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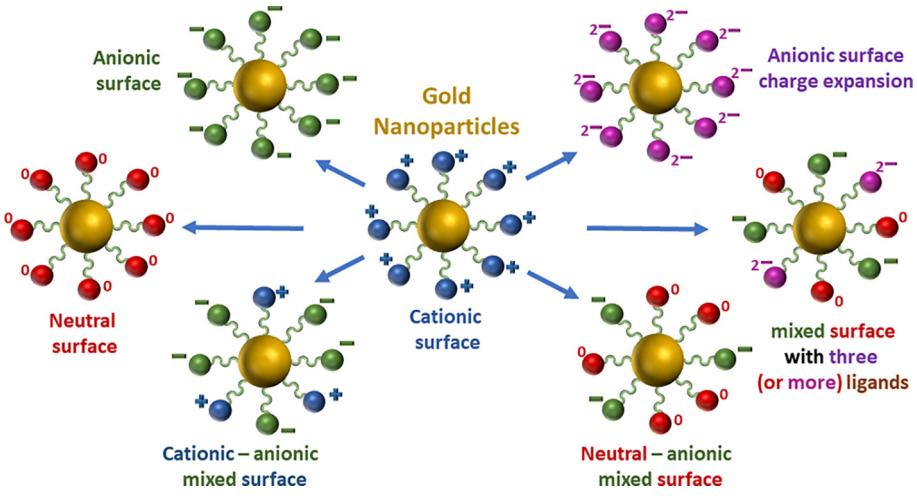
Facile generation of surface diversity in gold nanoparticles

Manuel M. Paz [a,b,*], Alberto Peinador Veiga [b], Tamara Regueira [b], Carlos Vázquez Vázquez [a,c], M. Arturo López Quintela [a,c]

[a] Instituto de Materiais (iMATUS), Facultade de Química, Universidade de Santiago de Compostela, 15782 Santiago de Compostela, Spain
[b] Departamento de Química Orgánica, Facultade de Química, Universidade de Santiago de Compostela, 15782 Santiago de Compostela, Spain
[c] Departamento de Química Física, Facultade de Química, Universidade de Santiago de Compostela, 15782 Santiago de Compostela, Spain

GRAPHICAL ABSTRACT



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ABSTRACT

Surface chemistry is a key determinant of the physico-chemical and biological properties of gold nanoparticles (AuNPs). The introduction of chemical diversity in the surface of AuNPs is usually accomplished by place-exchange reactions using incoming ligands containing the desired terminal functional groups. As an alternative approach, we present here a simple, practical methodology to modify the surface of gold nanoparticles that allows the preparation of AuNPs stabilized with polyethyleneglycol (PEG) ligands with different surface chemistries using AuNPs stabilized with thiol-PEG-amino ligands as starting material. The surface modification reaction involves the acylation of the terminal amino groups in the ligand with an organic acid anhydride in an aqueous buffer. In addition to a full surface modification, this method also allows the synthesis of AuNPs with tailored mixed surfaces, containing two or more different functional groups, each of them at the desired extent. The ease of the experimental conditions for the reaction, purification, and for determining the level of surface modification makes this strategy an attractive alternative to current methods for the preparation of AuNPs with diverse surface chemistry.

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* Corresponding author.

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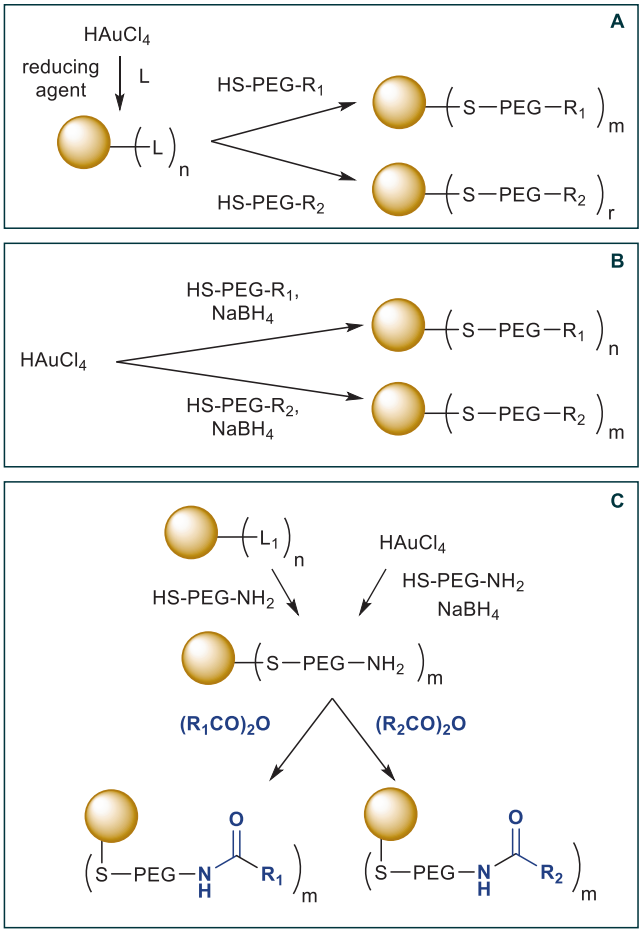
1. Introduction

Gold nanoparticles (AuNPs) are one of the most researched nanomaterials, particularly regarding their health-related applications. The key physico-chemical properties that control the biological activity of AuNPs are size, shape and surface chemistry,[1] the latter playing a crucial role in the pharmacokinetics, biodistribution, cellular internalization, and intracellular distribution of AuNPs.[2] Therefore, devising strategies that allow the generation of surface diversity is considered as a key factor in the development of nanomaterials for health-related applications.[3–5] As part of our ongoing research in the use of AuNPs as drug delivery vehicles we were interested in preparing AuNPs coated with polyethyleneglycol (PEG) ligands with diverse surface chemistry. PEG ligands were selected for the "stealth" properties they provide, preventing the nanoparticles from aggregation, opsonization, and phagocytosis, thus increasing their circulation time.[6] Two known approaches can be used to fabricate libraries of chemically diverse PEGylated AuNPs (Scheme 1A, 1B), depending on their size. In approach A, AuNPs are synthesized first (e.g., using either the Brust–Schiffrin [7] method or a version of the Turkevich method [8,9]), and then the original ligands (L) are displaced by reaction with thiol-PEG ligands containing the desired functional group. [10,11] Approach B would afford directly the PEGylated AuNPs by reducing a mixture of Au^{3+} and the appropriate thiol-PEG ligand with borohydride.[12] Both approaches present two disadvantages: they may result in AuNPs with different core size,[13–15] and they require the availability of as many PEG ligands as the number of desired surface functional groups. These heterobifunctional PEG ligands are quite expensive if commercially available or require a labor-intensive synthesis otherwise.[16,17].



Scheme 1. Strategies for the introduction of chemical diversity in PEGylated AuNPs.

We decided to explore an alternative strategy that would overcome these drawbacks: The modification of the cationic surface of AuNPs stabilized with HS-PEG-NH_2 ligands by acylation reactions with organic acid anhydrides in aqueous media (Scheme 1C). In this approach, the surface properties of the modified AuNPs will be determined by the nature of the anhydride and the reaction could be used, for example, to easily convert the cationic surface of the original AuNPs into neutral (e.g. by reaction with acetic anhydride) or anionic surfaces (e.g., by reaction with cyclic anhydrides).[18] This strategy is highly attractive for several reasons: First, it would allow access to a series of AuNPs with different surface chemistry starting with a single preparation of AuNPs stabilized with a thiol-PEG-amino ligand. Second, the acylation reaction is not expected to affect to a significant extent the core size of the original AuNPs, therefore this method should provide access to a series of AuNPs with different surface chemistry maintaining an identical core size. Also, as the acylation reactions will be performed in aqueous media, both the reaction and purification protocols will be quite simple. PEGylated AuNPs are usually stored and handled as suspensions in water, therefore performing the reactions in aqueous media should allow to use stock solutions of AuNPs directly as a reactant solution, without the need of further manipulation. After completion of the reaction, the purification should be easily executed by using centrifugal filtration or gel filtration chromatography. Additionally, we also want to explore reaction conditions that result in partial surface acylation, a reaction that will produce AuNPs with mixed surface functionality. AuNPs covered with mixed ligands, which show promising biological properties,[19] are usually synthesized by place-exchange reactions using mixtures of ligands.[20] Our methodology would provide an alternative synthesis of mixed-ligand AuNPs that will amplify the scope of surface chemistry available using current methods. The results of our research are presented below.

2. Experimental section

2.1. Materials and reagents

Thiol-PEG-amino MW 2000 g/mol (HS-PEG2K-NH_2,purity > 95%) was purchased from Sigma-Aldrich or Biopharma PEG Scientific Inc. HS-PEG_9-CH_2CH_2-NH_2·HCl (purity > 95%) was purchased from PurePEG LLC. Agarose (Low EEO, molecular biology grade) was supplied by Fisher Scientific. 2-(*N*-Morpholino)ethanesulfonic acid hydrate (MES, purity ≥ 99.5%), 4-(2-Hydroxyethyl)-1-piperazine propanesulfonic acid (EPPS, purity ≥ 99.5%), acetic anhydride (purity ≥ 99.0%), succinic anhydride (purity ≥ 99%), phthalic anhydride (purity ≥ 99%), *N*-carboxybenzoyl aspartic (*N*-Cbz Asp) anhydride (95%), $NaBH_4$ (purity ≥ 98.0%) and $HAuCl_4$, (purity ≥ 99.9%) were purchased from Sigma-Aldrich. Nicotinic anhydride (purity > 97.0%) was provided by TCI Chemicals.

2.2. Characterization techniques

UV–Vis spectra were measured in a Thermo Scientific Evolution 300 UV–Visible spectrophotometer using 1 cm path length semi-micro PMMA Brand cuvettes. Transmission electron microscopy (TEM) micrographs were obtained in a JEOL JEM1011 or a JEOL JEM-2010 electron microscopes, at an acceleration voltage of 100 kV or 200 kV respectively. Samples for TEM analysis were prepared by placing a drop (4 μL) of the aqueous solution (ca. 0.1 mg/mL) of gold nanoparticles onto a 400 mesh copper grid coated with an amorphous carbon film and left to evaporate at room tempera-

ture. Size distributions were evaluated using PEBBLES software package,[21] with 200–400 NPs sampled for each size determination. DLS and Zeta potential measurements were performed in a Malvern Zetasizer Nano ZS (Malvern Instruments, UK), using the Universal Dip Cell for the zeta potential measurements. IR spectra were obtained on a Varian 670-IR FT-IR Spectrometer using attenuated total reflection (ATR) sampling technique. ^{1}H NMR spectra were performed on a Bruker DRX-500 in AuNP dispersions in 10% D_2O in H_2O.

2.3. Gel electrophoresis

Samples containing 10 μL AuNP dispersion and 2 μL 60% glycerol in water were loaded into the wells of a 1% agarose gel, prepared by heating 200 mg of agarose in 200 mL of 50 mM phosphate buffer (pH 7.0). Electrophoresis was carried out for 15 – 30 min at 4 – 10 V/cm.

2.4. Synthesis of Au@PEG-NH_2 nanoparticles

Au2@PEG2K –NH_2: A solution of HS-PEG2K-NH_2 (60 mg, 60 μmol) in H_2O (1 mL) was added to a solution of tetrachloroauric acid trihydrate (4.0 mg, 10 μmol) in H_2O (15 mL). An aqueous solution of $NaBH_4$ (100 mM, 200 μL, 20 μmol) was added in small aliquots under rapid stirring. The dark-brown suspension obtained was further stirred for 2 h and incubated at 4 °C overnight. Purification was performed by centrifugal filtration with Amicon filters (10 kDa MWCO). The dispersion was finally concentrated to a volume of 1.0 mL to be used for acylation reactions. Au4@PEG-NH_2, Au5@PEG-NH_2 and Au13@PEG-NH_2 were prepared by ligand exchange with HS-PEG2K-NH_2 from the corresponding Au5@citrate NPs and Au13@citrate NPs. Briefly, an aqueous HS-PEG2K-NH_2 solution (5 μM, 1.0 mL, 5 μmol) was added to of an aqueous dispersion of Au@citrate nanoparticles (prepared from 10 μmol $HAuCl_4$). The reaction mixture was stirred gently for 24 h. Au4@PEG2K-NH_2 and Au5@PEG2K-NH_2 were washed and purified by centrifugal filtration (Amicon, 10 kDa MWCO) applying 3 cycles of centrifugation and collected in a final volume of 1 mL. Au14@PEG2K-NH_2 were lyophilized after ligand exchange, redissolved in 1 mL H_2O and purified by gel filtration chromatography using PD-10 columns. The purified AuNPs dispersions were finally concentrated to 5–10 mM Au (based on initial $HAuCl_4$) and stored at 4 °C. AuNP concentrations were determined using extinction coefficients calculated from their measured diameter according to Liu et al.,[22] and they were in as following ranges (±20%): 15 μM (Au2@PEG2K-NH_2), 3 μM (Au4@PEG2K-NH_2), 1.2 μM (Au5@PEG2K-NH_2), 0.10 μM (Au13@PEG2K-NH_2). The estimated concentration of surface amino groups was: 0.6 mM (Au2@PEG2K–NH_2), 0.5 mM (Au4@PEG2K-NH_2), 0.3 mM (Au5@PEG2K-NH_2) and 0.12 mM (Au13@PEG2K-NH_2). The calculation of ligand concentration assumes a footprint of ≈ 0.4 nm^2 per individual HS-PEG2K-NH_2 ligand.[23].

2.5. General procedures for acylation reactions

(a) Full surface modification (small scale): A dispersion of Au@PEGNH_2 nanoparticles (23 μL) was placed in a 1.5 mL microcentrifuge tube, followed by addition of a solution of EPPS buffer pH 9.0 (500 mM, 4 μL). The mixture was briefly stirred and a solution of the desired anhydride in dimethylformamide (DMF) was added (140 mM, 3 μL). The mixture was stirred in a vortex mixer equipped with a microcentrifuge tube adapter for 2 – 24 h. A solution of 60% glycerol in H_2O (6 μL) was added and a volume of 7 – 12 μL of the mixture was loaded in the gel. (b) Partial surface modification (small scale): An analogous protocol was used, except that MES or EPPS buffer at the desired pH was used and the concentration of anhydride was lowered (80 mM stock solution in DMF, 8 mM final concentration in the reaction mixture). (c) Larger scale reactions: They were performed similarly, using a microcentrifuge tube when the final reaction volume was lower than 0.5 mL, and a round bottom flask with magnetic stirring for larger volumes. For UV, DLS, Z-potential measurements, the acylated AuNPs were purified by gel filtration chromatography (NAP-5 or PD-10 columns, depending on the final reaction volume) or centrifugal filtration (Amicon filters, 10 kDa MWCO) applying 3 cycles of centrifugation. For IR and NMR measurements the modified AuNPs were purified by centrifugal filtration applying 5 cycles of centrifugation (12 mL to less than 0.5 mL in each cycle) to ensure that the ratio MES/EPPS buffer to AuNP-bound ligand was lower than 0.01.

3. Results and discussion

Amine groups can be acylated efficiently in aqueous media by organic acid anhydrides, as the reactivity of the amino groups towards the anhydride is much higher than that of H_2O or hydroxide and, therefore, the formation of the amide bond can compete efficiently with anhydride hydrolysis under adequate conditions. The acylation reactions are most frequently performed in alkaline media using a base such as NaOH or bicarbonate, although successful reactions in neutral or acidic aqueous media have also been reported.[24] For our work we studied the acylation of PEGylated AuNPs using the six anhydrides shown in Fig. 1.

Acetic anhydride should convert the cationic surface of Au@PEGNH_2 nanoparticles in a neutral surface containing terminal acetamido groups. Nicotinic anhydride will introduce terminal

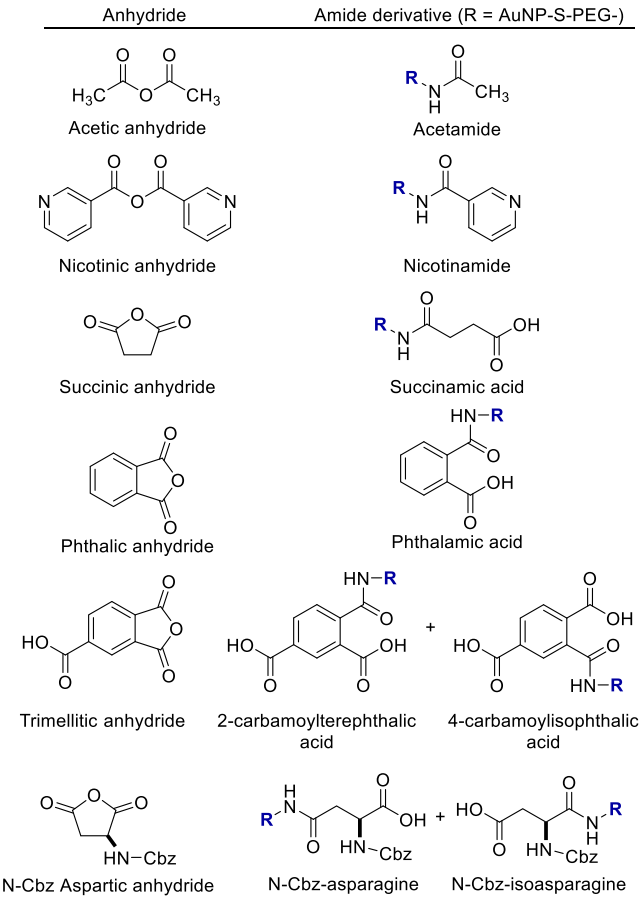


Fig. 1. Structure of the anhydrides used in this work and the products of their reaction with the amino group of AuNPs stabilized with HS-PEG-NH_2 ligands.

nicotinamide groups, a much weaker base than the original primary amines. The acylation with succinic, phthalic, or *N*-carboxybenzoylaspartic (*N*-Cbz Asp) anhydrides should result in surface charge-reversal, as each surface amino group would be converted to a carboxylate group. The acylation with trimellitic anhydride should result in surface charge reversal and expansion, a consequence of converting each cationic charge in the original AuNPs into two anionic charges.

3.1. Full surface modification

In order to find the most appropriate conditions for amine acylation reactions on AuNPs we started by investigating, as a model reaction, the acylation of the surface amino groups in AuNPs stabilized with HS-PEG2K-NH_2 ligands (Au_4@PEG2KNH_2) with succinic anhydride. The efficiency of the acylation reactions was assayed using an electrophoretic mobility shift assay: The acylation reactions should not significantly alter the size of AuNPs, therefore the electrophoretic mobility will be governed by surface charge. The reaction with succinic anhydride will convert the cationic amino groups in the original AuNPs into anionic groups and, therefore, the extent of the reactions should be easily determined by measuring the shifts in electrophoretic mobility of the reaction products. A quantitative acylation of the surface amino groups with succinic anhydride will result in surface charge reversal and, consequently, the electrophoretic mobility should be inverted. Incomplete surface acylation should result in AuNPs showing intermediate electrophoretic mobilities, proportional to the level of surface modification.

In our first experiments we investigated the influence of the pH on the outcome of the reaction (Fig. 2A). We used gold nanoparticles with an average diameter of 4.3 ± 0.7 nm (Au4@PEG2KNH_2), prepared by ligand exchange from citrate-stabilized AuNPs.[25] Acylation reactions were performed at pH values between 5.0 and 10.8, using four different buffers (MES, EPPS, borate, and carbonate, concentration 70 mM) to cover this pH range. The concentrations of anhydride and AuNPs were kept constant. The outcome of the reactions was analyzed by 1% agarose gel at pH 7.0. The acylation reactions do not significantly alter the size of the original AuNPs (*vide infra*), therefore the observed variations in electrophoretic mobility can be attributed to changes in surface charge. It was found that reactions performed at alkaline pH values (EPPS pH 9.0, borate pH 9–10, and carbonate pH 9.2) gave the highest mobility shifts. These reaction conditions gave AuNPs with inverted mobility, compared to the original cationic AuNPs, indicating that the nanoparticle surface had undergone charge-reversal. The use of MES buffer (pH 5.0 – 7.0) gave bands with lower mobility shifts, denoting a lower yield of surface acylation, an effect that was also observed using carbonate buffer at pH values higher than 10. The results of this experiment indicate that not only the pH, but also the nature of the buffer, influences the outcome of the reaction. For example, MES buffer at pH 7.0 (lane 4) results in a higher yield of acylation than EPPS buffer at the same pH (lane 5). Also, borate buffer at pH 10 (lane 10) gave a high yield of acylation, while carbonate buffer at the same pH gave a much lower yield and with a significant broadening of the band, suggesting a high polydispersity of surface charge. It should be noted that the reactions performed in MES or EPPS buffer that resulted in partial acylation of the AuNPs surface (Fig. 2A, lanes 2 to 5) do not show the band broadening observed with carbonate buffer (Fig. 2A, lanes 12 and 13). This suggests that the acylation under those conditions can be used to obtain AuNPs with mixed surface charges, a potential strategy that we also researched and will be discussed later.

We next studied the influence of the concentration of anhydride, using EPPS buffer at pH 9.0 (Fig. 2B). The maximum shift in electrophoretic mobility was observed with concentrations of anhydride between 7.5 and 20 mM. Higher concentrations resulted in bands with reduced mobility, probably because the acid generated by hydrolysis of the anhydride surpasses the buffer capacity, acidifying the media and reducing the efficacy of the acylation reaction. The experiments above indicate that the best conditions to achieve the maximum yield of acylation require the use of EPPS pH 9.0, borate pH 9–10, or carbonate pH 9.2 as buffer and a concentration of anhydride in the range 7.5–20 mM.

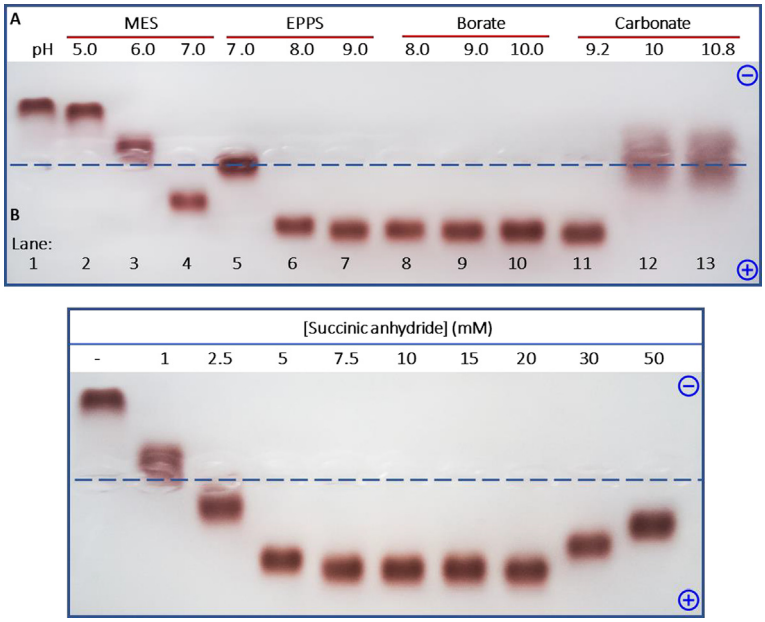


Fig. 2. Agarose gel electrophoresis of Au_4@PEG2KNH_2 after reactions with succinic anhydride. (A) Reactions at different pH values (final concentrations were 70 mM buffer, 14 mM anhydride). (B) Reactions at different concentrations of succinic anhydride (all reactions used 70 mM EPPS buffer, pH 9.0). Dashed lines represent the position of the wells.

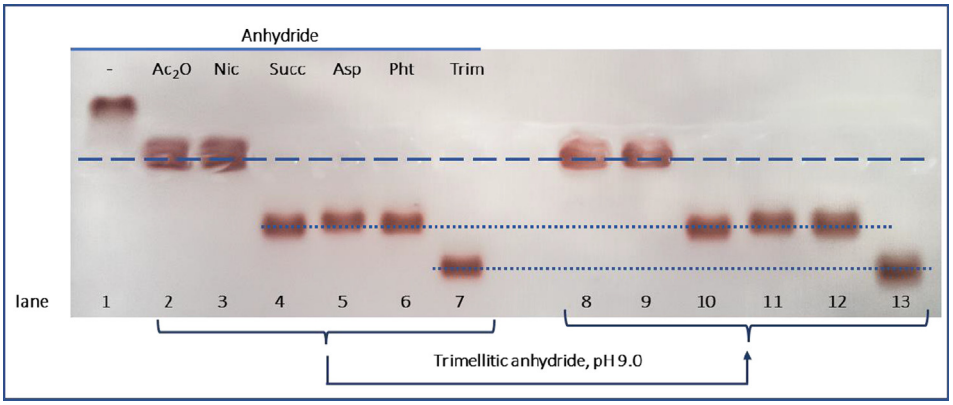


Fig. 3. Gel electrophoresis of Au4@PEG2KNH$_2$ (lane 1) and its reactions with six anhydrides in EPPS buffer pH 9.0 (lanes 2–7). The abbreviations Nic, Succ, Asp, Pht and Trim represent nicotinic, phthalic, *N*-Cbz-aspartic, and trimellitic anhydride respectively. Reaction of samples from lanes 2–7 with trimellitic anhydride (lanes 8–13). Dashed lines represent the position of the wells. Dotted lines are included as a reference to facilitate mobility comparisons.

To analyze if the reaction conditions developed for succinic anhydride can be generalized to other anhydrides, we studied the acylation reaction with five other anhydrides: acetic, nicotinic, phthalic, *N*-Cbz-aspartic, and trimellitic. The reactions were performed in 70 mM EPPS buffer (pH 9.0) with a final concentration of 14 mM anhydride. The results are shown in Fig. 3 (lanes 2 to 7). The reaction with acetic anhydride (lane 2) gave AuNPs with little electrophoretic mobility, indicating that the vast majority of the amino groups in the original AuNPs have been converted to neutral acetamide derivatives. The same result was observed, as expected, with nicotinic anhydride (lane 3). In this case the basicity of resulting nicotinamide groups is very low (the pK_a of nicotinamide is 3.35), therefore the number of protonated residues in the surface of nicotinamide modified AuNPs will be minimal at the neutral pH used in the electrophoretic analysis, resulting in a band with minimal mobility. Phthalic and Cbz-aspartic anhydrides (lanes 5 and 6) gave bands with a similar mobility to succinic anhydride (lane 5), which is to be expected as in all three cases each amino group in the original AuNPs is converted to one carboxylate group. For trimellitic anhydride (lane 7) the electrophoretic mobility is roughly twice that of succinic anhydride, supporting the conversion of each amino group in the original AuNPs to a dicarboxylate group. An additional experiment was performed with the products of these acylation reactions to determine whether all the reactive amine groups were acylated. AuNPs modified by reaction with the six different anhydrides were purified and reacted further with trimellitic anhydride at pH 9.0. If reactive amino groups had been present after the first acylation, a significant mobility shift should be observed after their reaction with trimellitic anhydride. The outcome of the reactions was analyzed by electrophoresis (Fig. 3, lanes 8–13). The electrophoretic mobility was not altered after the reactions with trimellitic anhydride, indicating that all the reactive amino groups had been acylated in the first reaction.

We also studied if the acylation can be applied to AuNPs of different sizes. We used AuNPs with diameters of 2.4 ± 0.4 nm (Au_2@-PEG2KNH$_2$), prepared by borohydride reduction of Au^{3+} in the presence of excess ligand,[12] AuNPs with 5.4 ± 1.4 nm diameter (Au_5@PEG2KNH$_2$) prepared by borohydride reduction of Au^{3+} in the presence of citrate followed by ligand exchange,[26] and AuNPs with 12.6 ± 1.8 nm diameter (Au_{13}@PEG2KNH$_2$) prepared by ligand exchange of citrate stabilized AuNPs synthesized by an optimized version of the Turkevich method.[27] Acylation reactions with acetic, succinic and trimellitic anhydrides were performed with the three sizes of AuNPs and analyzed by gel electrophoresis (Fig. 4). The reactions with acetic anhydride resulted in AuNPs with almost negligible mobility, indicating that their surface charge had been neutralized. Surface charge reversal was observed in acylation reactions with succinic and trimellitic anhydride. The reaction with trimellitic anhydride gave AuNPs with higher mobility than those reacted with succinic, as expected for charge reversal and expansion. It should be noted that Au_{13}@-PEG2KNH$_2$ shows an aberrant electrophoretic mobility, resulting in bands that do not move (or sometimes smear) on the gel. This phenomenon has been consistently observed for AuNPs>10 nm stabilized with HS-PEG-NH$_2$ ligands. When the surface of these cationic AuNPs was converted to anionic, their electrophoretic mobility regained the expected behavior.

One of the advantages of this methodology is that acylation reactions are not expected to alter the core size of the AuNPs. This was confirmed by UV–visible spectroscopy and transmission electron microscopy (TEM) measurements. Fig. 5 shows that the UV–Vis spectra of Au4@PEG2KNH$_2$ and the products of their acylation with the six different anhydrides are superimposable, with an unaltered wavelength for the SPR band, evidencing that the core size was preserved after the acylation. The same effect was observed for AuNPs of other sizes (Fig. S2). Preservation of the core size of the AuNPs after acylation was also confirmed by TEM, with a measured variation in diameter of less than 5% after acylation (Fig. S3).

The effect of acylation on the hydrodynamic size was determined by dynamic light scattering (DLS). The results (Table 2) show a moderate increase in size for the hydrodynamic diameters (0.7–3 nm), as expected from the addition of the intrinsic size of two acyl substituents (0.5 – 1.6 nm, depending on the anhydride) to the hydrodynamic size of the original AuNPs.

To further confirm the changes in surface charge after acylation reactions, we measured the Z potential of colloidal dispersions in diluted phosphate buffer at pH 7.0 for AuNPs of three different sizes acylated with either Ac_2O, succinic anhydride or trimellitic anhydride using electrophoretic light scattering. As shown above, the acylation reactions do not significantly alter the size of AuNPs, therefore the changes in Z-potential will be mostly a consequence of changes in surface charge. Also, the Z-potential values measured by this technique should correlate with the mobilities observed in gel electrophoresis, as the Z potential values are not measured directly, but they are deduced from the electrophoretic mobility of AuNP dispersions.[28] The correlation between electrophoretic mobility measured using these two methods was confirmed for the three sets of AuNPs (Table 1). The original AuNP@PEG2KNH$_2$ gave positive Z-potential values, acetylated AuNPs gave small neg-

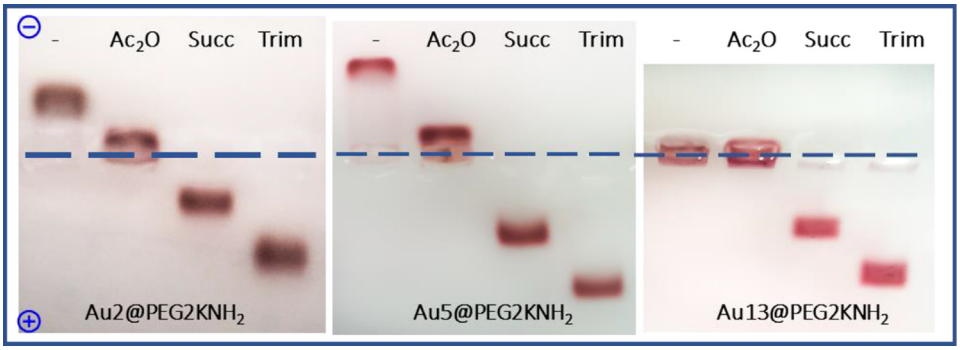


Fig. 4. Reaction of AuNPs stabilized with HS-PEG2K-NH_2 with different core diameters with acetic, succinic or trimellitic anhydrides in EPPS buffer at pH 9.0. Dashed lines represent the position of the wells.

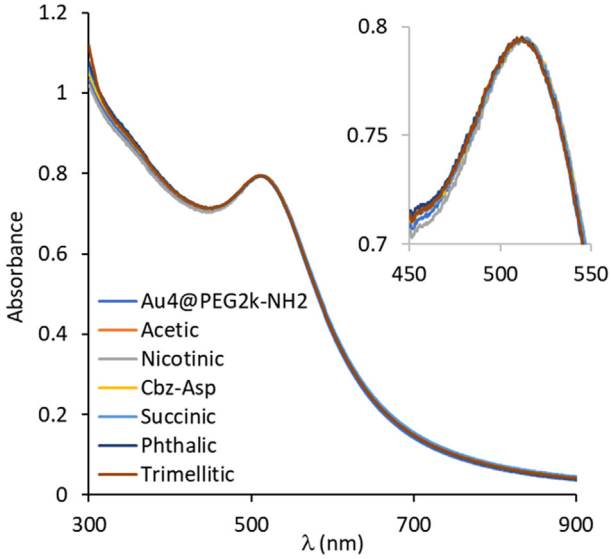


Fig. 5. UV–Vis spectra of Au4@PEG2KNH_2 and the products of their reaction with six different anhydrides (as indicated in chart).

Table 1
Z-potential measurements of Au@PEG2KNH_2 NPs and acylated derivatives measured in 5 mM Phosphate Buffer (pH = 7.0). Standard deviations were determined from at least ten measurements.

d (nm)	4.3 ± 0.7	5.4 ± 1.4	12.6 ± 1.8
Anydride	Z-potential (mV)		
–	+10.1 ± 0.7	+11.4 ± 0.9	+20.4 ± 1.8
Acetic	+1.8 ± 0.3	−4.3 ± 0.6	−5.3 ± 0.9
Succinic	−4.4 ± 0.5	−12.8 ± 1.6	−20.5 ± 1.6
Trimellitic	−6.8 ± 0.7	−18.8 ± 1.9	−29.4 ± 1.8

Table 2
Hydrodynamic diameters (number mean) of AuNPs stabilized with HS-PEG2K-NH_2 of three different core sizes and the products from their reaction with acetic, succinic or trimellitic anhydrides. Standard deviations were determined from at least five measurements.

Core d (nm)	4.3 ± 0.7	5.4 ± 1.4	12.6 ± 1.8
Anhydride	Hydrodynamic diameter (nm)		
–	11.8 ± 0.5	12.7 ± 0.6	20.1 ± 1.9
Acetic	13.9 ± 0.7	15.5 ± 0.8	20.8 ± 0.8
Succinic	13.2 ± 1.2	14.1 ± 0.7	21.6 ± 1.3
Trimellitic	14.3 ± 0.4	15.7 ± 1.2	22.4 ± 1.0

ative values, AuNPs acylated with succinic anhydride gave negative values and AuNPs modified by trimellitic anhydride showed Z potential values of higher magnitude than those modified by succinic anhydride.

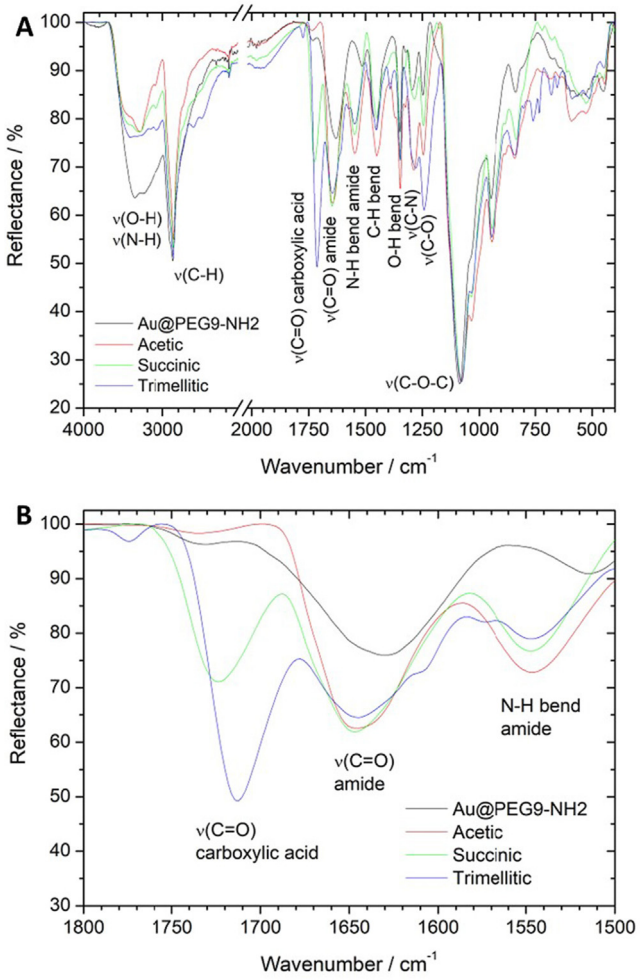


Fig. 6. (A) FTIR-ATR spectra of Au2@PEG9NH_2 and the products of its reaction with acetic, succinic or trimellitic anhydrides. (B) Expanded area of the FTIR-ATR spectra showing the key bands. Reflectance was normalized at the C—O—C stretch absorption band.

The introduction of the acyl substituents on the surface of AuNPs was also confirmed by infrared spectroscopy (IR) and nuclear magnetic resonance (NMR) experiments. AuNPs stabilized with a shorter ligand (HS-PEG_9-NH_2) with a diameter of 2.4 nm (Fig. S1E) were used in these experiments to facilitate interpretation of the spectra. The IR spectra of the original Au2@PEG_9NH_2 nanoparticles and the products of their reaction with acetic, suc-

cinic or trimellitic anhydride are shown in Fig. 6. The acylated AuNPs show typical characteristic vibrations for the amide functional group at 1645 cm^{-1} (C═O stretching band) and 1548 cm^{-1} (N—H bending for secondary amide). In the case of the two cyclic anhydrides, additional bands corresponding to the carbonyl stretching band of carboxylic acids appear at 1728 cm^{-1} (for succinic anhydride) and 1715 cm^{-1} (for trimellitic anhydride), the latter more intense as expected for the introduction of two carboxylate groups per ligand.

For ^{1}H NMR experiments we used nanoparticles modified with acetic anhydride or *N*-Cbz aspartic anhydride, as they contain methyl and benzoyl groups whose chemical shifts are distant from the PEG peaks, facilitating the interpretation of the spectra. The NMR data confirms the introduction of acetyl and asparagine groups in the surface of AuNPs (Fig. S4). The relative integration of the PEG peak and the methyl or phenyl peaks indicate that almost all the ligands are modified in both cases. This observation is in contrast with previously reported data, where it was determined that the number of reactive amines in AuNPs stabilized with PEG-amino ligands was less than 60% of the total amino groups. [23] There are three significant differences between the two experiments that help explain this discrepancy. First, in our experiment we used a short PEG (9 units, about 0.5 K), while the in the former experiments large PEGs (>3K) were used. Second, the amine-modifying reagent in our case are small, highly reactive anhydrides, while the previous work used larger and less reactive molecules. Third, our experiment was performed with AuNPs with a diameter of 2.4 nm, while the former employed AuNPs with diameters in the 30–60 nm range.

3.2. Synthesis of mixed-ligand AuNPs

The acylation reactions with succinic anhydride in MES buffer at neutral or acidic pH values shown in Fig. 2A (lanes 2–5) indicate that it is possible to control the extent of surface acylation, obtaining AuNPs with mixed surfaces. We performed a detailed study to test whether the pH-dependence of the acylation reaction can be used to synthesize AuNPs with tailored mixed surfaces. The results of a series of representative experiments for acylation reactions at different pH values with six anhydrides are shown in Fig. 7. We found that, indeed, using pH-controlled reactions, AuNPs with gradual shifts in gel mobility can be obtained with all the anhydrides. Acylation reactions with anhydrides that introduce a neutral group gave AuNPs with mobilities corresponding to mixed surfaces containing positively charged/neutral ligands (Fig. 7D, 7E). Cyclic anhydrides gave mobilities correlating with AuNPs containing mixed cationic/anionic ligands, with a global surface charge ranging from positive to negative, depending on the degree of acylation (Fig. 7A, 7B, 7C). Reactions with trimellitic anhydride gave AuNPs with surface charge ranging from positive to negative, with a wider range of negatively charged surfaces (Fig. 4D). The precise pH values to achieve a given degree of acylation vary slightly for each anhydride but, in general, good results can be obtained using MES buffer between pH 5 and 7 using 0.2 units increments. The specific pH used for the experiments shown in Fig. 7 are given in the supporting information (Fig. S5 to S10). It should be noted that AuNPs with mixed surfaces prepared by this method do not show a significant broadening of the bands in the gel, indicating that partial acylation reactions do not generate significant charge polydispersity.

Furthermore, the electrophoretic mobility of the modified AuNPs allows to estimate their level of surface modification using as calibration points the original AuNPs (0% substitution) and fully reacted AuNPs (100% substitution), assuming a linear relationship between electrophoretic mobility and surface charge. This is a fair assumption since electrophoretic mobility is proportional to the charge-to-mass ratio. As mentioned above, the acylation reactions do not alter the Au core size, therefore the mass of the AuNPs will be minimally changed after the acylation reactions and the observed shifts in electrophoretic mobility should be proportional to the changes in surface charge. As an example, the calculated extent of surface modification for the reactions with succinic anhydride and trimellitic anhydride shown in Fig. 7A and 7F are provided in the supporting information (Tables S1, S2).

The pH-dependent partial acylation reactions were also performed using AuNPs with other core sizes, and they showed a similar trend in their mobility shifts, independently of size (Fig. 8). As noted above, cationic AuNPs with a diameter of 13 nm showed an aberrant mobility, thus impeding the estimation of the level of surface modification. As we will discuss below, this issue can be

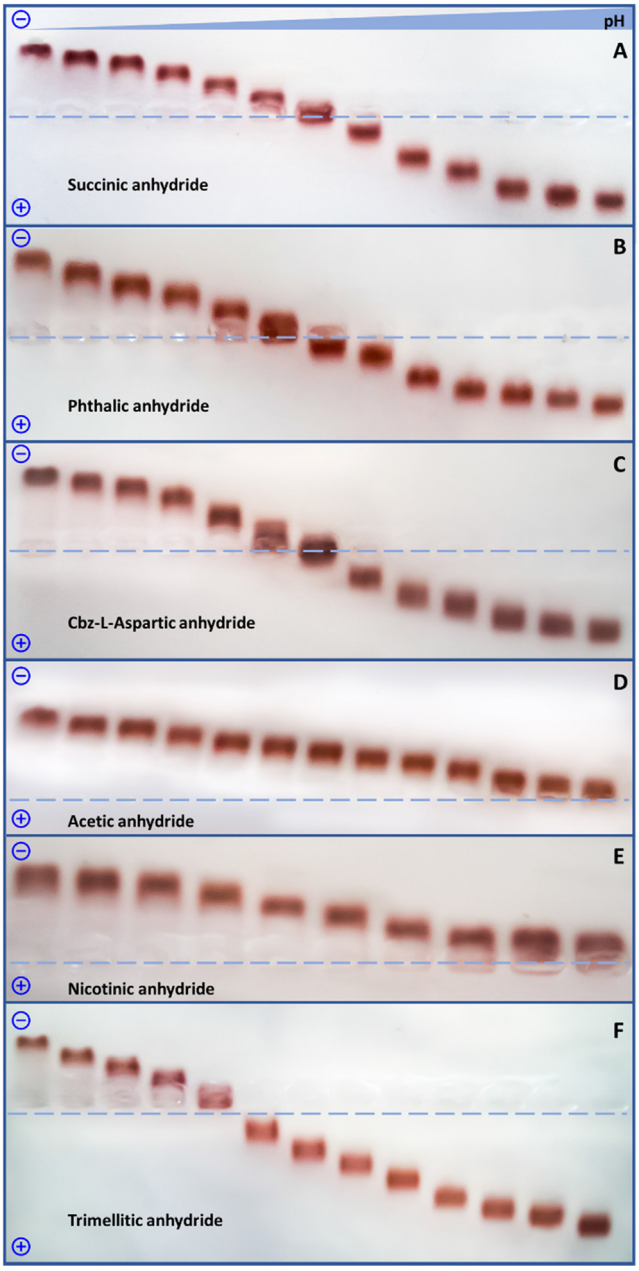


Fig. 7. Gel electrophoresis of pH-dependent reactions of Au4@PEG2KNH_2 with six anhydrides (representative experiments). Dotted lines represent the position of the wells. The specific pH values for the buffers used in the reactions are reported in the Supporting Information (Figs. S6 to S10).

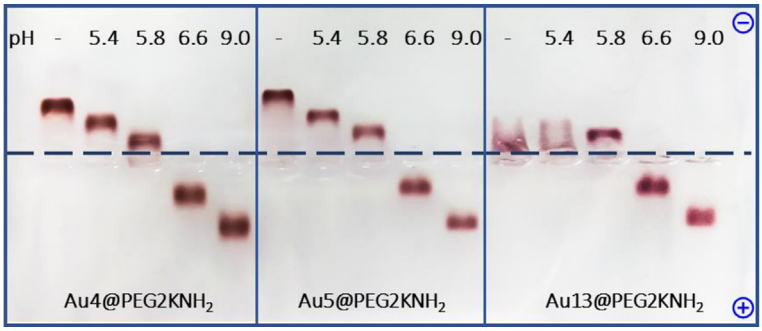


Fig. 8. Gel electrophoresis of pH-dependent reactions of succinic anhydride with Au@PEG2KNH$_2$ of three different core sizes. Dashed lines represent the position of the wells.

solved by performing a second acylation reaction that converts the unreacted amino groups to neutral groups.

Small variations in individual reaction yields have been obtained from one experiment to another in the course of this work. However, the pH-dependent gradual shifts in electrophoretic mobility have been repeatedly observed whenever a series of parallel acylation reactions were performed within the proper pH range.

The experiments discussed above demonstrate that it is possible to prepare AuNPs with tailored mixed surfaces, one of them being an anionic, neutral of weakly basic group, while the other is, in all cases, a cationic amino group. To complete this approach, we also studied the use of acylation reactions to synthesize AuNPs with mixed neutral/anionic surfaces. Two strategies were considered to achieve this aim: (1) Performing the acylation in a single step using a mixture of two anhydrides, and (2) using two sequential acylation reactions, first a partial acylation with one anhydride followed by the total acylation of the unreacted amino groups with a second anhydride. Both approaches proved successful (Fig. 9). In the one-step acylation using mixtures of acetic/succinic anhydrides, AuNPs with a gradual increase of surface charge from neutral to fully negative were obtained (Fig. 9A). It must be noted that the ratio of anhydrides does not correlate with the ratio of modified ligands, as the relative reactivity of the anhydride must be factored in. In this particular case, AuNPs containing 50% of each substituent are obtained using a mixture containing 30% acetic anhydride and 70% succinic anhydride, a consequence of the higher reactivity of acetic anhydride. The second strategy, using two sequential acylation reactions to prepare AuNPs with neutral/anionic mixed surface, is shown in Fig. 9B. First, three partial acylation reactions with acetic anhydride were performed to give AuNPs that gave the expected gradual decrease in electrophoretic mobility. Then, the purified AuNPs were treated with succinic anhydride at pH 9.0 to acylate the unreacted amino groups, giving AuNPs that show the expected gradual decrease in electrophoretic mobility, corresponding to AuNPs with surface charge ranging from fully negative to neutral. The UV–vis spectrum of the original and modified AuNPs from the experiments described above were superimposable, indicating that the core size was not affected during acylation (data not shown), therefore the observed shifts in electrophoretic mobility can be attributed to a change in net surface charge after acylation reactions.

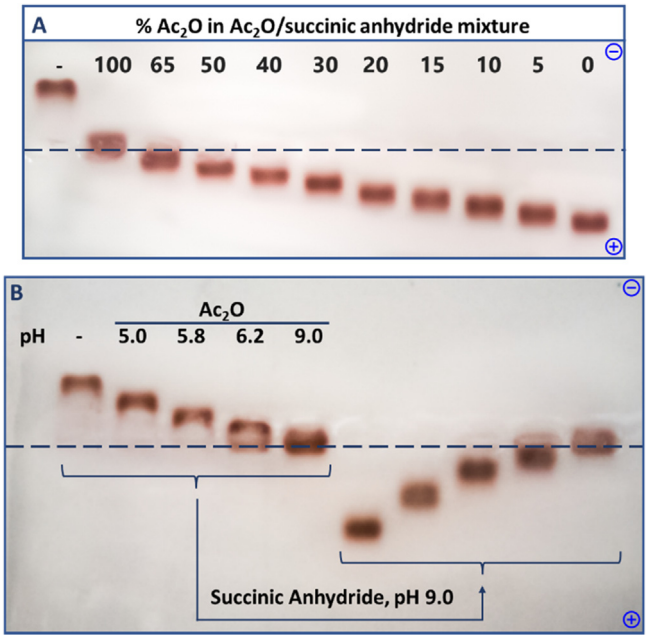


Fig. 9. Synthesis of AuNPs with mixed neutral/anionic surfaces. (A) By a one step reaction with mixtures of acetic and succinic anhydrides (proportions as indicated) at pH 9. The total concentration of anhydrides was 14 mM in all cases. (B) Using two sequential reactions: first, a pH-controlled partial acetylation with acetic anhydride (pH as indicated), followed by a second reaction with succinic anhydride at pH 9.0. Dashed lines represent the position of the wells.

The possibility of performing a second acylation reaction on AuNPs with partially modified surface also serves as a tool to determine the extent of acylation in AuNPs from reactions using large nanoparticles (13 nm diameter), a data that was not previously accessible due to the aberrant electrophoretic mobility of these large AuNPs in their cationic form. This issue can be solved by converting the unreacted amino groups to neutral acetamide groups in a second acylation reaction (Fig. 10). The first five lanes show the electrophoretic mobilities of the original AuNPs and four acylation reactions with succinic anhydride at different pH values. Both the original AuNPs (lane 1) and the reaction with succinic at pH 5.4 (lane 2) show abnormal mobility. Lanes 6 to 10 show these same five samples after acetylation reactions at pH 9.0. Upon acetylation, the surface of Au13@PEG2KNH$_2$ is converted to neutral, while AuNPs with mixed surface show mobilities corresponding to their mixed neutral/anionic surfaces. The extent of surface modification in the first reaction with succinic anhydride can now be estimated, using lanes 6 and 10 as 0% and 100% calibration points respectively. The yields for the first partial acylation determined using this method are analogous to the yields for acylation reactions performed at the same pH values using AuNPs of other sizes (Fig. S11), indicating that the level of surface modification obtained at a given pH value does not depend on core size.

3.3. AuNPs with three or more mixed ligands

The experiments shown in Figs. 8 and 9 demonstrate that two different functional groups can be introduced using sequential acy-

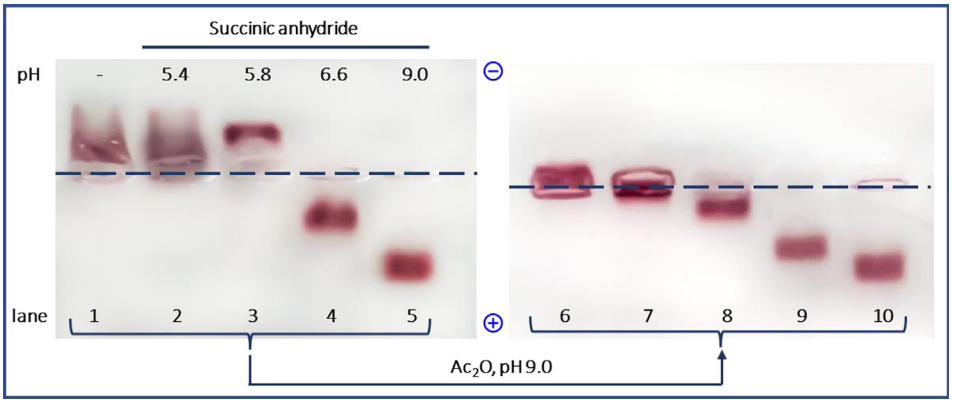


Fig. 10. Gel electrophoresis analysis of pH dependent partial acylation reactions of Au13@PEG2KNH$_2$ with succinic anhydride (pH as indicated in chart), and their subsequent acylation with acetic anhydride at pH 9.0. Dashed lines represent the position of the wells.

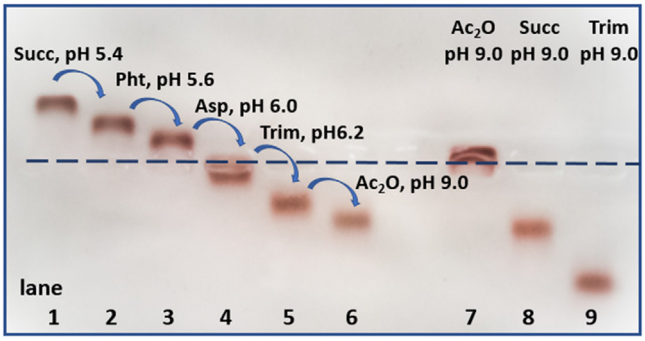


Fig. 11. Gel electrophoresis analysis of Au4@PEG2KNH$_2$ (lane 1) and their sequential acylation reactions with succinic (lane 2), phthalic (lane 3), Cbz-aspartic (lane 4), trimellitic (lane 5) and acetic (lane 6) anhydrides at the indicated pH values. Control reactions of Au4@PEG2KNH$_2$ with acetic (lane 7), succinic (lane 8) and trimellitic (lane 9) anhydrides at pH 9.0 are included for reference. Dashed lines represent the position of the wells.

lation reactions of Au@PEGNH$_2$, resulting in a series of AuNPs containing mixed neutral/anionic ligands with control over the ligand ratio. As a final experiment we studied whether the use of a larger number of sequential acylation reactions can be used to obtain AuNPs containing more than two different surface functional groups. To this end, a synthetic sequence of five steps was performed using Au4@PEG2KNH$_2$ as starting material (Fig. 11). First, the nanoparticles were partially acylated with succinic anhydride (lane 2), and then in three successive reactions they were partially acylated with phthalic (lane 3), Cbz-aspartic (lane 4), and trimellitic (lane 5) anhydrides. Finally, the remaining amino groups were fully acylated with acetic anhydride (lane 6) at pH 9.0. The mobility shifts observed in the gel electrophoresis analysis demonstrate that it is possible to introduce five different substituents in the surface of AuNPs with control over the extent at which each of the functional groups is present. The mobility shifts can be used to calculate the extent of surface modification with each of the substituents(Fig. S12).

4. Conclusions

We studied the use of organic chemistry for the introduction of surface diversity in PEGylated AuNPs, thus avoiding the use of ligand-exchange reactions. We showed that AuNPs stabilized with thiol-PEG-amino ligands are a versatile substrate for the introduction of chemical diversity using acylation reactions with anhydrides in aqueous media. We have exemplified this methodology by synthesizing AuNPs with six different surface functional groups, including neutral (acetamido groups), weak base (nicotinamide groups), aliphatic carboxylic acids (succinamic acid or asparagine groups), aromatic carboxylic acids (phthalamic groups) and dicarboxylic acids (carbamoylterephthalic/carbamoylisophthalic groups). The reactions are performed directly on aqueous dispersions of PEGylated AuNPs, and the modified nanoparticles can be easily purified by centrifugal filtration or gel filtration chromatography. The methodology is budget-friendly, as a large variety of AuNPs with different surface chemistry can be prepared using a single HS-PEG-NH$_2$ ligand. An additional advantage of this method is that it amplifies the scope of chemical diversity available, as ligand-exchange approaches are limited by the availability of ligands containing the desired functional groups.[10] Now, the available chemical diversity is, in principle, only limited by the commercial or synthetic availability of anhydrides providing the desired functional groups. The reactions that result in full surface modification of AuNPs require the use of an alkaline buffer, such as EPPS pH 9.0. Reactions performed at lower pH values give partial surface modification, allowing the synthesis of AuNPs with cationic/anionic or cationic/neutral mixed surfaces. This pH-dependence reactivity allows to control the ligand ratio in the mixed surfaces. AuNPs with mixed neutral/anionic surfaces can be synthesized by two methods: using a mixture of anhydrides or by using two sequential acylation reactions. These methods provide an attractive alternative to the use of place-exchange reactions using mixtures of ligands[20] for the fabrication of AuNPs with mixed surfaces. Finally, an example is given where up to five different ligands are introduced in the surface of AuNPs using a synthetic sequence of five acylation reactions. As surface chemistry is one of the key properties that determine the biological activity of AuNPs, the large diversity of surface functionality now accessible should allow more in-depth research on how surface chemistry influences the biological properties of gold nanoparticles.

CRediT authorship contribution statement

Manuel M. Paz: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Alberto Peinador Veiga:** Validation, Formal analysis, Investigation. **Tamara Regueira:** Formal analysis, Investigation. **Carlos Vázquez Vázquez:** Investigation, Resources, Writing – original draft, Writing – review & editing, Funding acquisition. **M. Arturo López Quintela:** Resources, Writing – original draft, Writing – review & editing, Funding acquisition.

Data availability

Data will be made available on request.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Carlos Vázquez Vázquez reports financial support was provided by Ministry of Science Technology and Innovations. Arturo López Quintela reports financial support was provided by Government of Galicia Department of Culture Education and Universities. Manuel M. Paz reports financial support was provided by Government of Galicia Department of Culture Education and Universities.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jcis.2023.03.043.

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