citrate present in solution. Such behavior has also been observed in macromolecules and biology, where low-affinity ligands create higher affinity via multivalent binding to cell-surface receptors.[34,35] Unfortunately, it has proved not feasible to remove the citrate prior to the exchange of surfactant; for example, dialysis of citrate-stabilized AuNPs against pure water induces immediate aggregation due to the inherent attraction of NPs by van der Waals forces. Citrate may only be removed once the AuNPs have been stabilized with another type of surfactant. However, in the case of the surfactant having a positively charged terminus, citrate acts to cross-link the conjugates, and subsequent purification just leads to partial

(34) Hong, S.; Leroueil, P. R.; Majoros, I. J.; Orr, B. G.; Baker Jr, J. R.; Banaszak Holl, M. M. *Chem. Biol.* **2007**, *14*, 107–115.
(35) Montet, X.; Funovics, M.; Montet-Abou, K.; Weissleder, R.; Josephson, L. *J. Med. Chem.* **2006**, *49*, 6087–6093.

redissolution of the aggregates. Therefore, other synthetic and purification protocols should be employed in order to have access to stable cationic functionalized AuNPs.

In conclusion, the results presented here confirm the DLVO model for nanoparticle interaction in the case of colloidal solutions of AuNPs in citrate, but adding a new situation where the repulsive forces between charged NPs can be overcome by the presence of small molecules with opposite charges which act as cross-linkers between the charged conjugates. Thus, the conjugating species ending in $R-COO^-$ form are stablized by electrostatic repulsive forces, which overcome van der Waals attraction. By contrast, for R−COOH- or $R-NH_3^+$-terminated species, additional attractive interactions may appear, resulting in aggregation despite the presence of the PEG moiety. In the first case, the absence of charge at low pH leads to domination of van der Waals and H-bonding attractive forces, while the instability in the second case is driven by electrostatic interaction mediated by citrate bridging molecules.

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Supporting Information Available: TEM and size distribution analysis of AuNPs, DLS and zeta-potential measurements of conjugates, UV−vis absorption spectra of CIPGNVG-PEG_a-AuNP after addition of ethanolamine, UV−vis absorption spectra of the conjugation of biomolecules **3** and **4**, and ^{1}H NMR spectrum of species in solution. This material is available free of charge via the Internet at http://pubs.acs.org.

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