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Electrospray Mass Spectrometry Study of Tiopronin Monolayer-Protected Gold Nanoclusters

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Abstract: Electrospray ionization time-of-flight mass spectrometry (ESI-TOF MS) and gel permeation chromatography (GPC) were used to study the synthesis of a series of tiopronin monolayer-protected gold nanoclusters (MPCs) and to monitor their postsynthesis peptide ligand place-exchange reactions. All mass spectra identified the presence of cyclic gold(I)−thiolates with a strong preference for tetrameric species. During the synthesis of pre-monolayer-protected nanoclusters (pre-MPCs), esterified gold(I)−thiolate tetramers were initially observed in minor abundance (with respect to disulfide bridged tiopronin species) before dramatically increasing in abundance and precipitating from solution. After conversion of pre-MPCs to MPCs, ESI-TOF mass spectra demonstrated an overall predominance of tetrameric species with conversion from ester-terminated end groups to carboxyl-terminated end groups. Further modifications were performed through postsynthesis ligand place-exchange reactions to validate the existence of the tetramers. This work suggests that monolayer protection is accomplished by cyclized gold(I)−thiolate tetramers on the gold core surface, and/or that gold(I)−thiolates are a basic building block within the nanoparticles.

Introduction

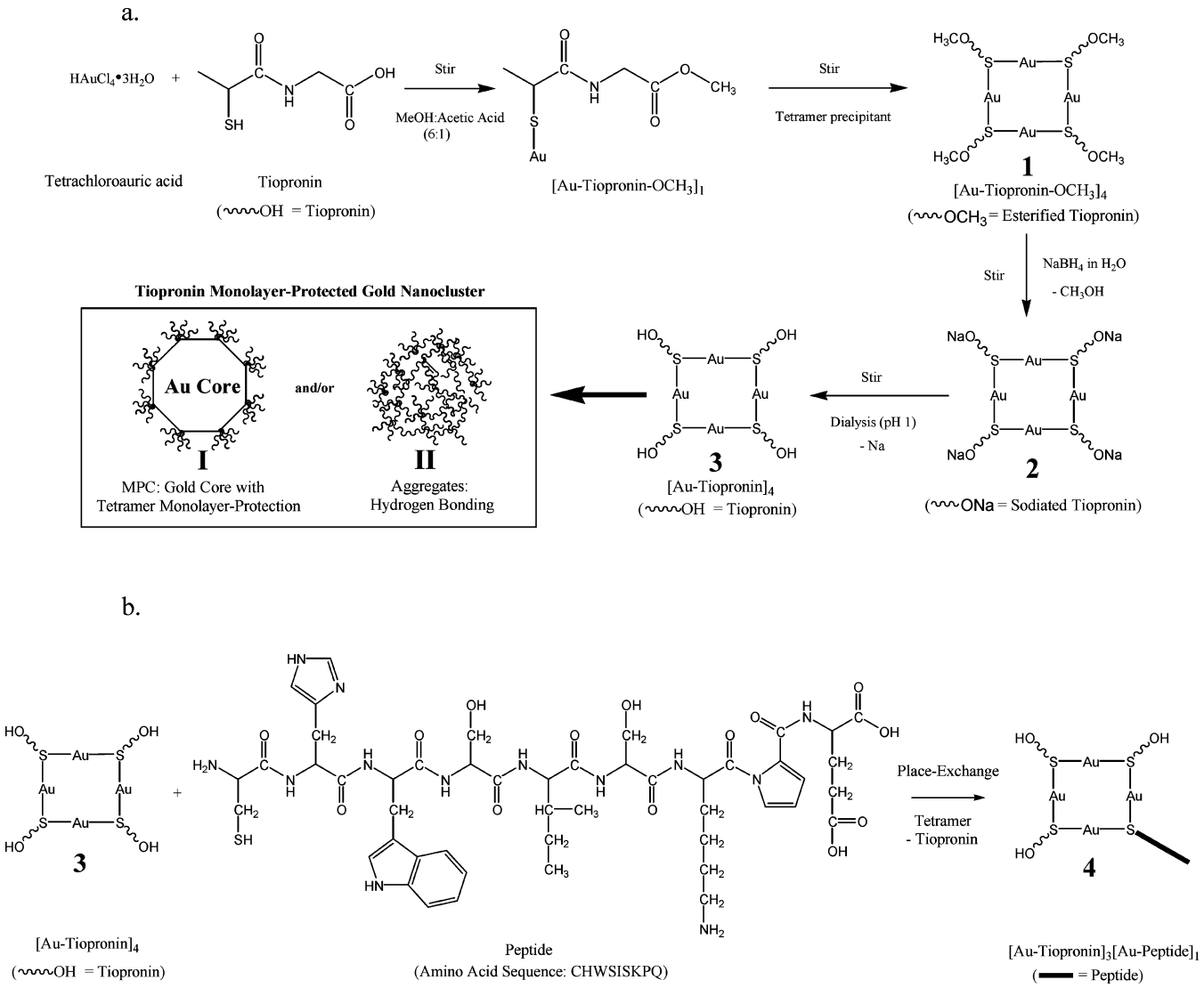
Monolayer-protected nanoclusters (MPCs) are nanocluster materials that show enormous potential for applications as redox-active storage devices, solution-based sensors, and highly efficient catalysts.[1] The ability to readily introduce molecular functionality onto well-defined nanometer-size metal cores makes MPCs great candidates for complex functional devices.[1] Gold MPCs combine a metallic (gold) core with a self-assembled organic shell (e.g., amino acid or peptide) to prevent agglomeration of the gold cores, and through postsynthesis (ligand place-exchange) modifications they can serve as platforms for the attachment of additional functional groups.[1] Moreover, monolayer-protection imparts to gold nanoparticles a variety of new properties (unavailable to their bulk metal counterparts) ranging from solubility in organic or aqueous solvents, to the introduction of redox active or specific molecular recognition elements.[1,2]

For MPC applications in which one wants to carefully control nanoparticle properties, quantification of ligand attachment, average particle size, and determination of the actual composition of a MPC sample are of the utmost importance.[1] The greatest obstacles to MPC characterization are pre-analysis mixture separation[3−5] (for conventional spectroscopy techniques) and rapid ligand exchange (at room temperature) that occurs with mixed-ligand $Au^{(I)}$ complexes in solution.[6] Unfortunately, due to their limitations as averaging techniques, conventional solution-phase methods of analysis (i.e., NMR, IR, UV−visible spectroscopy) have proven to be inadequate for providing detailed information about the mass distribution, chemical distribution, and structural heterogeneities present in MPCs.[3]

However, mass spectrometry is a non-averaging characterization technique that has the potential to yield a wealth of information about the molecular mass, structure/stoichiometry, and chemical modifications of species generated in a reaction, without prior separation of the mixture.[7] Furthermore, ESI-MS is an extremely sensitive technique that can analyze aggregate-free dilute solutions (i.e., concentrations too low for NMR measurements)[7] to reveal individual components of a system in which ligand exchange is rapidly occurring. This is possible because ligand exchange stops immediately when solution ions enter the gas phase; thus each ion is "locked" into its conformation at the moment of transfer to the gas phase,[6] and unlikely to react further due to gas-phase dilution.[6] Surprisingly, few mass spectral applications to gold MPCs are found in the literature. Of those, all have been unable to yield concise information relating to MPC structure/stoichiometry or ligand attachment due to poor resolution and S/N ratios. However, there is enough mass spectral evidence to suggest MPC preference

(1) Drechsler, U.; Erdogan, B.; Rotello, V. M. *Chem.-Eur. J.* **2004**, *10*, 5570.
(2) Templeton, A. C.; Hostetler, M. J.; Warmoth, E. K.; Chen, S. W.; Hartshorn, C. M.; Krishnamurthy, V. M.; Forbes, D. E.; Murray, R. W. *J. Am. Chem. Soc.* **1998**, *120*, 4845.
(3) Choi, M. M. F.; Douglas, A. D.; Murray, R. W. *Anal. Chem.* **2006**, *78*, 2779.
(4) Schaaff, T. G. *Anal. Chem.* **2004**, *76*, 6187.
(5) Templeton, A. C.; Chen, S.; Gross, S. M.; Murray, R. W. *Langmuir* **1999**, *15*, 66.
(6) Gatlin, C. L.; Turecek, F. In *Electrospray Ionization Mass Spectrometry: Fundamentals, Instrumentation, and Applications*; Cole, R. B., Ed.; John Wiley & Sons, Inc.: New York, 1997; p 561.
(7) Andersen, U. N.; Seeber, G.; Fiedler, D.; Raymond, K. N.; Lin, D.; Harris, D. *J. Am. Soc. Mass Spectrom.* **2006**, *17*, 292.

Scheme 1. (a) Tiopronin Monolayer-Protected Gold Cluster Synthesis and (b) Peptide Ligand Place-Exchange



for "magic number" clustering.[8−10] The majority of these applications have focused on solid-to-gas-phase ionization techniques such as laser desorption ionization (LDI)[9−12] (a hard ionization technique) and matrix assisted laser desorption/ionization (MALDI)[4,13,14] (a softer ionization technique). However, very few have employed the softest (solution-to-gas-phase) ionization technique of electrospray ionization,[8,13−15] which would be expected to yield intact ions most representative of the actual mixture (especially when interfaced to a time-of-flight mass analyzer). To date, there has been no definitive evidence to prove the case for gold colloid monolayer protection through tiopronin molecules[5,9] versus surface association of gold(I)−thiolate "oligomers".[9,16] Considering the intense interest in these materials, we believe a thorough soft ionization mass spectrometry study has been long overdue to investigate the distribution of species present throughout MPC synthesis and postsynthesis ligand place-exchange reactions.

In this work, we present the first application of electrospray ionization time-of-flight mass spectrometry (ESI-TOF MS) to the study of MPC synthesis and postsynthesis modifications through the collection of mass spectra at different time/reaction intervals and analysis conditions. Our studies have discovered preferential formation of cyclic gold(I)−thiolate tetramers in tiopronin monolayer-protected gold nanoclusters and have identified the structural/mass distribution changes that occur in the postsynthesis ligand place-exchange process. Furthermore, this work suggests that mass spectrometry and gel permeation chromatography (GPC) have great potential as rapid characterization techniques for the direct analysis of nanoparticle synthesis, ligand modification, and side product formation.

Experimental Section

Tiopronin Monolayer-Protected Cluster Synthesis. Tiopronin monolayer-protected gold nanoclusters (MPCs) were synthesized using

(8) Negishi, Y.; Takasugi, Y.; Sato, S.; Yao, H.; Kimura, K.; Tsukuda, T. *J. Am. Chem. Soc.* **2004**, *126*, 6518.
(9) Templeton, A. C.; Wuelfing, W. P.; Murray, R. W. *Acc. Chem. Res.* **2000**, *33*, 27.
(10) Tsunoyama, H.; Negishi, Y.; Tsukuda, T. *J. Am. Chem. Soc.* **2006**, *128*, 6036.
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(12) Jimenez, V. L.; Georganopoulou, D. G.; White, R. J.; Harper, A. S.; Mills, A. J.; Lee, D.; Murray, R. W. *Langmuir* **2004**, *20*, 6864.
(13) Schaaff, T. G.; Knight, G.; Shafigullin, M. N.; Borkman, R. F.; Whetten, R. L. *J. Phys. Chem. B* **1998**, *102*, 10643.
(14) Schaaff, T. G.; Whetten, R. L. *J. Phys. Chem. B* **2000**, *104*, 2630.
(15) Negishi, Y.; Nobusada, K.; Tsukuda, T. *J. Am. Chem. Soc.* **2005**, *127*, 5261.
(16) Shaw, C. F.; Schaeffer, N. A.; Elder, R. C.; Eidsness, M. K.; Trooster, J. M.; Calis, G. H. M. *J. Am. Chem. Soc.* **1984**, *106*, 3511.

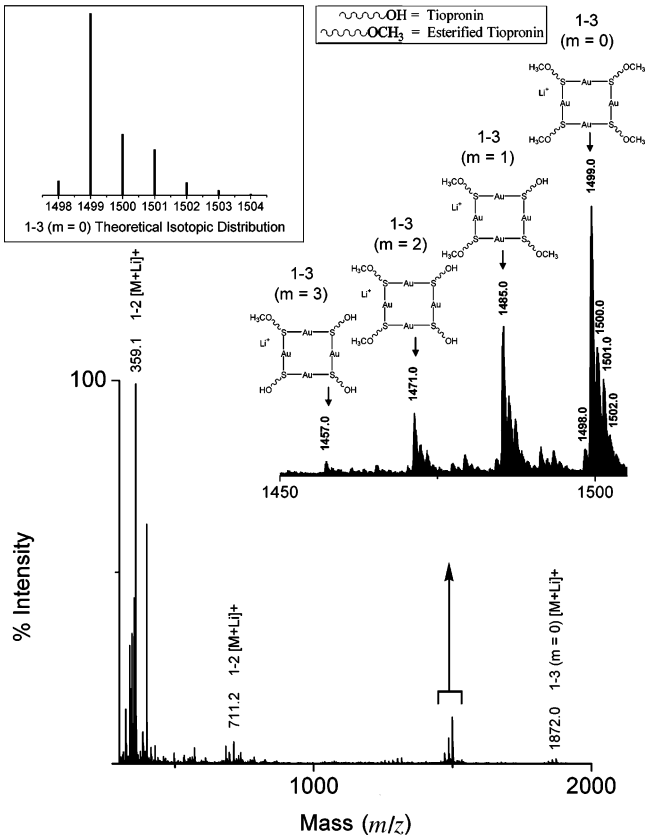


Figure 1. ESI-TOF mass spectrum (300−2000 Da) of tiopronin−gold solution, just as the solution became cloudy (pre-MPC: 2 h), doped with 50 μL of aqueous 0.1% LiBr.

a solution-based synthesis (Scheme 1a) previously reported.[5,17−19] Pre-MPC materials were synthesized as follows: Gold metal (99.99%) was initially converted to tetrachloroauric acid ($HAuCl_4 \cdot 3H_2O$) by boiling Au^0 in a 1:1 solution of HCl/HNO_3.[20] Tetrachloroauric acid ($HAuCl_4 \cdot 3H_2O$) (0.33 mmol) was added to a 0 °C solution of methanol/glacial acetic acid (6:1, v/v), which turned the solution from colorless to bright yellow (λ max = 320 nm). Upon addition of *N*-(2-mercaptopropionyl)-glycine (tiopronin) (Sigma, reagent grade) (1.1 mmol), the solution turned faint orange and faded to a colorless solution. The final conversion to MPC materials was conducted as follows: $NaBH_4$ dissolved in H_2O was added to the reaction mixture in a 10-fold molar excess ($NaBH_4$:gold) with rigorous stirring for 1 h. Solvent was then removed under vacuum at temperatures <40 °C, and the pH of the crude product was adjusted to ~1 by dropwise addition of concentrated HCl. Purification was achieved by dialysis with cellulose ester membranes (Spectra/Por CE, MWCO = 10 000 Da), which were stirred in 4 L of water for 3 days, changing the water twice per day. Initial UV−visible spectroscopy and transmission electron microscopy (TEM) characterization for all MPCs is provided in the Supporting Information. These characterization techniques suggest that the MPC materials possess, on average, diameters of 3.2 nm, molecular compositions of $Au_{617}Tiop_{237}$, and molecular weights of 160 kDa.

Peptide Monolayer-Protected Cluster Synthesis. Tiopronin monolayer-protected nanoclusters were peptide (amino acid sequence: CHWSISKPQ) functionalized via ligand place-exchange reactions (Scheme 1b) previously described.[21−23] Tiopronin-protected monolayer-protected gold nanoclusters (10.5 mg, batch 1 and 11.3 mg, batch 2) were dissolved in 5.0 mL of deionized H_2O to yield a dark solution. Peptide (amino acid sequence: CHWSISKPQ, 1.0 mg, batch 1 and 5.3 mg, batch 2) was added to the MPC solution and allowed to stir for 3 days at room temperature. Purification of the crude product was achieved by dialysis as described above for the MPC product except that the solution pH was not adjusted before dialysis.

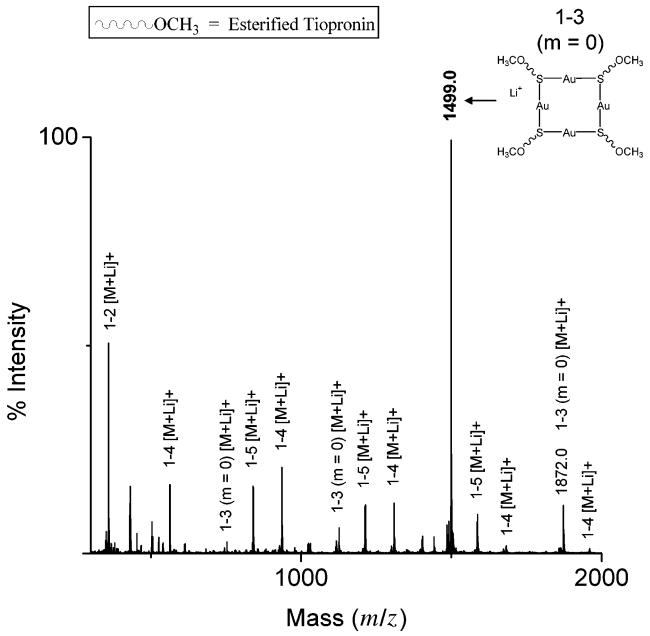
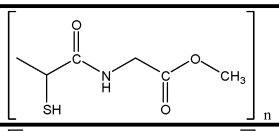
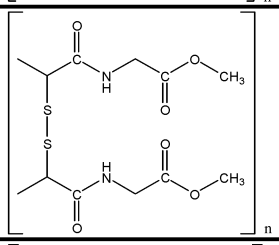
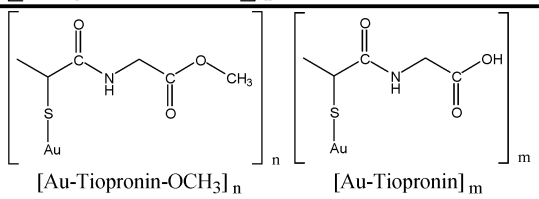
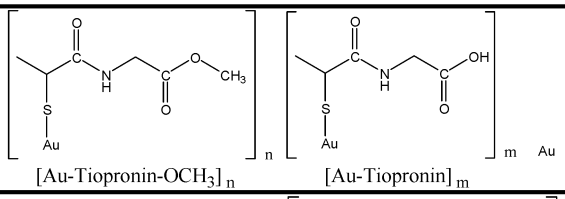
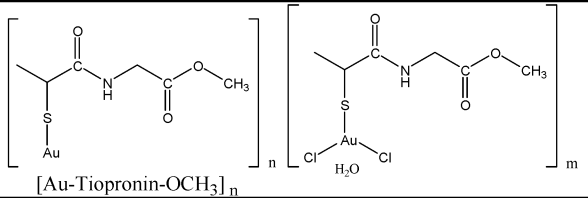


Figure 2. ESI-TOF mass spectrum (300−2000 Da) of gold(I)−thiolate precipitate (pre-MPC: 24 h) dissolved in DMF and doped with 50 μL of 0.1% LiBr in DMF.

ESI-TOF MS Measurements. All samples were analyzed using a Mariner ESI-TOF MS (Applied Biosystems, Framingham, MA). Mass spectra were obtained in the positive ion mode (and negative ion mode for the pre-MPC samples) with the spray tip, nozzle, skimmer, ion guide, and TOF mass analyzer potentials (along with nozzle temperature, 110 °C) optimized to achieve the best signal-to-noise ratio. A curtain of nitrogen drying gas was utilized to assist in the ESI process. All spectra were acquired in the reflectron mode of the TOF mass spectrometer (which has a mass range up to 10 000 Da) and had mass resolutions greater than 2500 fwhm; isotopic resolution was observed throughout the entire mass range detected. External mass calibration was performed using protein standards from a Sequazyme Peptide Mass Standard Kit (Applied Biosystems) and a three-point calibration method using Angiotension I (m = 1296.69 Da), ACTH (clip 1−17) (m = 2093.09 Da), and ACTH (clip 18−39) (m = 2465.20 Da). Internal mass calibration was subsequently performed using a PEG standard (Polymer Source, Inc.) to yield monoisotopic masses exhibiting a mass accuracy better than $\Delta m = \pm 0.1$ Da. The instrument was calibrated before every measurement to ensure constant experimental conditions. Pre-MPCs in methanol/acetic acid solution were analyzed neat or doped with 1% ammonium hydroxide (for the negative ion mode), and doped with 0.1% LiBr (for the positive ion mode). Pre-MPC precipitants had to be dissolved in *N*,*N*-dimethylformamide (DMF) (Sigma, 99.9+%, HPLC grade) (1 mg/mL) before they could be analyzed neat or doped with 1% ammonium hydroxide (for the negative ion mode), and doped with 0.1% LiBr (for the positive ion mode). MPC solutions were prepared in deionized H_2O (1 mg/mL) and dialyzed to remove excess salt. To aid in their ionization, 50 μL of H_2O:ACN (1:1 doped with 1% acetic

(17) Gerdon, A. E.; Wright, D. W.; Cliffel, D. E. *Anal. Chem.* **2005**, *77*, 304.
(18) Huang, T.; Murray, R. W. *J. Phys. Chem.* **2001**, *105*, 12498.
(19) Templeton, A. C.; Cliffel, D. E.; Murray, R. W. *J. Am. Chem. Soc.* **1999**, *120*, 4845.
(20) Brauer, G. *Handbook of Preparative Inorganic Chemistry*; AP: New York, 1965.
(21) Ingram, R. S.; Hostetler, M. J.; Murray, R. W. *J. Am. Chem. Soc.* **1997**, *119*, 9175.
(22) Hostetler, M. J.; Templeton, A. C.; Murray, R. W. *Langmuir* **1999**, *15*, 3782.
(23) Gerdon, A. E.; Wright, D. W.; Cliffel, D. E. *Biomacromolecules* **2005**, *6*, 3419.

Table 1. Structural and Mass Assignments for Peaks in the ESI-TOF Mass Spectra Reported in Figures 1 and 2[a]

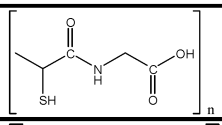
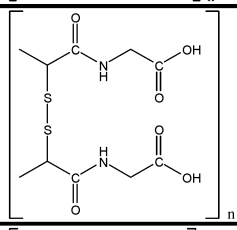
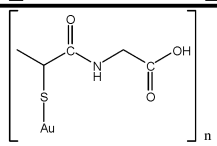
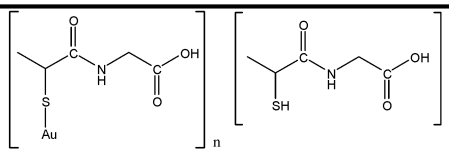
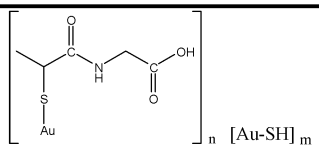
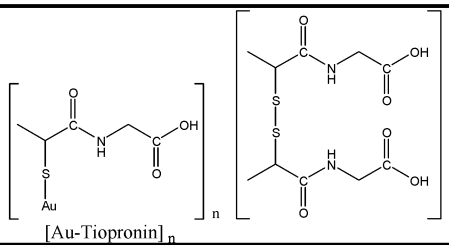
Species	Structure (M)	Li^+ M (Da)
1-1		**184.1** (n = 1) **361.1** (n = 2)
1-2		**359.1** (n = 1) **711.2** (n = 2)
1-3	 $[\text{Au-Tiopronin-OCH}_3]_n$ $[\text{Au-Tiopronin}]_m$	**(m = 0)** **380.0** (n = 1) **753.0** (n = 2) **1126.0** (n = 3) **1499.0** (n = 4) **1872.0** (n = 5) **(m = 1)** **739.0** (n = 1) **1112.0** (n = 2) **1485.0** (n = 3) **1858.0** (n = 4) **(m = 2)** **1098.0** (n = 1) **1471.0** (n = 2) **1844.0** (n = 3) **(m = 3)** **1457.0** (n = 1) **1830.0** (n = 2)
1-4	 $[\text{Au-Tiopronin-OCH}_3]_n$ $[\text{Au-Tiopronin}]_m$ Au	**936.0** (n = 1) **1309.0** (n = 2) **1682.0** (n = 3)
1-5	 $[\text{Au-Tiopronin-OCH}_3]_n$	**841.0** (n = 1) **1214.0** (n = 2) **1587.0** (n = 3) **1960.0** (n = 4)

[a] Mass values are for Li^+ cationized species.

acid solution (for the positive ion mode) or 1% ammonium hydroxide (for the negative ion mode)) was added into the MPC solutions before introduction into the ESI interface by a Cole-Parmer syringe pump at a flow-rate of 500 μL/h. Without dialysis, all MPC spectra displayed excessive sodium/hydrogen exchange, which greatly hindered ion formation and yielded poor S/N ratios in the mass spectra.

ESI-MS/MS Collision-Induced Fragmentation Measurements. Samples of the pre-MPC precipitant were analyzed using a ThermoFinnigan TSQ-7000 ESI-MS/MS tandem quadrupole instrument (Thermo Electron Corp., Waltham, MA). Positive ion mass analysis was performed using the product ion scan mode where the spray tip, nozzle, skimmer, ion guide, and quadrupole mass analyzer potentials (along with nozzle temperature, 200 °C) were optimized to achieve the best signal-to-noise ratio. The precursor ion of interest (1499.0 Da, the major ion produced in Figures 1 and 2) was mass-selected in the first mass analyzer, fragmented in the collision cell, and the product (fragment) ions were mass-analyzed by scanning the second mass analyzer. A curtain of nitrogen drying gas was utilized to assist in the ESI process, and argon gas was employed for CID experiments (using potentials ranging from 5 (for soft fragmentation) to 60 eV (for hard fragmentation)). Unit mass resolution was achieved through the mass range observed. External mass calibration was performed using a PEG standard (Polymer Source, Inc.) to yield monoisotopic masses exhibiting a mass accuracy better than $\Delta m = \pm 0.5$ Da. Pre-MPC precipitants were dissolved in DMF (Sigma, 99.9+%, HPLC grade) (1 mg/mL) doped with 0.1% LiBr, before introduction into the ESI interface by a Cole-Parmer syringe pump at a flow-rate of 500 μL/h.

Table 2. Structural and Mass Assignments for Peaks in the ESI-TOF Mass Spectra Reported in Figures 3 and 4[a]

Species	Structure (M)	H^+ M (Da)	Na^+ M (Da)
2-1		**164.0** (n = 1) **327.1** (n = 2)	**186.0** (n = 1) **349.1** (n = 2)
2-2		**325.0** (n = 1) **649.1** (n = 2)	**347.0** (n = 1) **671.1** (n = 2)
2-3	 [Au-Tiopronin] n	**360.0** (n = 1) **719.0** (n = 2) **1078.0** (n = 3) **1437.0** (n = 4) **1796.0** (n = 5) **2154.9** (n = 6) **2513.9** (n = 7) **2872.9** (n = 8)	**382.0** (n = 1) **741.0** (n = 2) **1100.0** (n = 3) **1459.0** (n = 4) **1817.9** (n = 5) **2176.9** (n = 6) **2535.9** (n = 7) **2894.9** (n = 8)
2-4	 [Au-Tiopronin] n	**523.0** (n = 1) **882.0** (n = 2) **1241.0** (n = 3) **1600.0** (n = 4) **1959.0** (n = 5) **2318.0** (n = 6) **2677.0** (n = 7)	**545.0** (n = 1) **904.0** (n = 2) **1263.0** (n = 3) **1622.0** (n = 4) **1981.0** (n = 5) **2340.0** (n = 6) **2699.0** (n = 7)
2-5	 [Au-Tiopronin] n [Au-SH] m	**(m = 1)** **589.9** (n = 1) **948.9** (n = 2) **1307.9** (n = 3) **1666.9** (n = 4) **2025.9** (n = 5) **2384.9** (n = 6) **2743.9** (n = 7) **(m = 2)** **819.9** (n = 1) **1178.9** (n = 2) **1537.9** (n = 3) **1896.8** (n = 4) **2255.8** (n = 5) **2614.8** (n = 6) **2973.8** (n = 7)	**(m = 1)** **611.9** (n = 1) **970.9** (n = 2) **1329.9** (n = 3) **1688.9** (n = 4) **2047.9** (n = 5) **2406.9** (n = 6) **2765.9** (n = 7) **(m = 2)** **841.9** (n = 1) **1200.9** (n = 2) **1559.8** (n = 3) **1918.8** (n = 4) **2277.8** (n = 5) **2636.8** (n = 6) **2995.8** (n = 7)
2-6	 [Au-Tiopronin] n	**684.0** (n = 1) **1043.0** (n = 2) **1402.0** (n = 3) **1761.0** (n = 4) **2120.0** (n = 5) **2479.0** (n = 6) **2838.0** (n = 7)	**706.0** (n = 1) **1065.0** (n = 2) **1424.0** (n = 3) **1783.0** (n = 4) **2142.0** (n = 5) **2501.0** (n = 6) **2860.0** (n = 7)

[a] Mass values are for H^+ and Na^+ cationized species.

Gel Permeation Chromatography Measurements. Pre-MPC precipitants and PEG standards were dissolved in DMF (Sigma, 99.9+%, HPLC Grade) (4 mg/mL) and analyzed using a HP series 1100 chromatograph (Hewlett-Packard, Palo Alto, CA) and DMF mobile phase. Three milliliters of filtered sample solution (0.45 μm PTFE Acrodisc, Waters Corp., Milford, Ma) was injected into a 100 μL Rheodyne sampling loop (Rheodyne, Cotati, CA) and pumped (1 mL/min) through a 7.8 × 300 mm Ultrastyragel 500 Å column (Waters Corp., Milford, MA), with an effective molecular weight range of 100−10 000 Da. Sequential detection was performed though a miniDAWN triple angle laser light scattering (TALLS) detector (Wyatt Technology, Santa Barbara, CA), a WellChrom K-2300 refractive index detector (Knauer, Berlin, Germany), and an HP series 1050 ultraviolet detector (Hewlett-Packard, Palo Alto, CA). Peak average molecular weights (M_p) were calculated against a calibration of five PEG standards (Polymer Source, Inc.).

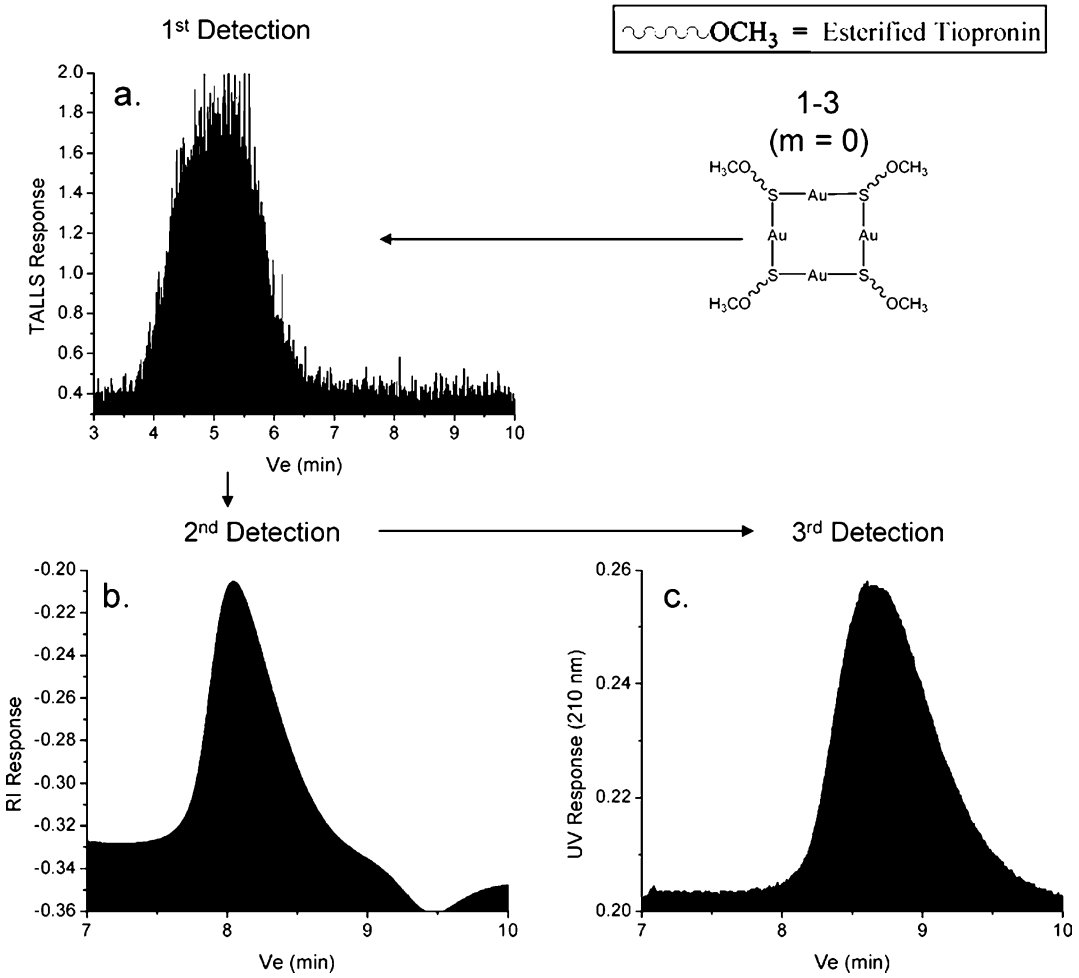


Figure 3. GPC chromatograms of gold(I)−thiolate precipitate (pre-MPC) dissolved in DMF (4 mg/mL). Tandem detection was performed through (a) triple angle laser light scattering, (b) refractive index, and (c) ultraviolet (210 nm) monitoring of the elutent. Lag-times between the chromatogram eluted volumes (V_e) are due to the tandem setup of the detectors.

Results and Discussion

Tiopronin monolayer-protected gold nanocluster (MPC) synthesis and postsynthesis peptide ligand place-exchange reactions were investigated by ESI-TOF MS in an effort to understand the chemical/structural changes occurring within these materials. Additionally, these efforts were to supply the exhaustive characterization information necessary for bioanalytical applications of peptide-functionalized MPCs. Studies included ESI-TOF MS analysis of the pre-MPC solution and precipitant (over a period of 3 days), MPC (undialyzed and dialyzed) materials, and postsynthesis tiopronin/peptide ligand exchange reactions (using various peptide:MPC mole ratios). It was envisioned that ESI-TOF mass spectra would reveal molecular weight distributions, intermediate tiopronin/peptide ligand exchange, and yield valuable information for optimization of MPC synthesis and application conditions.

All figures show structures and peaks labeled using the $x-y$ format (x = table number, y = structure number) subsequently labeled with the atom and/or ion that has been added to (or removed from) the species. For instance, a peak labeled 2-3 $[M - 4H + 5Na]^+$ corresponds to structure 3 found in Table 2, in which four sodium ions have been exchanged for four hydrogen ions and an additional sodium ion provides charge for the ESI process. Tables 1−3 show structures and masses of major species observed in Figures 1−9; the underlined masses correspond to the mass peaks shown in the figures, which indicate a tetrameric configuration.

Pre-MPC Spectra. Figure 1 shows the mass spectrum (300−2000 Da) of a pre-MPC sample (~2 h after synthesis) within minutes after the onset of precipitate formation in the solution (S/N = 790, for mass peak 1499.0 Da). Lithium bromide (0.1%) was added to the pre-MPC solution to aid in the production of positive ions and to increase the S/N ratio. This figure displays an overall predominance of esterified disulfide bridged tiopronin (species 1-2) with an increased abundance of ester terminated gold(I)−thiolate tetramer (species 1-3), relative to ESI-TOF MS analysis of pre-MPC samples prior to precipitate formation. The expanded mass region (1450−1510 Da) emphasizes the gold(I)−thiolate tetramer and displays four tetramers, which will be described in order of increasing abundance and esterification (i.e., increasing molecular mass). The peaks at 1457.0, 1471.0, 1485.0, and 1499.0 Da represent gold(I)−thiolate tetramers with 1, 2, 3, and 4 ester-modified carboxyl end groups (in Table 1, species 1−3 (m = 3), 1−3 (m = 2), 1−3 (m = 1), and 1−3 (m = 0)). Additionally, there is a similar pattern of peaks (at 1830.0, 1844.0, 1858.0, and 1872.0 Da), which represent the less favorable pentamer formation of species 1-3. The inset of

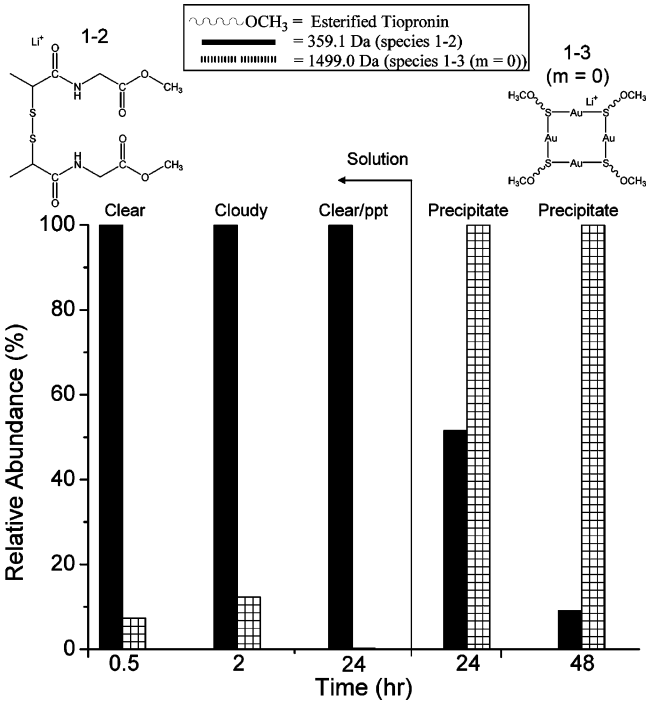


Figure 4. ESI-TOF MS monitoring of relative abundance (%) of disulfide bridged tiopronin (359.1 Da) versus gold(I)−thiolate tetramer (1499.0 Da) in solution, at 0.5 h (clear), 2 h (cloudy), and 24 h (clear with ppt), and for the precipitate, at 24 and 48 h. The most intense peak in each spectrum (359.1 or 1499.0 Da) is given a value of 100%.

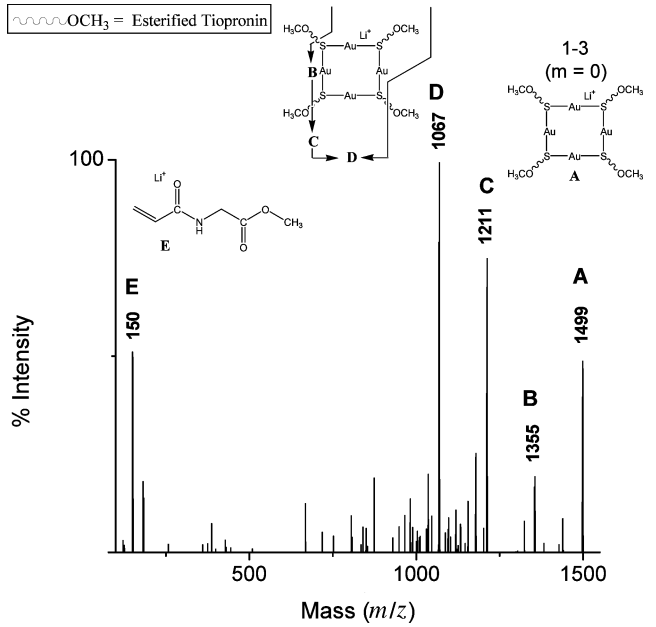


Figure 5. ESI-MS/MS collision-induced fragmentation (with argon, 50 eV) mass spectrum (100−1550 Da) of gold(I)−thiolate precipitate (pre-MPC gold(I)−thiolate tetramer: precursor ion, 1499.0 Da) dissolved in DMF and doped with 50 μL of 0.1% LiBr in DMF.

the theoretical isotopic distribution for the fully esterified tetramer is in agreement with a singly charged ($z = +1$) isotopic cluster around 1499.0 Da and further validates the formation of esterified gold(I)−thiolate tetramers. Moreover, ESI-TOF mass spectra of the pre-MPC samples prior to precipitation show an increasing abundance of gold(I)−thiolate tetramer with ester-modified end groups. Esterification would be accomplished by

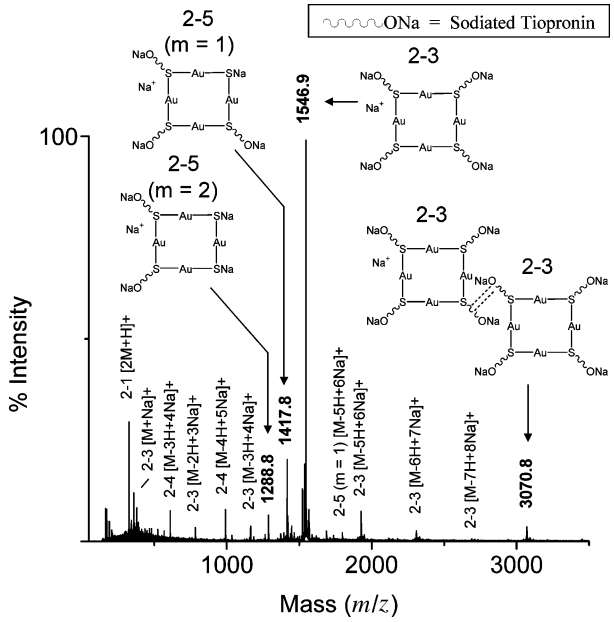


Figure 6. ESI-TOF mass spectrum (100−3500 Da) of tiopronin-protected gold MPCs doped with 50 μL of H_2O:ACN (1:1 and 1% glacial acetic acid). S/N = 2680 for mass peak 1546.9 Da.

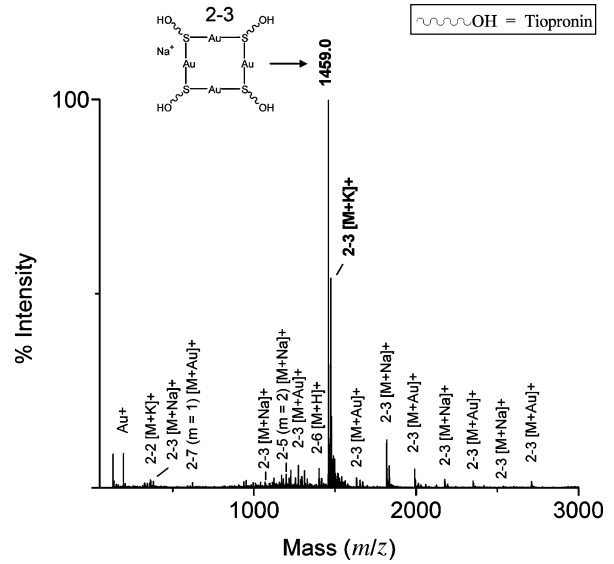
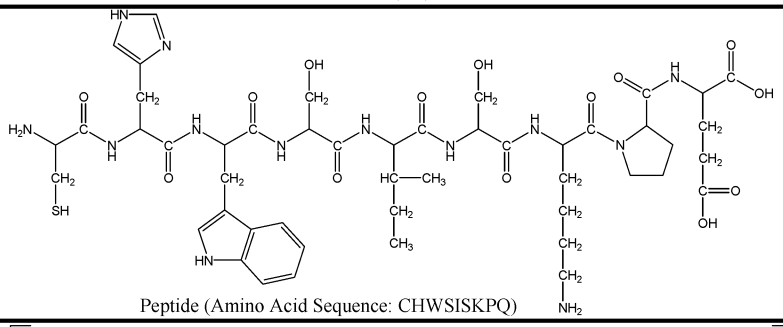
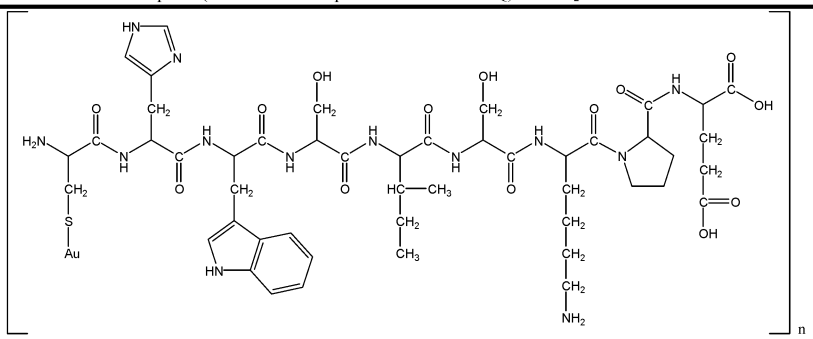
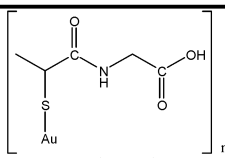
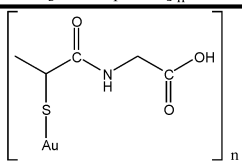


Figure 7. ESI-TOF mass spectrum (100−3000 Da) of dialyzed tiopronin-protected gold MPCs doped with 50 μL of H_2O:ACN (1:1 and 1% glacial acetic acid). S/N = 6210 for mass peak 1459.0 Da.

the methanol/acetic acid solvent,[24] explaining the increasing tetramer precipitation as the reaction proceeds.

Figure 2 displays a mass spectrum (300−2000 Da) of the pre-MPC (gold(I)−thiolate "oligomer") precipitate (~48 h after synthesis) dissolved in DMF and cationized with lithium (S/N = 3700, for mass peak 1499.0 Da). This mass spectrum displays one major peak at 1499.0 Da of the completely esterified gold(I)−thiolate tetramer (species 1-3) and a noticeably decreased presence of disulfide bridged tiopronin (species 1-2), which was the only species present in the reaction solution (at this point in the reaction). This mass spectrum shows that the precipitate is almost entirely composed of the gold(I)−thiolate tetramer, and it is easily isolated from the reaction mixture.

Table 3. Structural and Mass Assignments for Peaks in the ESI-TOF Mass Spectra Reported in Figure 5[a]

Species	Structure (M)	H^+ M (Da)	Na^+ M (Da)
3-1	 Peptide (Amino Acid Sequence: CHWSISKPQ)	**1086.5** (n = 1)	**1108.5** (n = 1)
3-2	 [Au-Peptide] n	**1282.5** (n = 1) **2564.9** (n = 2)	**1304.5** (n = 1) **2586.9** (n = 2)
3-3	 [Au-Tiopronin] n [Au-Peptide]	**1641.5** (n = 1) **2000.4** (n = 2) **2359.4** (n = 3) **2718.4** (n = 4)	**1663.4** (n = 1) **2022.4** (n = 2) **2381.4** (n = 3) **2740.4** (n = 4)
3-4	 [Au-Tiopronin] n [Au-SH] [Au-Peptide]	**1871.4** (n = 1) **2230.4** (n = 2) **2589.4** (n = 3) **2948.4** (n = 4)	**1893.4** (n = 1) **2252.4** (n = 2) **2611.4** (n = 3) **2970.4** (n = 4)

[a] Mass values are for H^+ and Na^+ cationized species.

Figure 3 shows gel permeation chromatograms of the pre-MPC gold(I)−thiolate "oligomer" precipitate (∼48 h after synthesis) analyzed in DMF. Because only one peak was observed in the chromatogram, the existence of a bimodal distribution of high and low mass species can be ruled out. The peak average molecular weight (M_p) was estimated to be M_p = 620 Da (against a five point PEG standard calibration curve). This estimated molecular weight of 620 Da certainly verifies that these are materials of low molecular mass. When taking into consideration the hydrodynamic volume (hvol):molecular weight (M_p) ratio differences (in DMF) between PEG and a gold(I)−thiolate tetramer (where the gold(I)−thiolate tetramer has a much lower hvol:M_p ratio than PEG due to the mass of the gold boosting the M_p), it is easily conceivable that this narrow peak (of the pre-MPC gold(I)−thiolate "oligomer" precipitant) is representative of the narrow distribution of structures surrounding the predominant tetramer peak observed in the ESI-TOF mass spectra in Figure 2. Further, ESI-TOF MS analysis of a collected GPC peak fraction gave a spectrum essentially identical to that of Figure 2.

Figure 4 summarizes ESI-TOF MS monitoring of the reaction changes over time as a function of relative abundance (%) of disulfide bridged tiopronin (359.1 Da) versus gold(I)−thiolate tetramer (1499.0 Da) in solution at 0.5 h (clear), 2 h (cloudy), and 24 h (clear with ppt), and in the precipitate, at 24 and 48 h. The intensity of the more abundant of the two peaks (359.1 Da vs 1499.0 Da) was set at 100, and the less abundant peak was scaled relative to this peak. The gold(I)−thiolate tetramer peak was observed to increase in abundance (relative to the disulfide bridged tiopronin peak) until precipitation, with subsequent depletion of all tetramer from solution after 2 days. Additionally, less disulfide bridged tiopronin was observed in the precipitate (i.e., it became purer) after 48 h. It should also be noted that esterification of the carboxyl end groups would

(24) Smith, M. B.; March, J. *March's Advanced Organic Chemistry: Reactions, Mechanisms, and Structure*, 5th ed.; John Wiley & Sons, Inc.: New York, 2001.

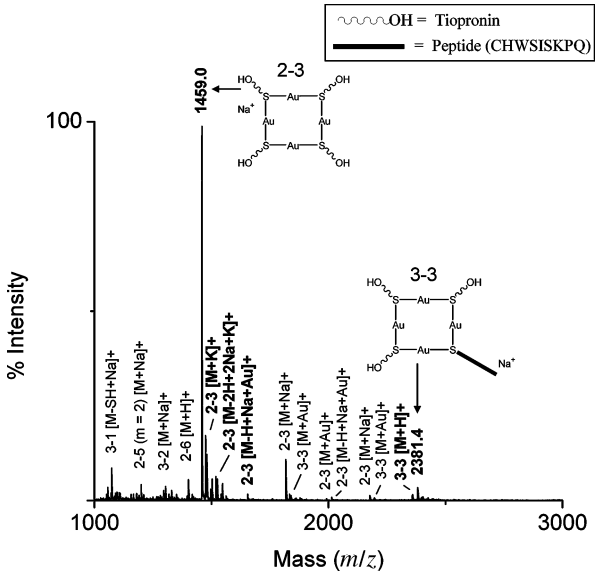


Figure 8. ESI-TOF mass spectrum (1000−3000 Da) of dialyzed peptide-substituted gold MPCs (10:1 MPC:peptide) doped with 50 μL of H_2O:ACN (1:1 and 1% glacial acetic acid).

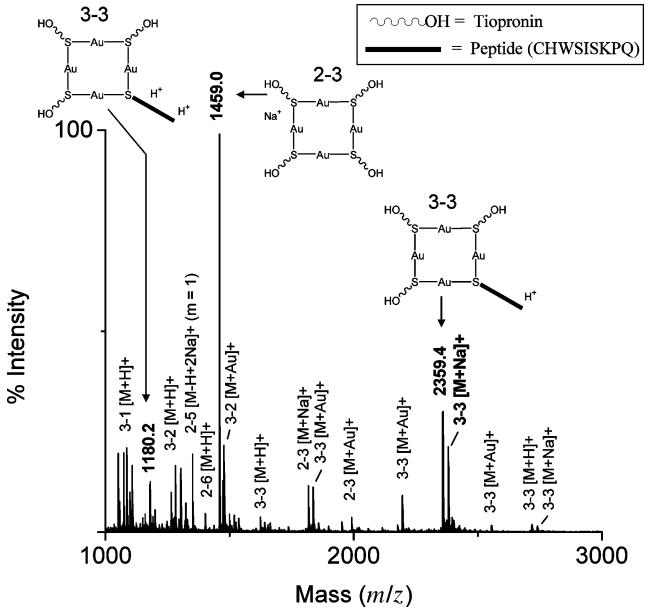


Figure 9. ESI-TOF mass spectrum (1000−3000 Da) of dialyzed (excess) peptide-substituted gold MPCs (2:1 MPC:peptide) doped with 50 μL of H_2O:ACN (1:1 and 1% glacial acetic acid).

certainly contribute to the white tetramer being insoluble in methanol and H_2O, yet readily soluble in DMF.

Figure 5 shows the ESI-MS/MS collision-induced fragmentation (50 eV) mass spectrum (100−1550 Da) of the gold(I)−thiolate precipitate (pre-MPC gold(I)−thiolate tetramer: precursor ion, 1499.0 Da) dissolved in DMF and doped with 0.01% LiBr. Inspection of the fragmentation pattern identifies the fully esterified tiopronin−gold tetramer (1499 Da, structure A, species 1−3 (m = 0)), the tetramer with loss of one tiopronin "arm" (1355 Da, structure B), the tetramer with loss of two "arms" (1211 Da, structure C), and the tetramer with loss of three "arms" (1067 Da, structure D); the lost "arm/s" were observed at 150 Da (structure E). The observance of the preferential "arm" loss and retention of the tetramer "core" (instead of random fragmentation) confirms that the esterified gold(I)−thiolate tetramer must have a cyclic structure. MALDI-TOF MS (using high laser settings to induce fragmentation) was also used to validate these findings. It should also be noted that the cyclic structure would explain the preferential tetramer formation observed in Figure 2: there must be a specific linking of the unimers before they can preferentially cyclize and precipitate from solution. The formation of a cyclic gold(I)−thiolate tetramer has been theorized previously through spectroscopic evidence[16] but until the present work has not been validated. During the editing of our work, a new paper by Hakkinen and co-workers theoretically predicted cyclic gold(I)−thiolate tetramers accomplishing monolayer-protection on the surface of the gold MPCs, based upon a $Au_{38}(SCH_3)_{24}$ model (i.e., Au_{14}-$[(AuSCH_3)_4]_6$).[25] Our results provide additional experimental evidence to support their claims, especially the exceptional stability of gold(I)−thiolate tetramers.

MPC Spectra. Figure 6 shows the ESI mass spectrum (100−3500 Da) of a tiopronin-protected MPC (S/N = 2680, for mass peak 1546.9 Da) with three predominant mass peaks indicating tetramer formation. These peaks (which will be described in order of relative intensity) result from the conversion of esterified (pre-MPC) gold(I)−thiolate tetramers (ex. 1499.0 Da in Figure 1) to carboxyl terminated (MPC) gold(I)−thiolate tetramers, in which four sodium ions have been exchanged for four hydrogen ions (on the carboxyl end groups). The peak at 1546.9 Da represents a gold(I)−thiolate tetramer (species 2-3) (Scheme 1a, structure 2) with a cluster of two tetramers found at peak 3070.8 Da (dicluster of species 2-3); the peak at 1417.8 Da represents a tetramer with partial loss of one tiopronin molecule (species 2−5 (m = 1)); and the mass peak 1288.8 Da represents a tetramer with partial loss of two tiopronin molecules (species 2−5 (m = 2)). This figure exhibits an overall predominance of sodium/hydrogen exchange on the carboxyl end groups (which greatly diminishes the S/N ratios) and verifies that $NaBH_4$ has effectively converted all ester end groups to carboxyl end groups, to yield H_2O soluble MPCs and gold(I)−thiolates. The excessive sodium displayed in this figure provides an explanation for the poor resolution and S/N ratios from previous mass spectral studies utilizing negative ionization,[8,11,14,15] as well as the source of "side peaks on the higher mass sides of the main peaks" in an electrospray MPC study reported shortly after the submission of this work.[26] We are displaying this figure for comparison with Figure 7 to stress the negative influence of excess salt on the mass spectral quality and S/N ratio. It should also be noted that ESI-TOF MS attempts using the negative ion mode were deemed unsuccessful with all MPC materials due to excessive sodium/hydrogen exchange, which resulted in peak splitting and poor S/N ratios.

Figure 7 displays a mass spectrum (100−3000 Da) of tiopronin-protected MPCs that have been dialyzed to remove excess sodium left over from the sodium borohydride termination (S/N = 6210, for mass peak 1459.0 Da). This mass spectrum displays one major peak at 1459.0 Da, which represents the gold(I)−thiolate tetramer (species 2-3) (Scheme 1a, structure 3). The mass spectrum shows that dialysis has

(25) Hakkinen, H.; Walter, M.; Gronbeck, H. *J. Phys. Chem. B* **2006**, *110*, 9927.

(26) Negishi, Y.; Takahashi, Y.; Sato, S.; Yao, H.; Kimura, K.; Tsukuda, T. *J. Phys. Chem. B* **2006**, *110*, 12218.

effectively improved spectral quality through reduction of sodium/hydrogen exchange on the carboxyl end groups and increased the S/N ratio by a factor of ~2.5, with respect to Figure 6; thus dialysis was used for the analysis of the place-exchanged MPCs, which will be discussed next.

Thus far, the overall predominance of the gold(I)−thiolate tetramers can still be seen after MPC synthesis termination, which indicates that the gold(I)−thiolates tetramer plays an important role in the stabilization of the gold nanocluster colloids (Scheme 1a, structure I) and/or are a basic building block within these nanoparticles (Scheme 1a, structure II). Moreover, this type of stabilization has been previously demonstrated through dendrimer stabilization of gold colloids,[27,28] so it is easily conceivable that a similar stabilization could occur with the gold(I)−thiolate tetramers. It is conceivable that the electrospray conditions may be encouraging the gold(I)−thiolate tetramers (loosely held on the surface, with respect to the Au−Au interactions of the core)[25] to spin off from the high molecular weight MPCs, thus favoring the appearance of gold(I)−thiolate tetramer ions in our ESI-TOF mass spectra. To provide further evidence for this proposal, we sought the use of ligand place-exchange reactions (Scheme 1b) to demonstrate the ability of ESI-TOF MS to quantitatively monitor peptide modification of the gold(I)−thiolate tetramers.

Figure 8 shows a mass spectrum (100−3000 Da) of peptide-modified MPCs (10:1 MPC:peptide) that were dialyzed to remove excess sodium (S/N = 5470, for mass peak 1459.0 Da). This mass spectrum displays one predominant mass peak at 1459.0 Da, which represents the gold(I)−thiolate tetramer (species 2-3) (Scheme 1a, structure 3), and a less abundant mass peak at 2381.4 Da, which represents the sodium cationized gold(I)−thiolate tetramer in which one tiopronin was ligand exchanged for one peptide molecule (species 3-3). This figure displays the overall predominance of gold(I)−thiolate tetrameric species with only ~7.6% displaying peptide modification.

Figure 9shows a mass spectrum (100−3000 Da) of peptide-modified MPCs (2:1 MPC:peptide) that were dialyzed to remove excess sodium (S/N = 3360, for mass peak 1459.0 Da). This mass spectrum displays two predominant mass peaks having a tetrameric configuration, which will be described in order of abundance. The mass peak at 1459.0 Da represents a gold(I)−thiolate tetramer (species 2-3) (Scheme 1a, structure 3), and the mass peak 2359.4 Da represents a gold(I)−thiolate tetramer in which one tiopronin was ligand exchanged for one peptide molecule (species 3-3) and the charge provided by protonation. Significant peak splitting is observed due to variations in cationization (sodiation at 2381.4 Da), species 3-3. A diprotonated species is also seen at 1180.2 Da (*m*/2). The probable reason for the peak splitting is that the protein contains lysine (a basic site), which makes it more likely to pick up protons (and encourage multiple cation association) than the unmodified (MPC) gold(I)−thiolate tetramer.[28] Inspection of the ^{13}C isotopic clusters of the peptide modified gold(I)−thiolate tetramers (species 3-3) allowed for the unambiguous determination of their charge states. For a singly charged tetramer (2359.4 Da; $[M + H]^+$), the splitting between the ^{13}C isotopes was 1.0 Da (similar to the ^{13}C isotopic *m*/*z* difference observed in Figure 1, theoretical isotope distribution inset). For the doubly charged tetramer (1180.2 Da; $[M + 2H]^{+2}$), the ^{13}C isotopic splitting was 0.5 Da, indicating a +2 charge. Figure 9 displays the overall predominance of tetrameric structures both with (~38.6%) and without peptide modification and shows that multiply charged species can be observed with the attachment of larger ligands. A very important aspect of this figure is the possibility of using ESI-TOF MS to follow MPC peptide modification. Peptide modification (of the gold(I)−thiolate tetramer) was observed to be ~38.6% for a 2:1 (MPC:peptide) place-exchange reaction versus ~7.6% for a 10:1 (MPC:peptide) place-exchange reaction. For example, the increase in relative intensity of the peptide-modified species increases by a factor of 5.1 for an increase of 5.0 in the ratio of peptide to MPC.

Conclusions

The important results of this paper are as follows: we present the first experimental validation of cyclic gold(I)−thiolate tetramer formation in tiopronin monolayer-protected nanoclusters and report the chemical pathway leading to their formation in MPCs; dialysis of MPC samples was shown to effectively reduce sodium/hydrogen exchange on the carboxyl end groups and significantly improved mass spectral S/N ratios by a factor of ~2.5; and postsynthesis (MPC) gold(I)−thiolate peptide modification was quantitatively monitored by ESI-TOF MS. Studies are currently underway to further exploit the use of ESI-TOF MS and GPC in MPC characterization.

Acknowledgment. We would like to thank David W. Wright for supplying the peptide used in this study. This work was performed under the auspices of the Vanderbilt Institute of Nanoscale Science and Engineering and the National Institutes of Health (R01 GM076479).

Supporting Information Available: Details of the UV−visible and TEM images acquired for the tiopronin monolayer-protected nanoclusters discussed in this work. This material is available free of charge via the Internet at http://pubs.acs.org.

JA0639057

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(28) Kinter, M.; Sherman, N. E. *Protein Sequencing and Identification Using Tandem Mass Spectrometry*; John Wiley & Sons, Inc.: New York, 2000.