Understanding gold nanoparticles interactions with chitosan: crosslinking agents as novel strategy for direct covalent immobilization of biomolecules on metallic surfaces

Germán López-Pérez[1,*], *Rafael Prado-Gotor*[1], *Jose Alberto Fuentes-Rojas*[1] *and María Jesús Martin-Valero*[2]

[1] Dpt. Physical Chemistry, Faculty of Chemistry, University of Sevilla.

[2] Dpt. Analytical Chemistry, Faculty of Chemistry, University of Sevilla.

C/ Profesor García González, 1, 41012, Sevilla, Spain.

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*Corresponding Author

Email: gerlopez@us.es

Abstract

The development of a new method for covalent immobilization of biomolecules on the surface of bare gold nanoparticles (AuNPs) by using crosslinking agents in one step is presented. A very compatible biopolymer such as chitosan has been used as a target molecule to probe the viability of the proposed methodology. Click chemistry, based on biocompatible reactions and coupling with 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride and N-hydroxysuccinimide allow to analyze the covalent interactions between the metal nanoparticles and the biopolymer. Spectra deconvolution technique and Zeta potential measurements confirm the covalent interaction. The present study allows to quantify the proportion of AuNPs covalently adhered to the chitosan, which depends on the solution pH. The obtained results indicates that covalent interactions can be increased up to 25% in relation to total system interactions, which are mostly electrostatic. The proposed strategy opens up a new pathway for biomedical applications because the control of the chemical linkage can be directly performed on the nanoparticle surface without using any molecular intermediate, which may improve the encapsulation efficiency on drug delivery therapies.

Keywords: gold nanoparticles, biopolymers, surface plasmon band, covalent interaction, zeta potential, crosslinking agents

1. Introduction

The study of the so called non-covalent interactions (that is, interactions between chemical species where covalent bonds are not present) is a subject of great interest [1]. In this sense, it is possible to mention between others, the union antigen/antibody [2] and their presence in the development of molecular machines [3], organic electronic materials [4] or sensors [5]. Considering these types of chemical unions, a systematic study of the non-covalent interaction between free anionic gold nanoparticles (AuNPs) and a very versatile biopolymer such as chitosan has been performed [6]. The fact that gold nanoparticles absorb and scatter light in the visible region makes nanometric gold a valuable optical probe to study biomolecular interactions. On the other hand, one of the most important characteristics of chitosan is that it is not toxic, being also biocompatible and biodegradable, what favors multiple applications of the biopolymer [7-9].

Recently, it has been shown that the interaction between chitosan and AuNPs is controlled electrostatically by direct interaction of the citrate anions present on the nanoparticle surface and the protonated amine groups located in the molecular structure of the polymer. The maximum electrostatic interaction takes place at a pH 6.4 with 10 nm citrate gold nanoparticles. In these conditions, low concentrations of chitosan can be detected in solution, being the detection limit (LOD) equals to 69 nM [6].

Aiming to go one step further in studying the direct interaction between chitosan and gold nanoparticles, a new method for chitosan covalent immobilization on the non functionalized surface of AuNPs using crosslinking agents in a single reaction is presented. Coupling biomolecules to nanoparticles is quite complex procedure because it has to be considered not only the chemical parameters affecting to the reaction kinetics but also take into account the stability of the final product. In this sense, the bonding reactions to connect substrates of interest with specific biomolecules, show that the

coupling with EDC (1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) and s-NHS (N-hydroxy succinimide) are one of the most important strategies for covalent immobilization of proteins.

Very few chemical groups are known to provide specific and practical conjugation to carboxylic acids (R–COOH), such as occur in proteins and many other biomolecules. Carbodiimide compounds provide the most popular and versatile method for labeling or crosslinking to carboxylic acids. The most readily available and commonly used carbodiimides are the water-soluble EDC for aqueous crosslinking and the water-insoluble DCC (Dicyclohexylcarbodiimide) for non-aqueous organic synthesis methods. 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) can also be used for the interaction between silica nanoparticles with acid groups and 20 hydroxyl-modified camptothecin **(**CPT**)**. This carbodiimide covalent functionalization pathway has also been employed in the presence of silver nanoparticles. In aqueous media silver NPs with a thiol such as tiopronine could covalently be bound to the vasoactive intestinal peptide (VIP) [10], a small peptide of 28 amino acids with modulating capacities in pathologies related with an inflammatory and/or autoimmune character [11]. However, despite the existence of multiple strategies of covalent immobilization, it is not found in the literature a key strategy of immobilization for biopolymers coupling, such as chitosan, directly onto the surface of AuNPs by using the crosslinking agents EDC and s-NHS.

In order to induce the covalent linkage between the citrate-AuNPs and chitosan, the coupling with EDC/s-NHS have been carried out directly on the nanoparticle surface without using any other molecular spacer. The interaction of the citrate-AuNPs with the crosslinking agents was confirmed by Zeta potential measurements. The addition of such crosslinking agents to the chitosan and AuNPs solution produces a broadening of the Surface Plasmon Band (SPB) between 600 and 700 nm, due to the existence of an

underlying band which is responsible for the covalent interactions. The UV-Vis spectra have been analyzed by means of the deconvolution technique allowing to quantify the proportion of the each interaction type involved on the aggregation process. A study as a function of the solution pH has been performed to evaluate the efficiency of the proposed strategy, showing that covalent interactions can be increased up to 25% in relation to the total system interactions.

2. Materials and Experimental Methods

2.1 Materials

All the chemical reagents used were of analytical grade. Gold nanoparticles (AuNPs) used for the experiments were synthetized following the Turkevich's method [12] by using tetrachloroauric acid (from Aldrich) as metallic precursor reagent and citric acid dibasic trihydrate (from Aldrich) as reduction agent (99% of purity).

Chitosan solutions were prepared by using polymer of low molecular weight (67 kDa) from Aldrich, and as crosslinking agents s-NHS (98%) and EDC (both from Sigma Aldrich) were used. The pH of chitosan solutions was controlled by sodium hydroxide from Aldrich and glacial acetic acid solution (1% V/V) from Probus, S.A. All solutions were prepared with deionized water, being its conductivity less than 10^{-6} S m^{-1}.

Chitosan stock aqueous solutions (22.85 μM) were prepared by weighting 22.96 mg and adding 15 mL of acetic acid (1% V/V) very slowly, using a syringe tip with continuous stirring. Once the solution was prepared, concentrated aqueous solutions of hydrochloric acid or sodium hydroxide were added with a micropipette until the working pH was obtained.

2.1.1 Crosslinking agent's preparation

Aqueous solutions of 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide (EDC) and N-Hydroxy-sulfosuccinimide sodium salt (s-NHS) were used for the covalent linkage of AuNPs and chitosan. The stock solutions were 50 nM and 25 nM for EDC and s-NHS, respectively.

2.1.2 Working solutions

Two different sets of samples were prepared in order to check the previously analyzed electrostatic interaction [6] and the new immobilization covalent protocol proposed by using crosslinking agents. The first set was prepared by mixing 3 mL of AuNPs (9.52 nM), 1mL of chitosan stock solution and 30 µL of water, without EDC and s-NHS crosslinking agents. The second set was prepared adding 3mL of AuNPs (9.52 nM), 1 ml of chitosan stock solution, 20µL of EDC and 10µL of s-NHS. Crosslinking agents were incubated with the AuNPs during an hour before chitosan aliquot was added.

2.2 UV–Vis spectra

A Shimadzu UV–Vis mini 1240 spectrophotometer with a spectral resolution of 1 nm was used in the wavelength range of 400-850 nm. Plastic disposable cells with an optic path of 10 mm were used for spectrophotometric measurements. The UV–Vis absorption spectra of the working solutions were acquired 10 min after mixing at 298.2 K. A blank spectrum of chitosan solution at the working pH was always recorded.

2.3 TEM measurement

The size and shape of AuNPs were confirmed by using TEM images, whose samples were prepared by placing a single drop of the AuNPs solution on a copper grid coated with a carbon film. This drop was evaporated during half an hour at room temperature and it was analyzed with the TEM microscope (Hitachi CM 200) working at 200 kV.

2.4 Zeta potential measurements

Zeta potential measurements were carried out with a Zetasizer Nano ZS Malvern Instruments Ltd (UK), which measured the electrophoretic mobility of the sample from the velocity of the particles using a Laser Doppler velocimeter (LDV). A DTS 1060 polycarbonate capillary cell was used at 298.2 K. Experiments were done by triplicate. The Zeta potential of the gold colloid is about -37 mV in water solutions, which is sufficient to keep the particles from interacting with each other and therefore maintain a stable particle size of the sample. In all the experiments performed, concentrations remain constant: AuNPs (9.52 nM), EDC (50 nM) and s-NHS (25 nM). Four different solutions were measured: free AuNPs, a mix of AuNPs with each crosslinking agents separately and the last one containing AuNPs with both crosslinking agents, EDC and s-NHS.

2.5 Refractive index measurements

The refractive index of the chitosan solutions were measured using an optic lyman system Abbe refractometer. Based on the calibration solutions of known refractive index, the absolute error for this magnitude is estimated at ± 0.0004 units. Experiments were carried out at 298.2 K.

2.6 Concentration of AuNPs solutions

Once the nanoparticle's core diameter is measured by TEM images, the mean number of Au atoms in each particle (n) can be calculated, assuming spherical particle shape, by using the following relationship [13]:

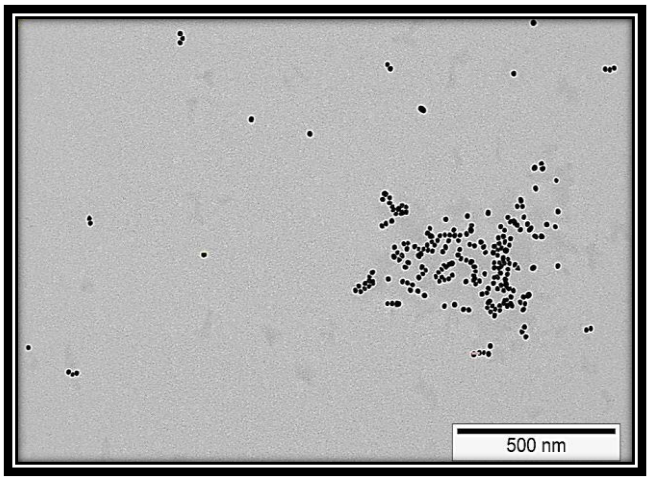
$$n = \frac{0.5\pi N_A d_M^3}{3V_M} \quad (1)$$

Where N_A stands for the Avogadros' number, d_m is the diameter of the nanoparticle expressed in cm and V_M corresponds to the molar volume of bulk gold (10.215 cm^3) [14]. For 15 nm gold nanoparticles, the mean number of Au Atoms in a particle corresponds to n = 30,867. Once the number of Au atoms in the gold nanoparticle solution is known, it is possible to obtain the average concentration of nanoparticles from the n value, resulting in a solution with a concentration of 9.52 nM in AuNPs.

3. Results and discussion

3.1 Characterization of the AuNPs

The size and shape of the synthetized AuNPS have been confirmed by using the corresponding TEM images. All of them present a circular shape (as can be seen in Fig. 1.a) with an estimated average diameter of 16±1 nm, obtained from the corresponding histogram (shown in Fig. 1.b). The average size of the AuNPs was also corroborated from the UV–Vis measurements showing a well-defined maximum of the surface plasmon band (SPB) band at 520 nm, which is in good agreement for the size and shape of the synthetized AuNPs [15].



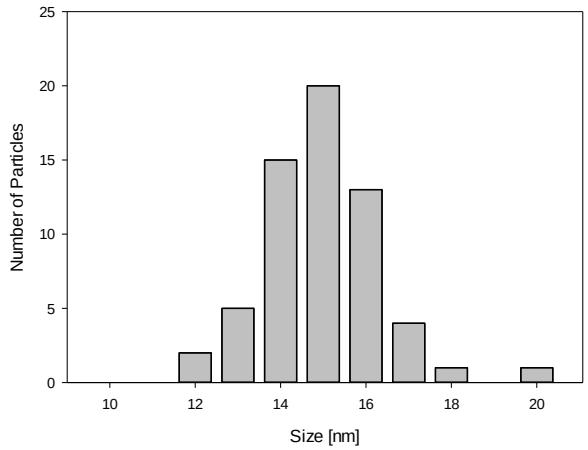
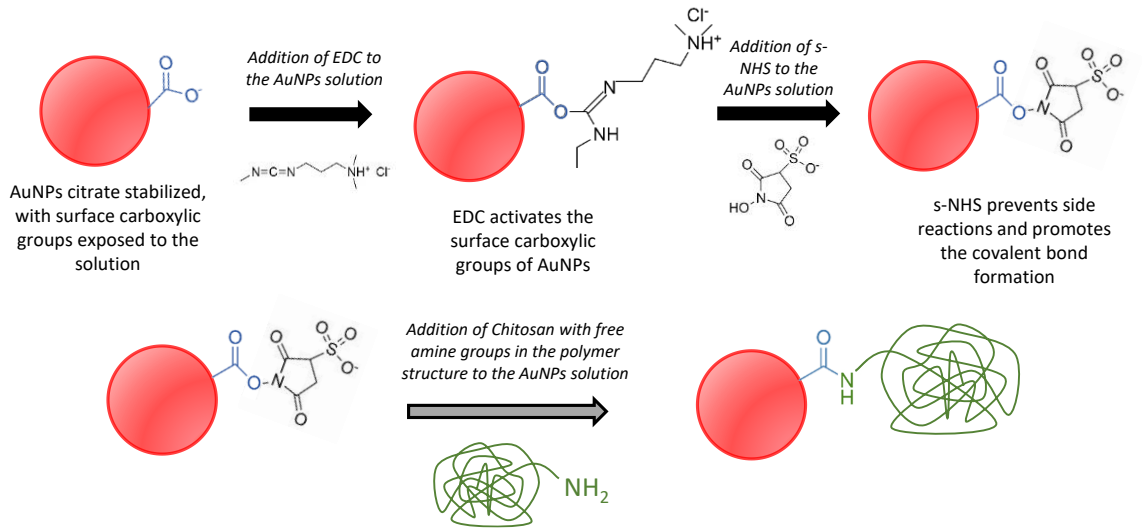


Fig. 1. a) TEM image showing the shape and the size of the synthetized gold nanoparticles. b) Calculated histogram to determine the average size of the AuNPS from the TEM image.

3.2 Covalent interaction of the AuNPs with Chitosan

Electrostatic interaction of AuNPs with chitosan has been previously investigated [6] showing a broadening in the UV–Vis spectrum, which can be explained in basis of two overlapping bands, one of them corresponding to the free AuNPs and the other one to the interacting AuNPs with chitosan. The electrostatic interaction is modulated by the medium pH because it allows to modify the surface charge proportion through the adsorbed citrate molecules on the AuNPs surface as well as the biopolymer and consequently the amount of charged groups available, which can interact on both sides.

Once the electrostatic interaction has been analyzed, a new challenge arises from the possibility to join covalently the AuNPs and the chitosan in a direct form. For this purpose a straightforward approach which allow the direct assembly of the AuNPs and chitosan by using crosslinking agents such as EDC/s-NHS is presented. The proposed procedure is shown in the reaction Scheme 1.



Scheme 1. Chemical steps for the covalent union between AuNPs and chitosan by using the crosslinking agents (EDC/s and NHS). It is only shown the formation of just one covalent bond between the AuNPs and biopolymer, though there are many linkage sites on the biopolymer chain.

This reaction mechanism involves two consecutive steps: the first one consists in activating the carboxylic groups of the surficial citrate adsorbed onto the gold nanoparticles by using EDC. The second step involves the participation of the s-NHS, which promotes the amide bond formation preventing side reactions. Thus, it is possible to perform the covalent linkage between the carboxylic groups of the adsorbed citrate groups located on the AuNPs surface and the amine groups of the chitosan structure to

form the corresponding amide bond. It is important to remark, from the experimental point of view that this mechanism occurs in a single step, being a direct, fast and easy method. Accordingly, it has been possible to investigate the direct covalent attachment of the chitosan to the AuNPs, as well as the possible differences between electrostatic and covalent interactions. A similar strategy based on EDC/s-NHS coupling has been proposed to bind covalently peptides to colloidal gold functionalized with a carboxylic thiol chain, used as molecular bridge [16]. However, the new proposed strategy allows to link covalently gold nanoparticles in a direct form without using any molecular spacer, employing for this purpose the surficial citrate adsorbed onto the nanoparticles through their carboxylate groups. The main advantage of the proposed method is the elimination of using any chemical modifiers, which facilitates the chemical linkage between AuNPs and many biomolecules containing free-amine groups, such as aminoacids, proteins, nucleotides and various biopolymers including DNA.

The UV–Vis spectroscopy is a powerful tool to study the aggregation process of AuNPs because the SPB band position is highly affected when the particles have agglomerated in some extent. Normally, the aggregation process shows a significantly broadening of the SPB band as well as a red shift of the band maximum. In some cases, it is possible to find some changes on the spectral characteristics of the system due to the surrounding media on the AuNPs surface environment. For this purpose, refractive index measurements have been carried out on chitosan solutions using an optic Lyman system Abbe refractometer. If the surrounding media have a significant effect on the SPB band displacement, the refractive index of the chitosan solutions should show significant differences related to the pure solvent. Having performed the corresponding refractive index measurements, there is no significant differences between the solvent and chitosan solutions, showing only a slight variation of 0.0007 units in this magnitude. Underwood

et al. [17] show that only a 4-8 nm SPB displacement is found when the solution refractive index change, at least, on 0.05 units [17], considering the dielectric permittivity data of the literature [18,19]. Taking into account the values of the refractive indexes for chitosan solutions and the displacement of the SPB maximum found in the experimental spectra, it can be concluded that the observed colorimetric changes in the colloidal gold are not due to a change in the surrounding medium of the nanoparticles.

Furthermore, the spectra deconvolution permits to obtain the contribution of each underlying band to the experimental spectrum. For pure electrostatic interaction between AuNPs and chitosan as a function of the media pH, it is shown that experimental spectra have two main contributions: the first one is due to the free AuNPs and the second one is caused for the AuNPs particles which interact with the biopolymer. The pH plays a key role in this process because it modulates the interaction from very acid to neutral media, corroborating the electrostatic nature of the linkage.

From this point of view, it is also interesting to analyze the covalent union between the AuNPs and the chitosan, which allows to compare the extension and differences between these kinds of chemical interaction. For this purpose, a strategy based on reaction Scheme 1 is proposed in this work. Two different samples were prepared in presence and absence of crosslinking agents at pH 6.3 (as described in experimental section) in order to check the covalent linkage. The obtained UV–Vis spectra are shown in Fig. 2.

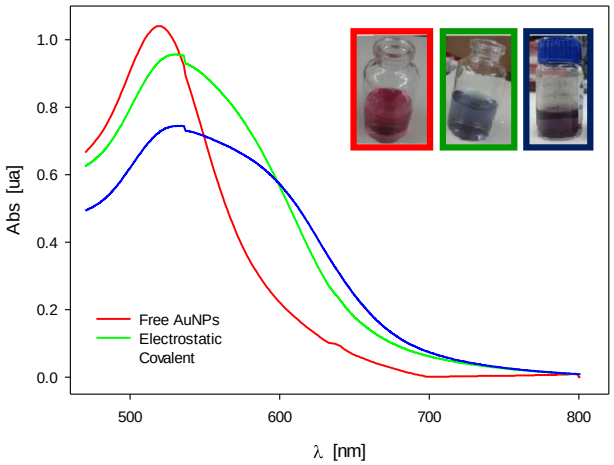


Fig. 2. UV-Vis spectra showing the SPB for free AuNPs (solid red line), AuNPs and chitosan (solid green line) and AuNPs and chitosan in presence of EDC/s-NHS (solid blue) at pH 6.3, respectively. The boxed inset images correspond to the experimental test solutions for each experiment showing their characteristic colors.

In absence of the crosslinking agents, there is a displacement of the SPB band towards higher wavelengths indicating the aggregation process occurs, as it is expected for an electrostatic interaction (green solid line). However, a noticeable broadening and a greater displacement of the SPB (located between 600-700 nm) is found in the presence of EDC and s-NHC (blue solid line), as it can be noted. The change is even appreciable in the color of the solution, which shows a clear change from blue to violet in the absence and the presence of the proposed agents, respectively (see inset images in the picture). In this way, the color changes observed in the worked solutions also demonstrate a greater aggregation state of the AuNPs compared to the electrostatic interaction. Therefore, the obtained results in the SPB in presence of the EDC and s-NHC confirm the validity of the proposed reaction scheme and it is also an evidence of the covalent linkage between the carboxylic surficial groups of the citrate residues onto the AuNPs and the free amine groups of the D-glucosamine monomer in the biopolymer. The addition of EDC, NHS or a mixture of both of them to a sample of free AuNPs does not produce any significant

303 change during one hour in the measured SPB, indicating that crosslinking agents do not
304 promote the aggregation process by themselves.

305 The obtained changes on the experimental spectra of AuNPs interacting with chitosan can
306 be analyzed in depth by using a deconvolution procedure for the SPB. Thus, it is possible
307 to divide the experimental spectra to obtain the contribution of each underlying band,
308 determining their characteristic parameters such as the maximum absorbance (A_{max}), the
309 wavelength corresponding to the maximum absorption (λ_{max}) and the half width of the
310 band (w_v) [20]:

311 $$A = A_{max} \exp\left\langle -\ln 2\left\{ \ln\left[1+\frac{2(\lambda_{max}-\lambda)}{\lambda_{max}\,\lambda}\frac{k\sinh(b)}{w_v}\right] \Big/ b \right\}^2 \right\rangle \qquad (2)$$

312 The deconvolution of the experimental UV–Vis spectrum for the covalent attachment at
313 pH 6.3 is shown in Fig. 3. Following the previous findings, it allows the assignation of
314 four different bands responsible for the background absorption (short dashed line), the
315 free AuNPs (solid red line), the electrostatic interaction (solid green line) and a new band
316 accountable for the covalent linkage (solid blue line), centered at λ_{max} = 623 nm. The
317 solid black line corresponds to the sum of the four underlying bands (simulated spectrum),
318 which matches perfectly with the experimental data (white circles).

319 It is clear the existence of an electrostatic band even when the crosslinking agents are
320 added to the solutions to favor the covalent interaction. This fact can be explained taking
321 into account the pK_a of the ammonium groups located into the chitosan chains. The
322 estimated pK_a value of 6.1–6.7 depends on the degree of N-deacetylation in chitosan [21].

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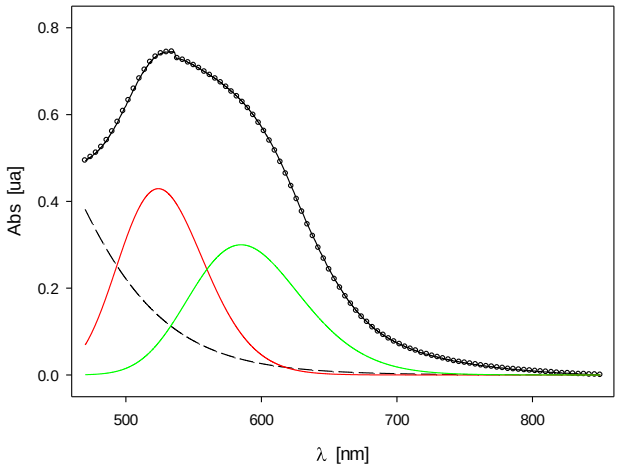

Fig. 3. Deconvolution of the UV–Vis experimental spectrum for the SPB in the presence of EDC/s-NHS for the AuNP–chitosan covalent interaction at pH 6.3. White circles: experimental data, solid black line: simulated data, dashed black line: background absorption, solid red line: free AuNPs (λ_{max} = 524 nm), solid green line: electrostatic interaction (λ_{max} = 585 nm), solid blue line: covalent interaction (λ_{max} = 623 nm).

Assuming a pK_a value of 6.7 and according to the corresponding distribution function, in the worked solution of pH 6.3 there are approximately a 75% of positive charged groups on the polymeric chain, which are available to interact electrostatically with the negative charged AuNPs. Consequently, the corresponding band for the electrostatic interaction is found in the deconvoluted spectrum, (green solid line). Furthermore, it is important to point out that only the uncharged amine groups of the chitosan chain (approximately the 25% at pH 6.3) are available for covalent attachment, according to the proposed methodology shown in Scheme 1, so a band with lower intensity should be obtained for the chemical interaction (blue solid line). Thus, the experimental conditions tested in this experiment should lead to the maximum proportion of covalent attachment between AuNPs and the biopolymer. To corroborate this assumption, a study of the interaction employing this methodology has been performed as a function of the solution pH, in the range from 3.5 to 6.3. Fig. 4 shows the obtained experimental spectra for pH 5.1 (left

344 panel) and pH 4.0 (right panel), as well as their corresponding simulated data by using
345 the deconvolution analysis, chosen as representative cases.

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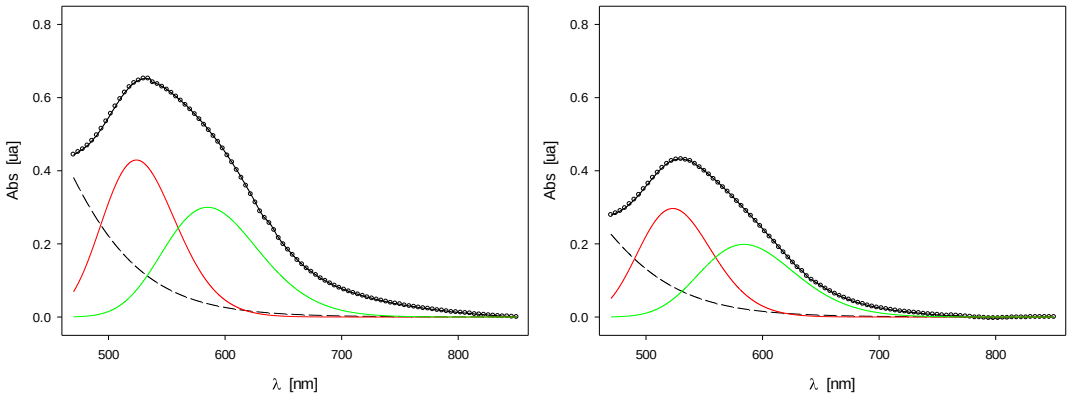

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348 **Fig. 4.** Deconvolution of the UV–Vis experimental spectra for the SPB in the presence of
349 EDC/s-NHS for the AuNP–chitosan interaction at pH 5.1 (left panel) and pH 4.0 (right
350 panel), respectively. Symbols have the same meaning than in Fig. 3.

351

352 As the solution pH is reduced, the band responsible for the covalent interaction (solid
353 blue line) gradually decreases up to its almost total disappearance at a pH 3.5 in favor of
354 the band responsible for the electrostatically interaction (solid green line), which becomes
355 the more important contribution in the aggregation process of the AuNPS with the
356 biopolymer. Table 1 gathers the parameters for the deconvolution analysis of the
357 investigated solutions, where the relative absorbance is calculated as the quotient between
358 the fraction of AuNPs that interact covalently (A_{REL}^{Cov}) and electrostatically (A_{REL}^{Elec}) respect
359 to the total, according to:

360
$$A_{REL}^{Cov} = \frac{A_{REL}^{Cov}}{A_{REL}^{Elec} + A_{REL}^{Cov}} \quad , \quad A_{REL}^{Elec} = \frac{A_{REL}^{Elec}}{A_{REL}^{Elec} + A_{REL}^{Cov}} \qquad (3)$$

while A_{REL}^{Free} stands for the fraction of free AuNPs present in solution which are not interacting, calculated as:

$$A_{REL}^{Free} = \frac{A_{REL}^{Free}}{A_{REL}^{Free} + A_{REL}^{Elec} + A_{REL}^{Cov}} \quad (4)$$

Table 1.

Influence of the pH solution on the deconvolution parameters for the experimental spectra of AuNP–chitosan in the presence of the crosslinking agents. The superscripts *Free*, *Elec* and *Cov* refer to free AuNPs, electrostatically and covalent interaction respectively and the absorbance's measurements refer to relative values of each population. The rest of parameters where: $\lambda_{\max}^{Free}$ =524 nm, w_v^{Free} =2.7 kK, $\lambda_{\max}^{Elec}$ =584 nm, w_v^{Elec} =2.8 kK, $\lambda_{\max}^{Cov}$ =622 nm, w_v^{Cov} =3.0 kK (Eq. 2).

Solution pH	A_{REL}^{Free} (au)	A_{REL}^{Elec} (au)	A_{REL}^{Cov} (au)
3.5	0.487	0.933	$6.63\ 10^{-2}$
4.0	0.503	0.929	$7.10\ 10^{-2}$
4.5	0.519	0.838	0.162
5.1	0.584	0.754	0.246
5.5	0.637	0.751	0.249
6.3	0.751	0.748	0.252

Fig. 5 shows the relative dependence of the free AuNPs fraction (red) as a function of the solution pH. As can be noted, there is a continuous depletion in the fraction of free nanoparticles as the solution pH is raised, verifying their ability to interact with the biopolymer. Furthermore, the relative dependences of the electrostatic (green) and covalent (blue) fractions are also represented in Fig. 5.

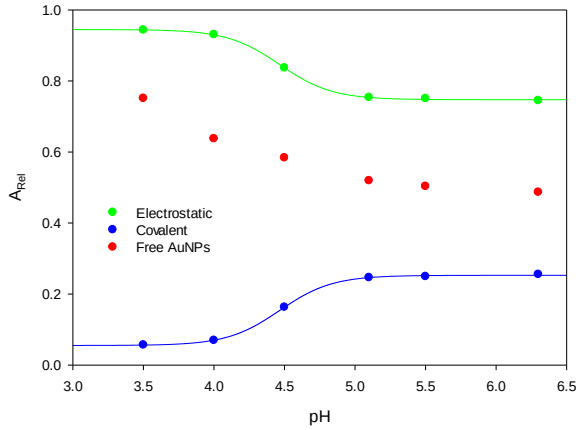

Fig. 5. Fractions of the free AuNPs (red) in solution, nanoparticles interacting electrostatically (green) and covalent linked nanoparticles (blue) as a function of the solution pH, obtained from the deconvolution analysis.

The maximum covalent attachment is obtained at pH 6.3, where $A_{REL}^{Elec} = 0.75$ and $A_{REL}^{Cov} = 0.25$ due to the maximum population of free amine groups on the chitosan structure, as stated before. As the solution pH is reduced the amount of free amine groups is also diminished, being the covalent bonding much more unfavorable, until pH 3.5 where it is almost negligible ($A_{REL}^{Cov} = 0.05$). In these conditions, the only possible interaction between AuNPs and the biopolymer is caused by the surface electrostatic charges of both components. From these limit situations, the solution pH 4.5 represents the situation where the 50% of free amine groups on the biopolymer are covalently attached to the surface of the AuNPs.

3.3 Zeta potential measurements

In an applied electric field, charged species are attracted to the electrode of the opposite polarity resulting in an electrostatic potential, called zeta potential. The system is more stable as the Zeta potential is larger due to heavy coulomb repulsion forces between particles of the colloid, which prevents their coagulation in solution. Table 2 shows Zeta

potential values obtained using a Malvern Zetasizer. Gold nanoparticles stabilized by citrate ions have a negative potential higher than -30 mV, being perfectly stable in solution. By adding the crosslinking agent EDC (cationic carrier) such potential decreases, reflecting EDC interaction with the surface of the nanoparticles. Furthermore, the addition of s-NHS agent also causes a reduction of the Zeta potential, but less marked.

Table 2.

Zeta potential measurements of free AuNPs and in the presence of crosslinking agents (EDC and s-NHS).

Solution composition	Zeta Potential (mV)		
	Run 1	Run 2	Run 3
Free AuNPs	-37	-37	-38
AuNPs + EDC	-26	-27	-24
AuNPs + s-NHS	-29	-30	-29
AuNPs + s-NHS + EDC	-24	-24	-22

The s-NHS, unlike the EDC, presents only a negative charge and therefore a clear decrease in the zeta potential would be expected. However, it should be taken into account the fact that a possible replacement of surficial citrate by the s-NHS would bring a load step from -3 to -1 negative charge, naturally from a global point of view and a complete replacement of the citrate ligand by s-NHS. From the zeta potential measurements, it can be concluded that AuNPs interacts with both EDC and s-NHS, increasing the zeta potential values. Although the presence of EDC and s-NHS partially reduces the measured potential, it remains to be a high enough negative value to maintain stable the colloid.

4. Conclusions

The proposed methodology by using crosslinking agents allows the covalent linkage of the AuNPs with chitosan. The proportion of AuNPs covalently bonded to the polymer can be quantified through the Zeta potential measurements and spectra deconvolution technique. The maximum proportion obtained is found to be limited by the free amine groups located in the biopolymer chain. For the system studied and taking into account the size of the nanoparticle as well as the degree of acetylation of the polymer, covalent interactions can increase up to 25% of the total interactions, which are mostly electrostatic.

There are two main factors which could affect to the procedure efficiency. First, the possibility of structural changes on the polymer when the pH tends to neutral media, which can favor the formation of hydrogen bonds between the chitosan chains, limiting the availability of the amine groups to react covalently. On the other hand, the amount of crosslinking agents with respect to the chitosan concentration, could be also a limiting factor if not all the amine groups in the chitosan chains are activated to form the amide bond. This point is extraordinarily important and it opens up new avenues of future research in relation to covalent immobilization on the surface of AuNPs using crosslinking agents. The described strategy can be very useful for biomedical applications, such as drug delivery, improving the encapsulation efficiency of therapeutic drugs through the control of the chemical linkage.

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