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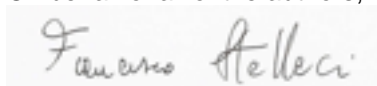*Director of the Integrative Food and Nutrition Center*

31 July 2018

Dear Benjamin:

Following your invitation we are hereby submitting a paper entitled "Synthesis and Characterization of Amphiphilic Gold Nanoparticles" for consideration as an Article in JOVE.

This paper describes in detail the synthesis, purification, and characterization of some key nanoparticles that we have been synthesising since 2004.

On behalf of all of the authors,

Francesco Stellacci
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1   **TITLE:**
2   **Synthesis and Characterization of Amphiphilic Gold Nanoparticles**
3

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27   **KEYWORDS:**
28   amphiphilic gold nanoparticles, sulfonated ligands, synthesis, characterization, binary ligand
29   coating, self-assembled monolayer
30

31   **SUMMARY:**
32   Amphiphilic gold nanoparticles can be used in many biological applications. A protocol to
33   synthesize gold nanoparticles coated by a binary mixture of ligands and a detailed
34   characterization of these particles is presented.
35

36   **ABSTRACT:**
37   Gold nanoparticles covered with a mixture of 1-octanethiol (OT) and 11-mercapto-1-undecane
38   sulfonic acid (MUS) have been extensively studied because of their interactions with cell
39   membranes, lipid bilayers, and viruses. The hydrophilic ligands make these particles colloidally
40   stable in aqueous solutions and the combination with hydrophobic ligands creates an amphiphilic
41   particle that can be loaded with hydrophobic drugs, fuse with the lipid membranes, and resist
42   nonspecific protein adsorption. Many of these properties depend on nanoparticle size and the
43   composition of the ligand shell. It is, therefore, crucial to have a reproducible synthetic method
44   and reliable characterization techniques that allow the determination of nanoparticle properties

45 and the ligand shell composition. Here, a one-phase chemical reduction, followed by a thorough
46 purification to synthesize these nanoparticles with diameters below 5 nm, is presented. The ratio
47 between the two ligands on the surface of the nanoparticle can be tuned through their
48 stoichiometric ratio used during synthesis. We demonstrate how various routine techniques,
49 such as transmission electron microscopy (TEM), nuclear magnetic resonance (NMR),
50 thermogravimetric analysis (TGA), and ultraviolet-visible (UV-Vis) spectrometry, are combined to
51 comprehensively characterize the physicochemical parameters of the nanoparticles.
52
53 **INTRODUCTION:**
54 The ligand shell of gold nanoparticles can be engineered to exhibit several different properties
55 that can be applied to address challenges in biomedicine[1-4]. Such versatility allows for the control
56 of the intermolecular interactions between nanoparticles and biomolecules[5-7]. Hydrophobicity
57 and charge play a decisive role, as well as other surface parameters that affect how nanoparticles
58 interact with biomolecules[5,8,9]. To tune the nanoparticles' surface properties, the choice of
59 thiolate molecules that make up the ligand shell offers a myriad of possibilities, according to the
60 characteristics sought. For example, a mixture of ligand molecules with hydrophobic and
61 hydrophilic (*e.g.*, charged) end groups are often used to generate amphiphilic nanoparticles[10,11].
62
63 One prominent example of this type of nanoparticles is protected by a mixture of OT and MUS
64 (hereafter called MUS:OT nanoparticles) that has been shown to possess many relevant
65 properties[12-14]. First, with a ligand shell composition of 66% MUS (hereafter 66:34 MUS:OT), the
66 colloidal stability of the nanoparticles is high, reaching up to 33% in weight in deionized water,
67 as well as in phosphate-buffered saline (1x, 4 mM phosphate, 150 mM NaCl)[15]. Moreover, these
68 particles do not precipitate at relatively low pH values: for example, at pH 2.3 and with salt
69 concentrations of 1 M NaCl[15], these nanoparticles remain colloidally stable for months. The
70 stoichiometric ratio between the two molecules on the ligand shell is important because it
71 dictates the colloidal stability in solutions with a high ionic strength[16].
72
73 These particles have been shown to traverse the cell membrane without porating it, *via* an
74 energy-independent pathway[1,12]. The spontaneous fusion between these particles and lipid
75 bilayers underlies their diffusivity through cell membranes[17]. The mechanism behind this
76 interaction is the minimization of contact between a hydrophobic solvent-accessible surface area
77 and water molecules upon fusion with lipid bilayers[18]. Compared to all-MUS nanoparticles
78 (nanoparticles having only the MUS ligand on their shell), the higher hydrophobicity on mixed
79 MUS:OT nanoparticles (for example, at a 66:34 MUS:OT composition) increases the span of the
80 core diameter that can fuse with lipid bilayers[18]. Different self-assembly organizations of the
81 ligand shell correlate to distinct binding modes of 66:34 MUS:OT nanoparticles with various
82 proteins, such as albumin and ubiquitin, when compared to all-MUS particles[19]. Recently, it has
83 been reported that 66:34 MUS:OT nanoparticles can be utilized as a broad-spectrum antiviral
84 agent that irreversibly destroys the viruses because of multivalent electrostatic bindings of MUS
85 ligands and nonlocal couplings of OT ligands to capsid proteins[14]. In all these cases, it has been
86 found that the hydrophobic content, as well as the core size of the nanoparticles, determines
87 how these bio-nano interactions take place. These diverse properties of MUS:OT nanoparticles
88 have prompted many computer simulation studies that aimed to clarify the mechanisms

underpinning the interactions between MUS:OT particles and various biological structures such as lipid bilayers[20].

The preparation of MUS:OT-protected Au nanoparticles poses a few challenges. First, the charged ligand (MUS) and the hydrophobic ligand (OT) are immiscible. Thus, the solubility of the nanoparticles and of the ligands needs to be taken into account throughout the synthesis, as well as during characterization. Additionally, the purity of the MUS ligand molecules—specifically, the content of inorganic salts in the starting material—influences the quality, reproducibility, as well as the short- and long-term colloidal stability of the nanoparticles.

Here, a detailed synthesis and characterization of this class of amphiphilic gold nanoparticles protected by a mixture of MUS and OT are outlined. A protocol for the synthesis of the negatively charged MUS ligand is reported to ensure the purity and, hence, the reproducibility of different nanoparticle syntheses. Then, the procedure to generate these nanoparticles, based on a common one-phase synthesis, followed by thorough purification, is reported in detail. Various necessary characterization techniques[21], such as TEM, UV-Vis, TGA, and NMR, have been combined to obtain all the necessary parameters for any further biological experiments.

PROTOCOL:

1. Synthesis of 11-mercapto-1-undecanesulfonate (MUS)

NOTE: This protocol can be used at any scale desired. Here, a 10 g scale-of-product is described.

1.1. Sodium undec-10-enesulfonate

1.1.1. Add 11-bromo-1-undecene (25 mL, 111.975 mmol), sodium sulfite (28.75 g, 227.92 mmol), and benzyltriethylammonium bromide (10 mg) to a mixture of 200 mL of methanol (MeOH) and 450 mL of deionized (DI) water (4:9 v/v MeOH:H_2O ratio) in a 1 L round-bottom flask.

1.1.2. Reflux the reaction mixture at 102 °C for 48 h. Cap the system with a pressure relief mechanism—for example, a balloon with a needle, or simply a needle. This reaction is not sensitive to atmospheric gases.

NOTE: The solution becomes colorless when the reaction is complete.

1.1.3. Connect the reaction mixture to a rotary evaporator to evaporate MeOH and reduce the volume to approximately 300 mL.

1.1.4. Transfer the remaining solution to a 1 L separatory funnel.

1.1.5. Extract the remaining aqueous solution 5x with diethyl ether, using the separatory funnel. Unreacted 11-bromo-1-undecene stays in the diethyl ether phase and the sulfonated product in H_2O.

133

134   CAUTION: Release any pressure buildup frequently during the extraction, and consult the correct
135   usage of separatory funnels.

136

137   1.1.6. Collect the final extracted water solution into a 1 L single-neck round-bottom flask.

138

139   1.1.7. Connect the reaction flask to a rotary evaporator by putting a bit of grease (or Teflon ring
140   strips or any other sealant) between the flask and the trap.

141

142   1.1.8. Decrease the vacuum slowly to evaporate the aqueous phase in a rotary evaporator.
143   Because the product is a surfactant, foaming will occur during the evaporation. To circumvent
144   this problem, follow the instruction in the next step.

145

146   1.1.8.1. Add ethanol to the mixture to accelerate the evaporation of H_2O and prevent foaming.
147   When foaming restarts because of the decrease in ethanol content, stop the evaporation,
148   remove the flask from the rotary evaporator, add more ethanol (about one-third of the total
149   volume), and reconnect the flask to the rotary evaporator. Repeat this process until the solution
150   mixture decreases significantly and does not form bubbles.

151

152   1.1.9. Dry the white powder directly by connecting the flask to a high vacuum. The drier the
153   powder, the less inorganic salts will creep into the subsequent steps.

154

155   NOTE: Heat can be used to dry the product—for example, by keeping the flask under vacuum in
156   a 60 °C bath and left overnight.

157

158   1.1.10. Suspend the white powder in 400 mL of methanol in a flask. Sonicate to dissolve the
159   maximum amount of product.

160

161   NOTE: The goal of this step is to dissolve the product but not the inorganic byproducts, such as
162   excess sodium sulfite and sodium bromide, that have limited solubility in methanol. Use
163   methanol with the lowest water content possible, because water in the methanol will increase
164   the solubility of the inorganic byproducts in the solvent.

165

166   1.1.11. To increase the solubility of the product, methanol can be gently heated close to its
167   boiling point (~64 °C).

168

169   CAUTION: Make sure to work under a fume hood during the heating of the flask. The fumes of
170   the evaporated methanol are dangerous.

171

172   1.1.12. Filter the solution to remove the methanol insoluble inorganic byproducts. Use a filtering
173   flask connected to a vacuum pump and a filtering funnel with quantitative filter paper, or a
174   borosilicate filter. Both the product and the inorganic salts are white powders when dry: the
175   product is soluble in methanol, while the salts are not.

176

177   1.1.13. Transfer the filtered solution from the filtering flask to a 1 L round-bottom flask.
178
179   1.1.14. Connect the flask to a rotary evaporator and evaporate the methanolic solution at 45 °C,
180   redissolve the white powder in methanol, and filter the solution (protocol steps 1.1.7, 1.1.8, and
181   1.1.9). Repeat this process at least 2x, to decrease the amount of inorganic salt.
182
183   1.1.15. Collect the white, methanol soluble powder (approximately 30 g, at this scale).
184
185   1.1.16. Dissolve approximately 10 mg of product in 500 µL of D_2O and transfer the solution to an
186   NMR tube.
187
188   1.1.17. Perform ^1H NMR spectrometry on the product in D_2O at 400 MHz with 32 scans.
189
190   NOTE: The peak assignments for ^1H NMR (D_2O) are 5.97 (m, 1H), 5.09 (m, 2H), 2.95 (t, 2H), 2.10
191   (m, 2H), 1.77 (q, 2H), 1.44 (br s, 12H).
192
193   **1.2. Sodium 11-acetylthio-undecanesulfonate**
194
195   1.2.1. Dissolve the approximate 30 g of sodium undec-10-enesulfonate (the reaction product of
196   section 1.1) in 500 mL of methanol inside a 1 L round-bottom flask. Add a 2.6x excess of thioacetic
197   acid to the solution and stir it in front of a UV lamp (250 W) overnight (~12 h). In case a UV lamp
198   is not available, the reaction can be performed by refluxing using a radical initiator, such as
199   azobisisobutyronitrile (AIBN); however, the use of a UV lamp is strongly recommended.
200
201   CAUTION: Make sure to work under the fume hood at all times. If the flask needs to be
202   transported to another space where the UV lamp is located, seal the flask to avoid spreading the
203   strong odor of thioacetic acid. Exercise care when operating a UV lamp: completely block the
204   space where the lamp is located and consult the institution's safety guidelines on how to operate
205   a UV lamp.
206
207   1.2.2. Monitor the reaction by taking ~2 mL aliquots from the reaction, evaporate solvent, and
208   add deuterated water to check with ^1H NMR. Once the peaks corresponding to the double bond
209   disappear, stop the reaction.
210
211   NOTE: Usually, after 12 h in front of the UV lamp, the reaction is complete. If the reaction mixture
212   becomes turbid, add more MeOH and continue the exposure to the UV light for six additional
213   hours.
214
215   1.2.3. Evaporate all of MeOH in a rotary evaporator until the solid residue becomes orange-red.
216   If left long enough, the product becomes brown to black.
217
218   CAUTION: Work mindfully because of the strong odors from the thioacetic acid. The strong odors
219   of any thiolate spills can be neutralized using an aqueous solution of bleach (sodium
220   hypochlorite).

1.2.4. Using a filtering flask, wash the product with diethyl ether to remove any excess thioacetic acid, until no more colored (orange-yellow) substances appear in the diethyl ether supernatant. Dry the solid under high vacuum and, then, dissolve it in methanol, yielding a yellow to orange solution.

NOTE: The color may vary at this step.

1.2.5. Add 3 g of carbon black to the solution, mix vigorously, and filter the mixture through filtration medium (see **Table of Materials**) covering two-thirds of a fluted filter paper.

NOTE: The porous structure of carbon black captures the colored side-product material (and some of the product). The filtered solution should be clear. If the filtered solution is still colored (yellow), repeat this process.

1.2.6. Evaporate the solvent completely in a rotary evaporator and collect approximately 35 g of white powder.

1.2.7. Dissolve ~10 mg of the product in ~500 µL of D_2O and transfer the solution to NMR tubes.

1.2.8. Perform ^1H NMR on the product in D_2O at 400 MHz with 32 scans.

NOTE: The peak assignments for the ^1H NMR (D_2O) are 2.93 (t, 4H), 2.40 (s, 3H), 1.77 (m, 2H), 1.62 (m, 2H), 1.45 (br s, 14H).

1.3. MUS

1.3.1. Reflux sodium 11-acetylthio-undecanesulfonate at 102 °C in 400 mL of 1 M HCl for 12 h to cleave the thioacetate group and obtain a thiol.

1.3.2. Transfer the product to a 1.5 L or 2 L round-bottom flask. Add 200 mL of 1 M NaOH to the final solution and top it with 400 mL of DI water to have a final volume of 1 L. This will keep the solution acidic and prevent the crystallization of inorganic salts as product.

NOTE: A complete neutralization of the solution to pH 7 will result in the crystallization of a product insoluble in methanol.

1.3.3. Keep the clear solution at 4 °C and it will crystallize overnight. This step yields a pure product. The product crystallizes as fine crystals that are viscous when wet.

NOTE: To accelerate the crystallization, add presynthesized MUS to the solution, if available.

1.3.4. Decant the clear supernatant and centrifuge down the viscous white product in 50 mL centrifuge tubes for 5 min at 4,000 x *g*.

265

1.3.5. Decant the supernatant into another flask and dry the white pellets under high vacuum—depending on the centrifuge available, this can be 2 - 16 tubes or more.

NOTE: Filtering is not advised because of the surfactant nature of the product; excessive foaming will occur and most of the product will be lost.

1.3.6. Collect approximately 12 g (about 30% yield) of methanol-soluble MUS from this purification step.

NOTE: Be mindful that the powder is fine and electrostatic—it tends to stick to spatulas and the surfaces of containers. Also, more material can be extracted from the supernatant of the centrifugation step by reducing the volume (to about a third of its original value) and keeping it at 4 °C. Decrease the volume even more (by 75%) to increase the yield at this step.

1.3.7. Dissolve ~10 mg of the product in ~500 μL of D_2O and transfer the solution to NMR tubes.

1.3.8. Perform ^{1}H NMR on the product in D_2O at 400 MHz with 32 scans.

NOTE: The peak assignments of ^{1}H NMR (D_2O) are 2.93 (t, 4H), 2.59 (t, 3H), 1.78 (m, 2H), 1.65 (m, 2H), 1.44 (br s, 14H). The calculated molar mass (including the sodium counterion) of the product is 290.42 g/mol.

2. Nanoparticle Synthesis: Preparation of the Reagents

2.1. Clean all glassware (one 250 mL and one 500 mL single-neck round-bottom flask, a 125 mL separatory funnel, and a small funnel) with fresh *aqua regia* (three parts hydrochloric acid to one part nitric acid). Rinse the glassware with an excess amount of water inside a fume hood and remove all the fumes. Then, rinse the glassware with ethanol and dry it in a laboratory glassware oven (40 - 60 °C is recommended).

2.2. Weigh 152.9 mg (0.45 mmol) of gold (III) chloride trihydrate ($HAuCl_4 \cdot H_2O$) in a small glass vial (10 or 20 mL clean glass vials, or on weighing paper).

2.3. Weigh 87 mg (0.3 mmol) MUS in a glass vial of 20 mL.

2.4. Add 10 mL of methanol to dissolve the MUS. Sonicate it in an ultrasonic bath until no solid material is visible, to ensure complete dissolution.

NOTE: Alternatively, using a heat gun or a warm bath (~60 °C), heat the solution gently. When heated, run cold water through the outside of the flask to bring it back to room temperature.

2.5. Add 26 μL (0.15 mmol) of OT to the methanol solution and agitate it to mix the ligands.

309   2.6. Weigh 500 mg (13 mmol) of sodium borohydride (NaBH$_4$) and add it to 100 mL of ethanol in
310   the 250 mL round-bottom flask. Stir vigorously using magnetic stirring (600 - 800 rpm). (The
311   NaBH$_4$ takes 10 to 20 min, depending on the grade, to form a clear solution in ethanol.)

313 **3. Synthesis of Gold Nanoparticles**

315   3.1. Dissolve gold salt in 100 mL of ethanol in the 500 mL round-bottom flask and start stirring at
316   800 rpm with a magnetic bar on a stirring plate. Make sure the gold salt dissolves completely.

318   3.2. Place a 100 mL separatory funnel above the round-bottom flask. Put a funnel on the top of
319   the separatory funnel with a quantitative paper filter inside. When the NaBH$_4$ is dissolved in
320   ethanol, start filtering the solution into the separatory funnel through the filter paper in the
321   funnel.

323   3.3. Add the ligand solution to the reaction mixture. Wait 15 min for the formation of gold-
324   thiolate complex. The color change of the reaction mixture from translucent yellow to turbid
325   yellow indicates the formation of gold-thiolate complex.

327   3.4. Start adding the filtered NaBH$_4$ solution from the separatory funnel dropwise. Adjust the
328   interval time of the drops so that the addition of NaBH$_4$ takes about 1 h.

330   3.5. After the complete addition of NaBH$_4$, remove the funnel. Keep stirring the reaction for
331   another hour. At the end of the reaction, remove the magnetic stirring bar using a magnet placed
332   on the outside of the flask.

334   3.6. Use a septum to close the flask and pierce a needle into the septum to release the H$_2$ gas
335   that will evolve after the reaction.

337   3.7. Keep the reaction mixture inside a laboratory refrigerator (4 °C) to precipitate the
338   nanoparticles overnight.

340 **4. Workup of the Synthesis**

342   4.1. Decant the supernatant ethanol to reduce the volume.

344   4.2. Transfer the remaining precipitant to 50 mL centrifuge tubes and centrifuge for 3 min at
345   4,000 x *g*.

347   4.3. Decant the supernatant, disperse the nanoparticles again with ethanol by vortexing, and
348   centrifuge them again. Repeat this washing process 4x.

350   4.4. Dry the nanoparticles under vacuum to remove the residual ethanol.
351

352   4.5. To clean the nanoparticles from free hydrophilic ligands/molecules, dissolve the precipitates
353   in 15 mL of DI water and transfer them to the centrifuge tubes with a filtration membrane of 30
354   kDa cutoff molecular weight. Dialysis is also amenable for this procedure.

356   4.6. Centrifuge these tubes for 5 min at 4,000 x g to concentrate the nanoparticle solution.

358   4.7. Add 15 mL of DI water to this solution and centrifuge to concentrate again. Repeat this
359   cleaning process at least 10x.

361   NOTE: One indication that the water-soluble impurities have been removed is the absence of
362   foaming when agitating the aqueous waste; after all, most of the impurities are disulfides of MUS
363   with itself or with OT (this can be determined by collecting the material and performing ^{1}H NMR).

365   4.8. After the centrifugation, transfer the concentrated nanoparticles to a 15 mL centrifuge tube.
366   To turn the nanoparticles into a manageable powder, either precipitate them in a solvent such
367   as acetone or freeze-dry the remaining aqueous solution. When freeze-dried, the nanoparticles
368   tend to form a loose powder that sticks to surfaces and may be difficult to manipulate.

370   **5. Characterization of the Nanoparticles**

372   **5.1. Purity**

374   5.1.1. To check whether the nanoparticles are free from unbound ligands, dissolve 5 mg of dry
375   nanoparticles in 600 µL of D_2O and perform a ^{1}H NMR measurement of the particles. If there are
376   no sharp peaks of the ligands, it means the nanoparticles are free from small organic molecules.

378   **5.2. Ligand ratio**

380   5.2.1. Prepare a 150 mg/mL methanol-d_4 solution of iodine. Add 600 µL of this solution to the ~5
381   mg of nanoparticles in a glass vial, to etch the nanoparticles.

383   5.2.2. Wrap the cap of the vial with paraffin film and sonicate it in an ultrasonic bath for 20 min.
384   Transfer the solution to an NMR tube and acquire a ^{1}H NMR (400 MHz) spectrum with 32 scans.

386   **5.3. Ligand density**

388   5.3.1. Transfer 2 to 8 mg of nanoparticles to a TGA crucible. Choose a temperature range from
389   30 °C to 900 °C and a speed of 5 °C per minute under N_2 gas.

391   **5.4. Size distribution**

393   **5.4.1. TEM**

395   5.4.1.1. Prepare 0.1 mg/mL nanoparticle solution in DI water. Drop 5 μL of the prepared solution
396   onto the 400-mesh carbon-supported copper grid. Wait until it dries.

397

398   5.4.1.2. Transfer the grid in a TEM holder and insert it into the microscope. Acquire 5 - 10 images
399   with a magnification of at least 64,000X, operated at 200 kV.

400

401   NOTE: To increase the contrast, an objective aperture of 20 nm can be inserted.

402

403   **5.4.2. UV-Vis spectra**

404

405   5.4.2.1. Prepare a 0.2 mg/mL nanoparticle solution in DI water.

406

407   5.4.2.2. Put the required amount of this solution in the quartz cuvette and scan from 200 nm to
408   700 nm.

409

410   **REPRESENTATIVE RESULTS:**
411   The reaction steps to synthesize MUS are shown in **Figure 1**. The ^{1}H NMR spectra of the product
412   of each step are represented in **Figure 2**. The synthesis workflow of the binary MUS:OT
413   amphiphilic gold nanoparticles is described in **Figure 3**. Following the synthesis, the workup of
414   the nanoparticles consisted of washing the particles several times with ethanol and DI water.
415   Prior to any characterization of nanoparticles, the cleanliness of the nanoparticles from unbound
416   free ligands was monitored by ^{1}H NMR in D_2O, as shown in **Figure 4**. The size distribution of the
417   nanoparticles was characterized by TEM (**Figure 5a,b**). Localized surface plasmon resonance
418   absorption was measured by acquiring UV-Vis spectra (**Figure 5c**).

419

420   The ratio of the two ligands was determined by etching the gold core using iodine, acquiring the
421   ^{1}H NMR, and calculating the relative amounts of each ligand using the integrated values. **Figure
422   6** shows the representative spectra, as well as the procedure of NMR peak assignments. To find
423   the ligand ratio between MUS and OT, we calculated the integrals of the peaks between 0.8 - 1
424   (I_1), 1.12 - 1.55 (I_2), 1.6 - 1.9 (I_3), and 2.6 - 3 (I_4) ppm. The I_1 peak contains signal from three OT
425   hydrogens, the I_2 peak from a combination of 14 MUS hydrogens and 10 OT hydrogens, and the
426   I_3 and I_4 peaks from four MUS hydrogens and two OT hydrogens (for each peak). Therefore, to
427   find the OT percentage, it is necessary to normalize I_1 to 3 and apply the following expressions.
428   For I_2,

429

430    $$OT\% = \frac{1}{\left(\frac{I_2-10}{14}\right)} + 1$$

431

432   For I_3 and I_4,

433

434    $$OT\% = \frac{1}{\left(\frac{I_{3,4}-2}{4}\right)} + 1$$

435

436   These calculations indicate the ratio of OT to MUS, assuming there is one arbitrary unit of OT in

437 the system. For **Figure 6B**, the three integrals gave similar values for OT percentage (*i.e.*, 15.3,
438 15.9, and 15.9 from I_2, I_3, and I_4, respectively).

439

440 The surface coverage of the nanoparticles is examined by TGA as shown in **Figure 7**. TGA, NMR,
441 and TEM data (**Figure 3**) are combined to calculate the ligand density, which is the number of
442 ligands on a unit of surface area, approximating the particles to a sphere. (This calculation
443 assumes that Na boils as $NaHSO_3$.) TEM data shows that the average diameter of the
444 nanoparticles is 2.4 nm, pointing to approximately 18.08 nm^2 ($A_{par} = 4\pi r^2$) of surface area (A_{par})
445 and 7.23 nm^3 ($V_{par} = 4\pi r^3/3$) of volume per particle (V_{par}). The density of gold is 19.9 g/cm^3 and
446 the mass of one particle is 1.3969 x 10^{-16} mg ($Mass_{particle} = V_{par}$ x the density of gold = 7.23 nm^3 x
447 19.9 g/cm^3 x 10^{-18} mm^3/nm^3). The remaining mass around 800 °C corresponds to the gold core,
448 and there are approximately 3.7 x 10^{16} particles (N_{par}) that are estimated using N_{par} =
449 ($Mass_{gold}/Mass_{particle}$) = 5.17 mg / 1.3969 x 10^{-16} mg. The total surface area (A_{tot}) of the particles
450 is 6.69 x 10^{17} nm^2 ($A_{tot} = N_{par}$ x A_{par} = 3.69 x 10^{16} x 18.08 nm^2). The NMR of iodine-etched
451 nanoparticles showed that the MUS:OT ratio is 85:15 and the amount of organic content in TGA
452 is 0.00146 g. Therefore, there are 3.26 x 10^{18} ligands (N_{ligand}) following the formula of N_{ligand} =
453 [$Mass_{organic}$ / ((R_{OT} x M_wOT) + (R_{MUS} x M_wMUS)) / (R_{MUS} + R_{OT})] x $N_{Avogadro}$ = [0.00146 g / ((15 x 146
454 g/mol) + (85 x 267.42 g/mol)) / (85 + 15) x (6.02 x 10^{23}) = 3.26 x 10^{18}. Finally, the ligand density is
455 4.8 ligands/nm^2, calculated by dividing the N_{ligand} by A_{tot} (4.8 = 3.26 x 10^{18}/6.69 x 10^{17} nm^2). The
456 stoichiometric ratios *vs.* the NMR ratios of the OT, resulting from various syntheses, are
457 compared in **Figure 8**.

458

459 **FIGURE AND TABLE LEGENDS:**

460

461 **Figure 1: Schematic of MUS synthesis.** MUS synthesis is the key point for the reproducibility of
462 amphiphilic nanoparticle synthesis. If MUS has a high salt content, the stoichiometric ratio of the
463 ligands may deviate. X = 9.

464

465 **Figure 2: NMR spectra of the molecules after each step in the MUS synthesis (400 MHz). (A)**
466 This panel shows the ^1H NMR spectrum of sodium undec-10-enesulfonate in D_2O. (**B**) This panel
467 shows the ^1H NMR spectrum of sodium 11-acetylthio-undecanesulfonate in D_2O. (**C**) This panel
468 shows the ^1H NMR spectrum of 11-mercapto-1-undecanesulfonate in D_2O. In all spectra, *
469 indicates the solvent peaks.

470

471 **Figure 3: Schematic of the amphiphilic nanoparticle synthesis. (A)** This panel shows the
472 preparation of the one-phase chemical reduction reaction using ethanol as solvent. (**B**) Gold-
473 thiolate complex is allowed to form before the addition of a reducing agent. At this stage, the
474 solution of gold salt became turbid. (**C**) During the dropwise addition of the reducing agent, gold
475 nanoparticles are formed.

476

477 **Figure 4: Cleanliness of the nanoparticles from unreacted free ligands. (A)** This panel shows the
478 ^1H NMR spectrum of the nanoparticles right after the synthesis and vacuum-drying. D_2O is used
479 as the solvent for the ^1H NMR analysis. Sharp peaks shown by red arrows indicate the existence
480 of free unbound ligands. (**B**) This panel shows the ^1H NMR spectrum of the nanoparticles after a

481   thorough purification (*i.e.*, washes and centrifugation with ethanol and DI water). The red arrow
482   points to the magnified part of the spectrum, in which the peaks are broad, not sharp as before
483   indicating the absence of free ligands. In both spectra, * indicates the solvent peaks.

485   **Figure 5: Size distribution of nanoparticles.** (**A**) This panel shows a representative TEM image of
486   MUS:OT nanoparticles. The scale bar is 20 nm. (**B**) This panel shows a histogram of the core size
487   of nanoparticles based on several TEM images. (**C**) UV-Vis spectra of the nanoparticles showed
488   the characteristic surface plasmon resonance peak of the nanoparticles at around 520 nm.

490   **Figure 6: Ligand ratio calculation.** (**A**) This panel shows representative NMR spectra of
491   combinations of disulfides (as references for ligands after core etching) and peak assignments for
492   different protons in MeOD-d4. (**B**) This panel shows ^{1}H NMR spectra of etched nanoparticles in
493   MeOD-d4. In all spectra, * indicates the solvent peaks.

495   **Figure 7: Ligand density analysis.** A TGA measurement of the nanoparticles was done to
496   determine the ratio and density of organic material (ligands). The graph of the measurements is
497   plotted as the weight percentage *vs*. the temperature. OT desorbs first, between 176 °C to 233
498   °C (vertical lines). MUS degrades to smaller molecules and is totally burned at around 800 °C. The
499   remaining weight percentage corresponds to the gold core of the nanoparticles.

501   **Figure 8: Comparison of stoichiometric and NMR ratios of OT on the particles.** It is possible to
502   tune the amphiphilicity of the nanoparticles by changing the starting stoichiometric ratio
503   between MUS and OT in the reaction. The error bars show the upper and lower limit of OT
504   content acquired using the indicated stoichiometric ratios. Stoichiometric ratios of 10%, 20%,
505   *etc.*, up to 90% OT, were synthesized to observe the limits of OT content on the nanoparticle
506   surfaces.

508   **DISCUSSION:**
509   This protocol describes first the synthesis of MUS ligand and, then, the synthesis and
510   characterization of amphiphilic MUS:OT gold nanoparticles. Synthesizing MUS with minimal salt
511   content enables a better reliability of the stoichiometric ratio between the ligands during the
512   nanoparticle synthesis, which is a key factor for the reproducible synthesis of MUS:OT
513   nanoparticles with a target hydrophobic content (**Figure 8**). The use of methanol as a common
514   solvent for MUS and OT, along with the synthesis of the particles in ethanol, allows for a reliable
515   synthesis of MUS:OT gold nanoparticles. The methods of characterization presented here make
516   up a minimal list of experiments necessary to acquire sufficient information on the nanoparticles
517   to verify the outcome of their synthesis.

519   There are four critical steps in this protocol: (i) the synthesis of MUS with a low salt content along
520   with the removal of colored impurities in the second step and a crystallization of pure MUS in the
521   end; (ii) tuning and determining the stoichiometric ratio between MUS and OT; (iii) the workup
522   of the nanoparticles; and (iv) the characterization of the nanoparticles.

524   During the formation of nanoparticles, MUS preferentially binds to the nanoparticle surfaces,

525 which might be related to the solubility of the resulting nanoparticles. For example, a 2:1
526 stoichiometric feed ratio between MUS and OT results in 15% of OT on the surface when
527 calculated using the data from the ^{1}H NMR of etched gold nanoparticles. Therefore, a higher
528 content of OT must be used during the synthesis of the particles (**Figure 8**) to obtain a
529 nanoparticle with a lower MUS to OT ratio; in other words, a more hydrophobic particle. In order
530 to assess the stoichiometric ratio between the ligands on the surface of the nanoparticles, it is
531 necessary to ensure there are no unbound ligands in the solution. The presence of unbound
532 ligands affects the determination of the ligand ratio on the nanoparticles and the density, along
533 with subsequent tests and experiments that may lead to incorrect interpretations. Repetitive
534 cleaning cycles with different solvents (such as ethanol and DI water) are required to eliminate
535 all unbound ligands and other impurities (byproducts of sodium borohydride, gold ions, *etc.*). [1]H
536 NMR is important to confirm the purity of the nanoparticles. The line-broadening effect of ligands
537 owing to the complex chemical environment on the nanoparticles broaden the peaks
538 corresponding to the ligands, while any sharp signal comes from unbound molecules[22].
539 Furthermore, due to restricted mobility, the NMR peaks corresponding to the methylene
540 adjacent to the thiol groups cannot be detected, which is another signature of the nanoparticle
541 when inspected using ^{1}H NMR. Once the nanoparticles are clean, then the metal core is etched
542 with iodine. Iodine etching is a well-established method to quantify the ligand ratio on
543 nanoparticles. For instance, two decades ago, Murray *et al.* reported the determination of
544 monolayer composition on gold nanoparticles after iodine etching, in which the iodine
545 decomposes the gold core and releases the thiolate ligands as disulfides[23]. The reliability of the
546 iodine etching method has been established using other methods; for example, Harkness *et al.*
547 reported that the ligand ratio obtained from NMR is within 1% deviation from mass spectroscopy
548 measurements[24].
549
550 TGA is a straightforward method to calculate the organic content on the nanoparticles. The
551 estimation of the surface ligand density assumes that all thiolate ligands bind to surface gold
552 atoms and all free ligands have been removed during purification. To determine the ligand
553 density, several assumptions are made, mainly that the particles are spherical, which is used to
554 calculate the surface area, as well as the packing density, of the gold core. TEM provides a size
555 distribution of the nanoparticle gold cores that can be used to calculate the approximate surface
556 area of a nanoparticle. The nanoparticle synthesis described here produces a polydisperse
557 population of particles with an average diameter of 2 - 3 nm and a size deviation of up to 30%.
558 Also, the average radius, used to calculate the average volume of one particle (approximating the
559 particles to spheres), combined with the density of gold, enables the calculation of the mass of
560 one nanoparticle. Then, the mass measured by TGA over 800 °C enables the calculation of the
561 number of particles initially present. By using this value and the average core size, the total
562 surface area of the gold nanoparticles can be estimated. The ligand ratio calculated from the data
563 acquired with ^{1}H NMR spectroscopy allows for the calculation of the number of moles of the
564 ligands on the surface of the nanoparticles. The molar ratio between the ligands over the surface
565 area of gold nanoparticles provides the ligand density (**Figure 7**). Clean nanoparticles have
566 approximately 4 ligands per nm^2. TGA data can also be used to estimate the ligand ratio, if the
567 temperature interval in which they desorb from the gold surface is known for each ligand, and
568 desorption occurs at separate temperature ranges.

569

570   In summary, this protocol provides a straightforward way to synthesize the MUS ligand with a
571   low salt content and MUS:OT amphiphilic gold nanoparticles. One of the key factors of the
572   reproducibility of these nanoparticles is the low inorganic salt content in the MUS used. These
573   nanoparticles are stable both as powder and in solution (*e.g.*, H_2O and physiologically-relevant
574   ones), which should be emphasized as a prerequisite for many applications. Thorough
575   characterization of the size and the surface properties of amphiphilic nanoparticles is essential
576   for future applications in which the degree of amphiphilicity may play a key role.

577

583

584   **DISCLOSURES:**
585   The authors have nothing to disclose.

586

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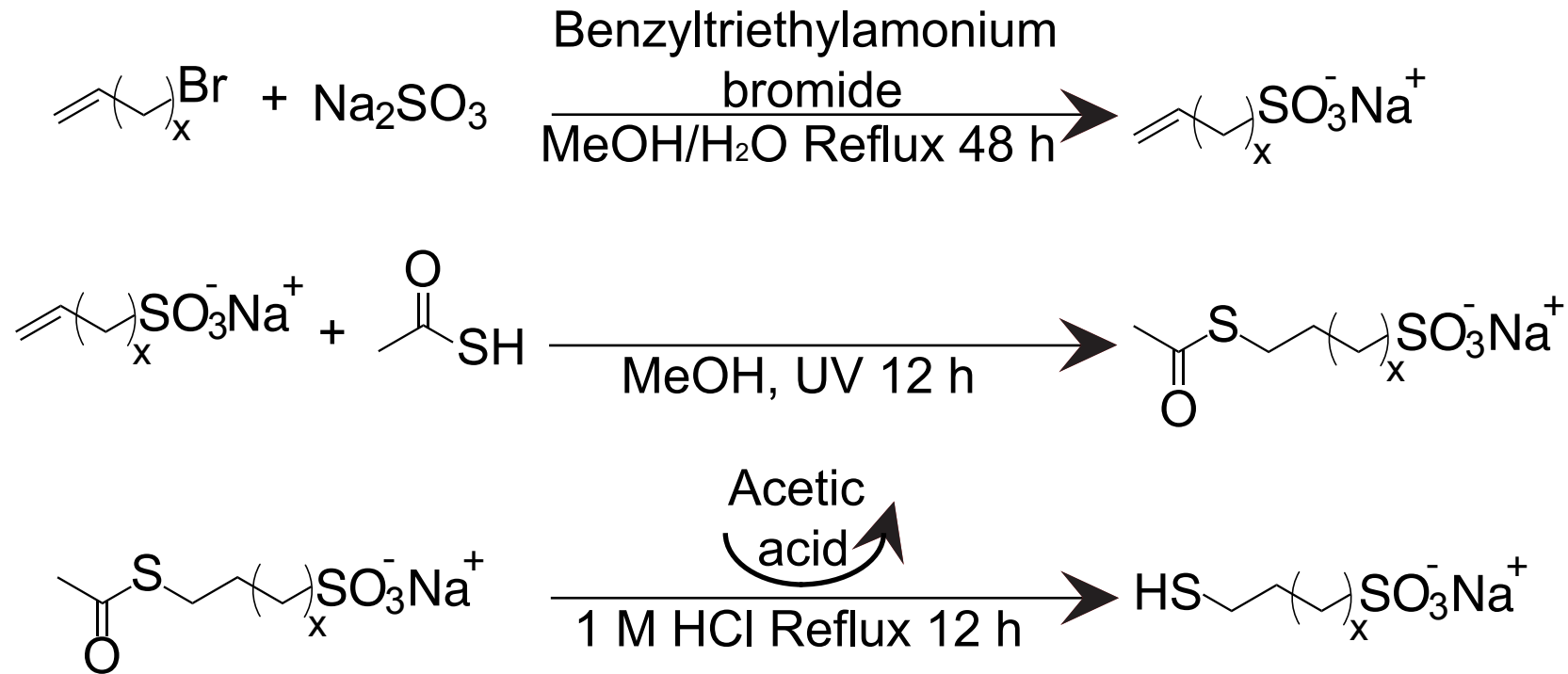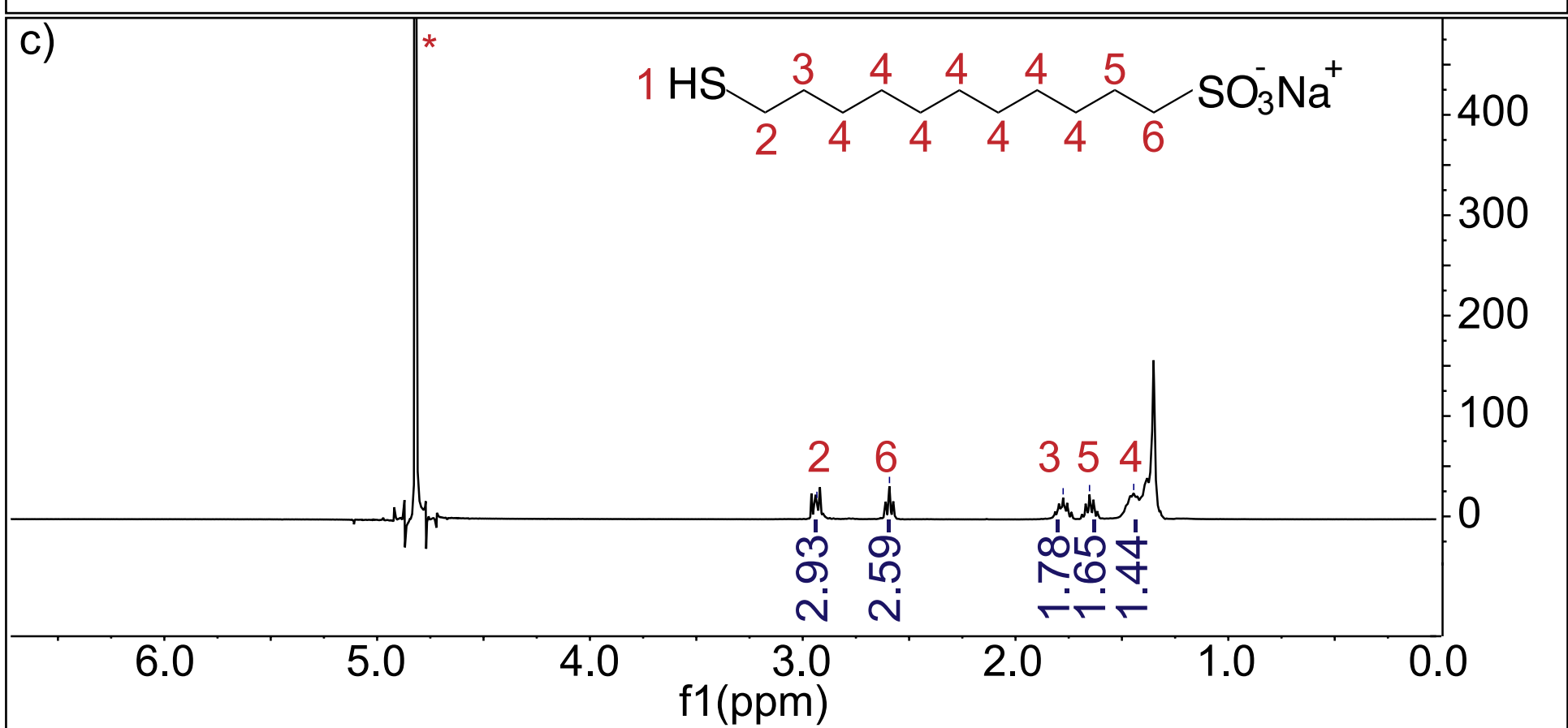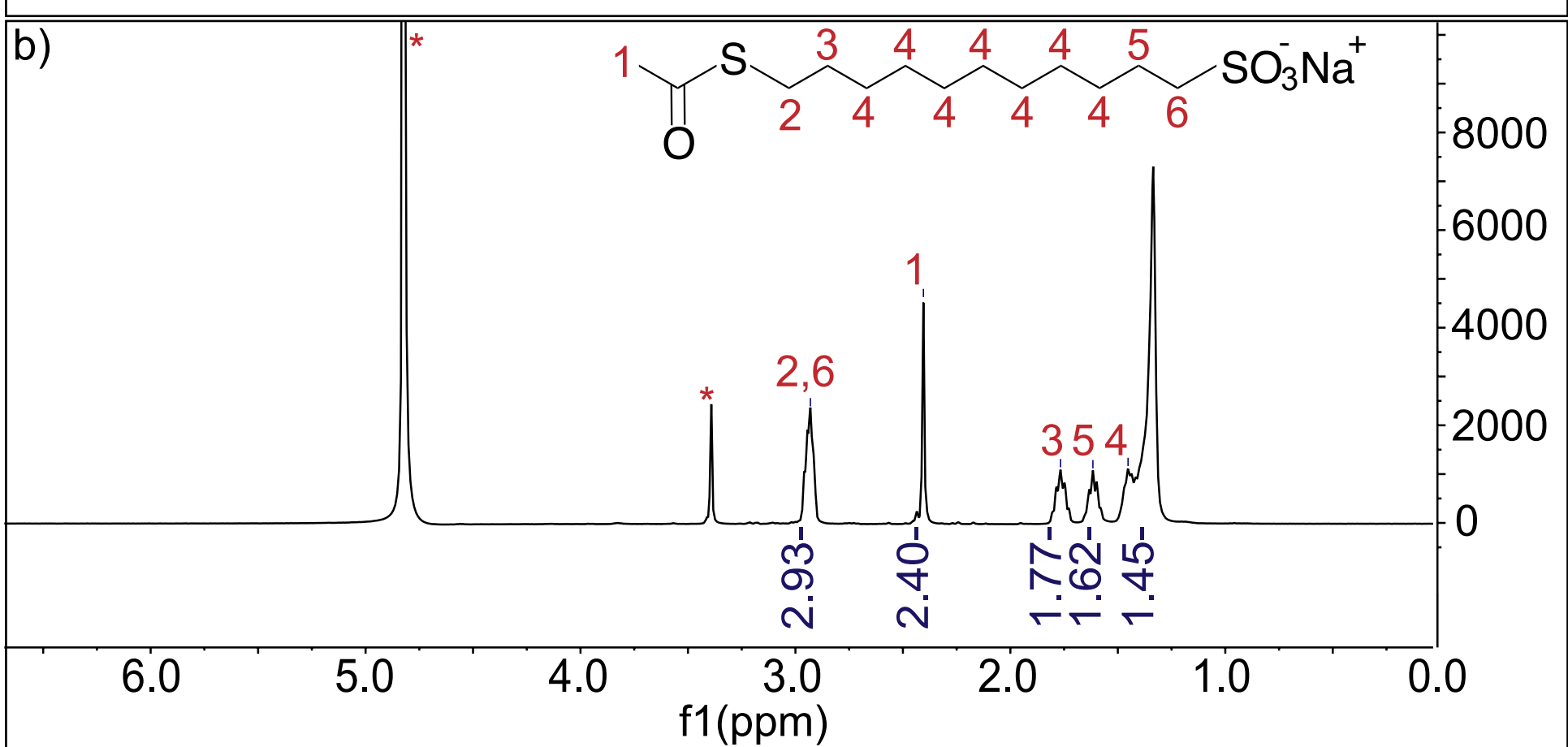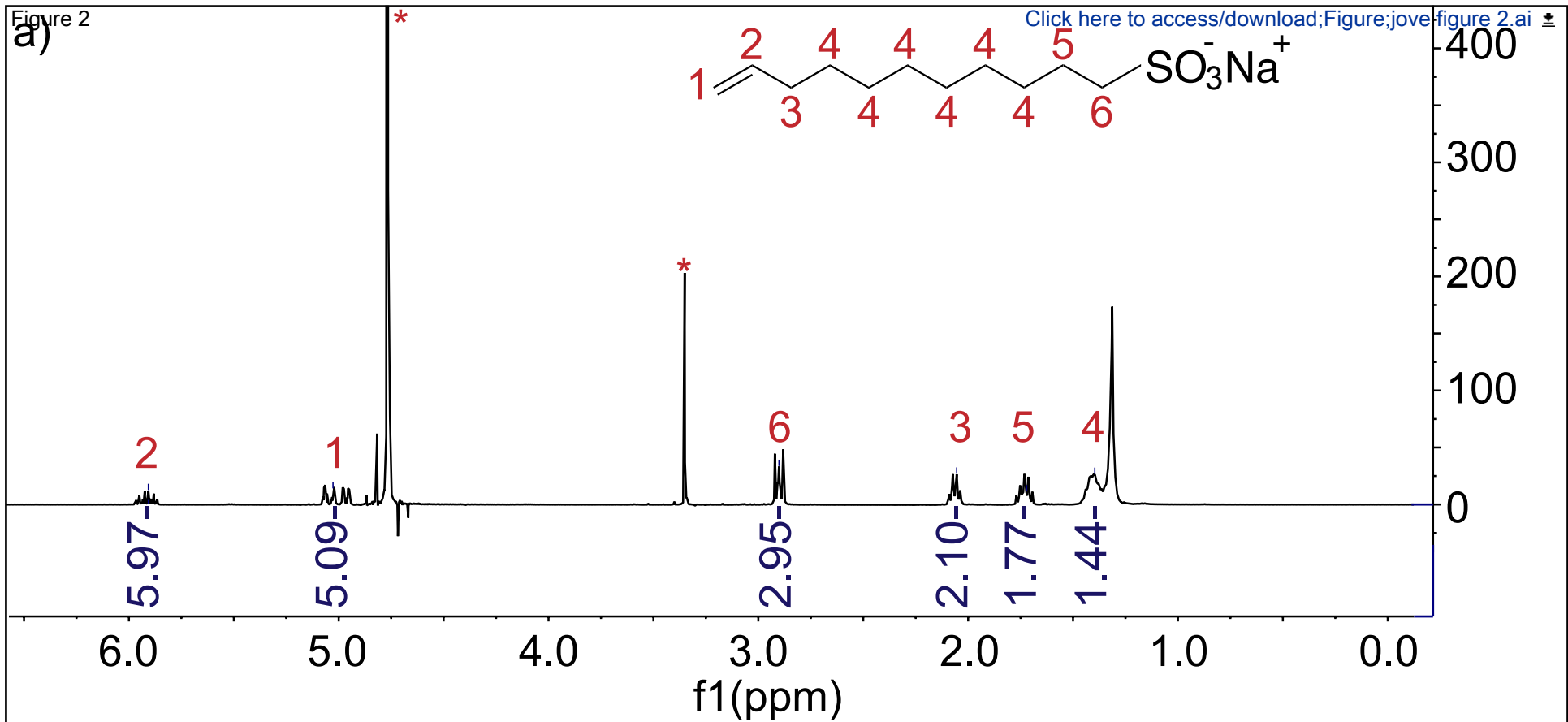
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662

Figure 1

Figure 1

Figure 2

Figure 3

a Addition of gold precursor and ligands

Cl⁻ Cl⁻

Au^{3+} H⁻O⁻H

Cl⁻

SH‿‿‿‿‿‿

Octanethiol (OT)

SH‿‿‿‿‿‿SO_3^- $=O$

Gold(III) chloride trihydrate

11-mercapto-1 -undecanesulfonate (MUS)

Ethanol

b Formation of gold-thiolate complex

NaBH₄ Sodium borohydride

(Au–S–Au–S–Au)
   R   R
   SR SR SR

c Formation of gold nanoparticles
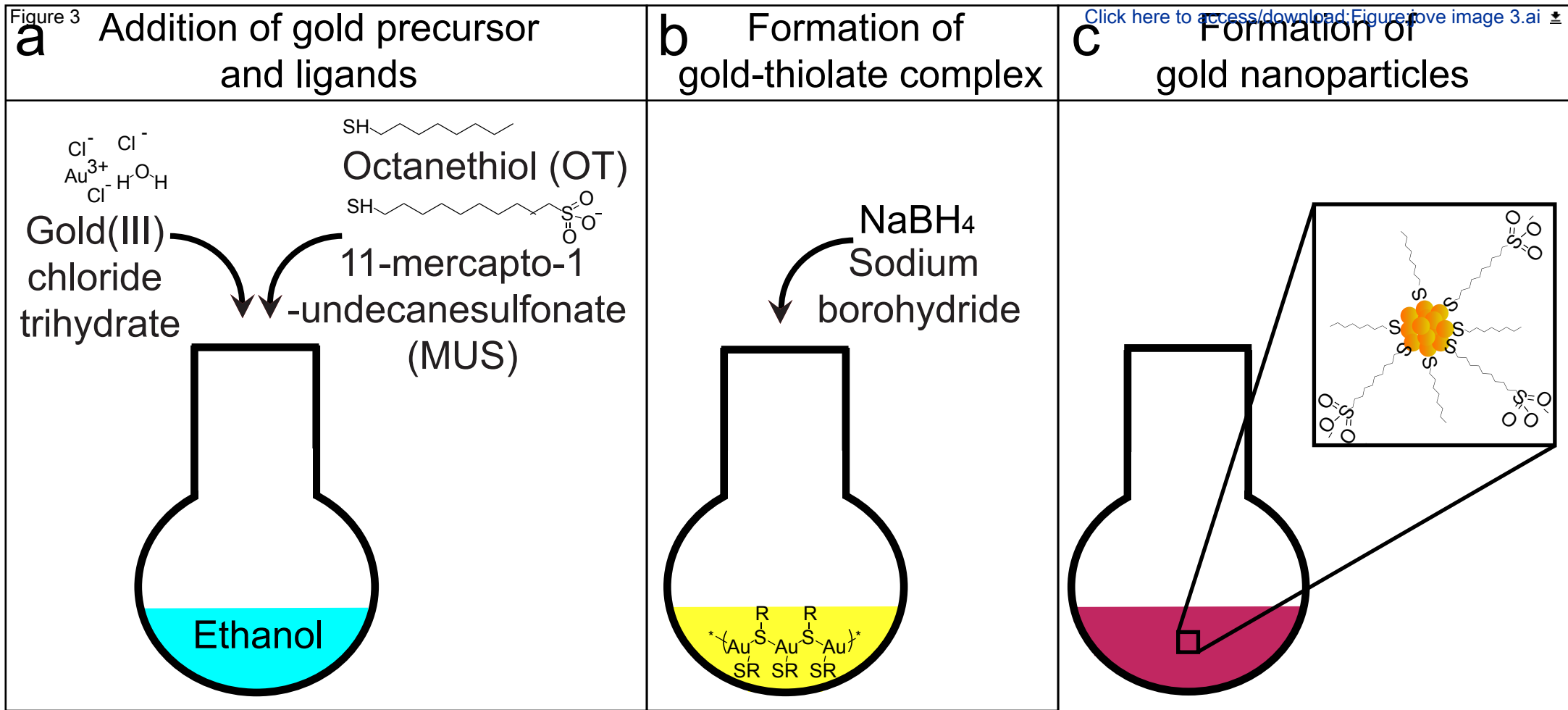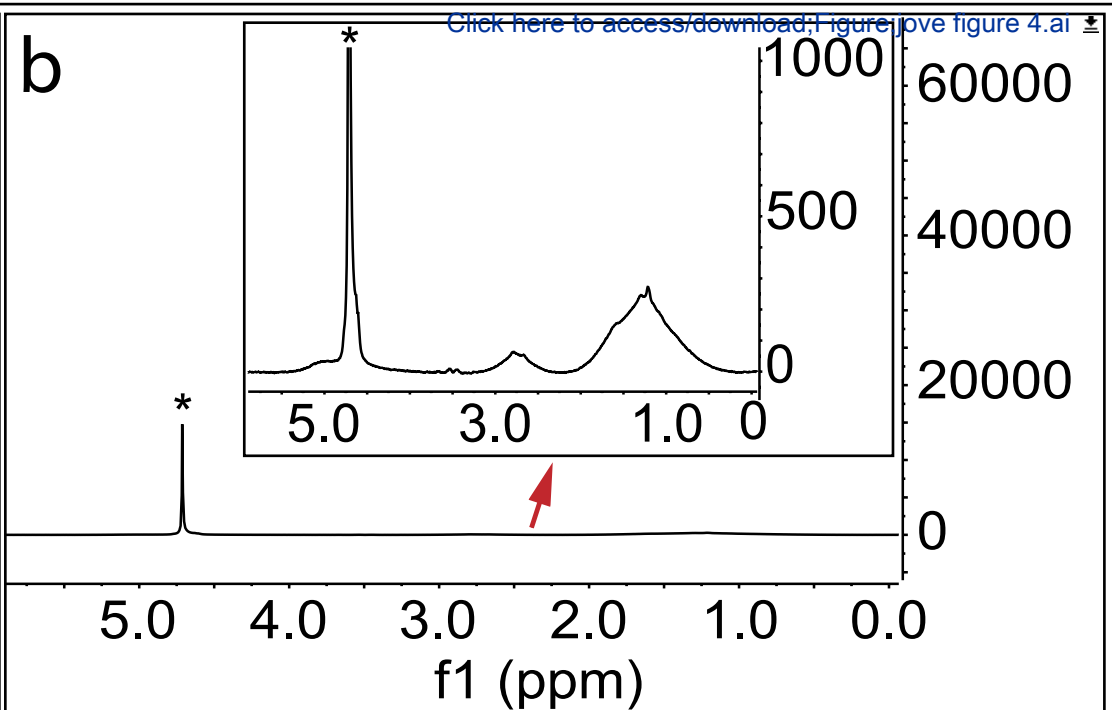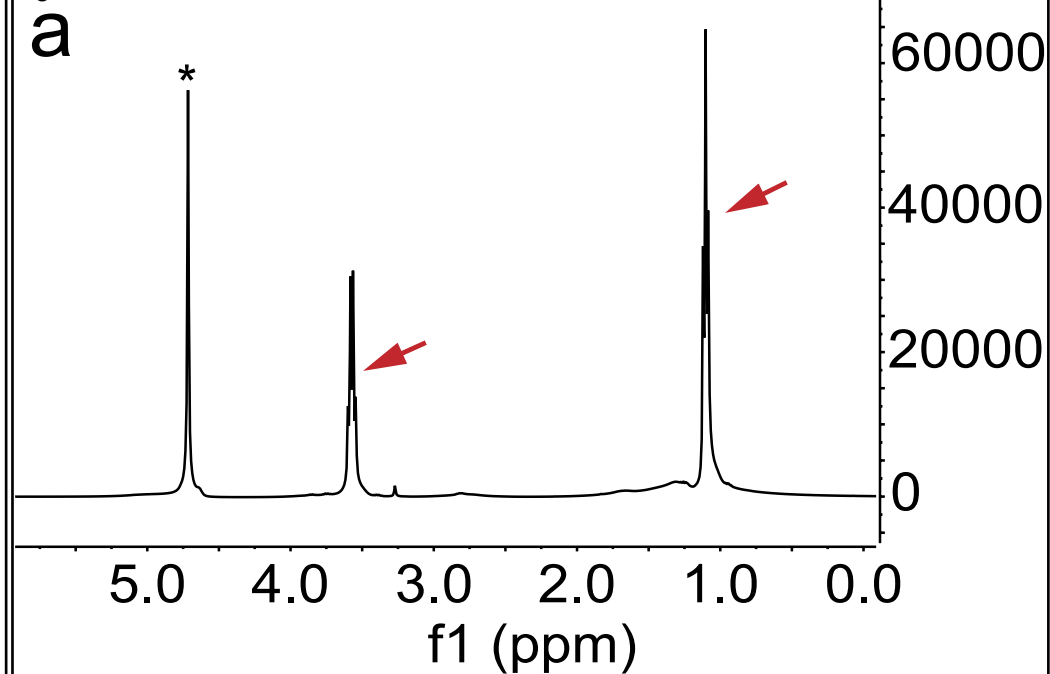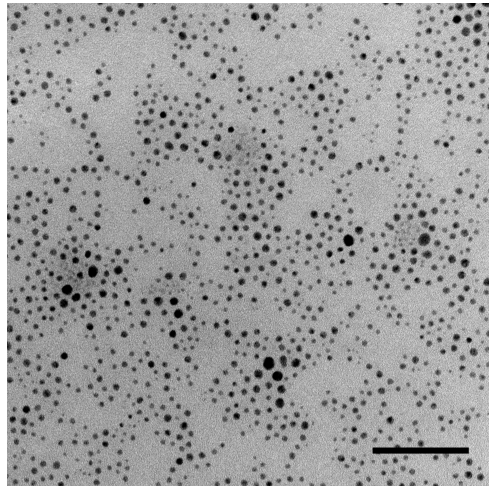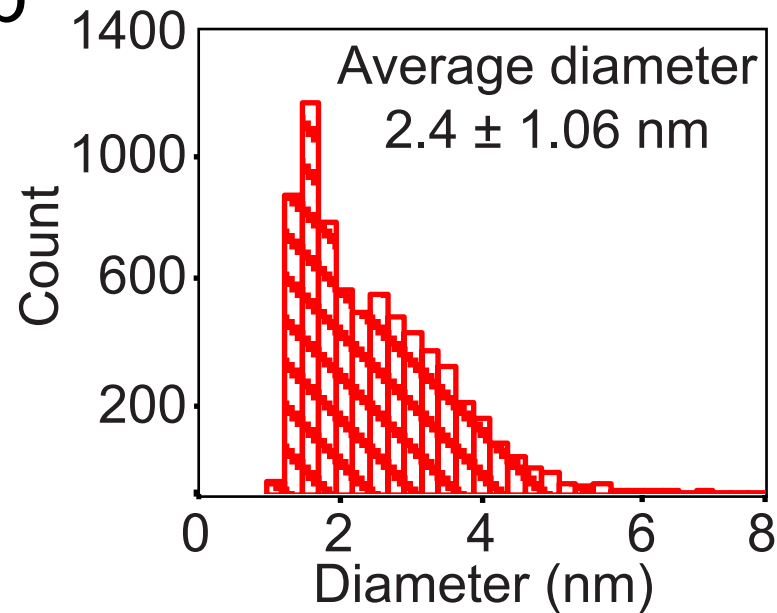
Figure 4

Figure 5

Figure 6
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Figure 7

Figure 8

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
11-bromo-1-undecene	Sigma Aldrich	467642-25 ml	
Sodium Sulfite	Sigma Aldrich	S0505-250 g	
Benzyltriethyl-ammonium bromide	Sigma Aldrich	147125-25 g	
Methanol	VWR	BDH1135-2.5 LP	
DI water	Millipore	ZRXQ003WW	Deionized water
1 L round bottom flask	DURAN	24 170 56	
Diethyl ether	Sigma Aldrich	1.00930 EMD Millipore	
Stirring bar	Sigma Aldrich	Z329207,	
Dow Corning High Vacuum Grease	Sigma Aldrich	Z273554 ALDRICH	
Filtering flask	DURAN	20 201 63	
Filtering Buchner Funnel	FisherSci	11707335	
Ethanol >99.8%, ACS, Reagent	VWR	2081.321DP	
Deuterium dioxide	Sigma Aldrich	151882 ALDRICH	
Thioacetic acid 96%	Sigma Aldrich	T30805 ALDRICH	
Carbon black	Sigma Aldrich	05105-1KG	
Celite	Sigma Aldrich	D3877 SIGMA-ALDRICH	Filtration medium
Condenser	Sigma Aldrich	Z531154	
Hydrochloric acid, ACS reagent 37%	Sigma Aldrich	320331 SIGMA-ALDRICH	
Sodium Hydroxide, BioXtra, pellets (anhydrous)	Sigma Aldrich	S8045 SIGMA-ALDRICH	
Centrifuge tubes	VWR	525-0155P	
250 mL round bottom flask	DURAN	24 170 37	
500 mL round bottom flask	DURAN	24 170 46	
Nitric acid, fACS reagent 70%	Sigma Aldrich	438073 SIGMA-ALDRICH	
Gold(III) chloride trihydrate >99.9% trace metal basis	Sigma Aldrich	520918 ALDRICH	
1-octanethiol >98.5%	Sigma Aldrich	471836 ALDRICH	
Sodium Borohydride purum p.a.>96%	Sigma Aldrich	71320 ALDRICH	
Separatory funnel	SIgma Aldrich	Z330655 SIGMA	
Funnel	DURAN	21 351 46	
2V folded filtering papers	Whatman	1202-150	

Amicon filters	Merck	UFC903024
Iodine, ACS reagent, >99.8%, solid	Sigma Aldrich	207772 SIGMA-ALDRICH
5 mm NMR-Tubes, Type 5HP (high precision)	Armar	32210.503 Length 178 mm
Methanol-d4 99.8 atom%D	Armar	16400.2035
TGA crucible	Thepro	9095-9270.45
400 mesh carbon supported copper grid	Electron Microscopy Science	CF400-Cu
quartz cuvette	Hellma Analytics	100-1-40

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Reviewer 1

The authors present a preparation of gold nanoparticles with a mixed ligand shell of a
hydrophobic and hydrophilic ligand at a carefully selected ratio intended to give stability
under biological conditions and to also be useful for delivering drugs to cell membranes.
This is an important topic of broad utility. A good detailed synthesis of the 11-
mercaptoundecane sulfonic acid ligand is provided in addition to synthesis and
modification procedure for the nanoparticles. It seems enough information is provided for
others to try the procedure.

Please provide more references or a little bit more discussion on how the iodine etching
method for determining the ligand ratio works and how reliable it is. Why does it say a
ratio of 66:34 is desired but on line 90 a 50:50 ratio is mentioned? Please run spell check,
for example on line 113 we have 'undec-10-enefulfonate'.

We thank Reviewer 1 for the comments. A more detailed discussion about iodine etching is
added to the discussion part of the paper. Line 449 - 455.

The ratios of nanoparticles were corrected. Line 100.

Spell check was run on the manuscript.

Reviewer 2

Guven et al. demonstrated a protocol to synthesize of amphiphilic gold nanoparticles by
using MUS and OT as ligand shell. Then the prepared nanoparticles are characterized by
NMR, TGA, TEM, UV-Vis techniques to identify the ligand ratios of MIS and OT. As
authors mentioned, the use of MUS ligand with low inorganic salt content is a key point
to prepare the reproducibility of the gold nanoparticles. Thus, the protocol of the
synthesis of MUS ligand with low content of inorganic salt is the major part in this
manuscript. The reproducibility, high salt and pH tests for the prepared AuNPs are
lacked in the manuscript. Overall, this manuscript provided a reasonable procedure of
the synthesis of MUS and eventually can be published after major corrections.

Could the provided protocol (MUS/OT as ligand shell and NaBH4 as reducing agent) be
also used for the preparation of amphiphilic silver nanoparticles?

How about using 11-Mercaptoundecanoic acid to replace 11-mercapto-1-
undcanesulfonate? It can reduce the synthesized procedure of 11-mercapto-1-
undcanesulfonate.

Figure 5 is missed in the manuscript. According to manuscript, the diameter of the
prepared AuNPs are about 2-3 nm. As we known, the smaller size of AuNPs, the less

We thank Reviewer 2 for the comments. The reproducibility of the nanoparticle synthesis protocol was provided in the Figure 8. 3 different batches of MUS:OT 2:1 nanoparticles were synthesized and resulted in nanoparticles with an average of 12% OT on the surface. The reaction is supposed to be performed under the conditions specified avoiding changes in salt and pH, this is implicitly discussed when we say that the starting materials (MUS) should be free of extra salt.

1-Silver nanoparticles were synthesized following the same protocol. Particles with a core diameter of 2.8 ± 1.3 nm were obtained using this synthesis. The ^{1}H-NMR characterization revealed the amount of OT on the surface was 20%, slightly higher than equivalent gold nanoparticles. However, contrary to the gold nanoparticles, silver nanoparticles were less water soluble. All the characterization results are depicted in Figure 1.
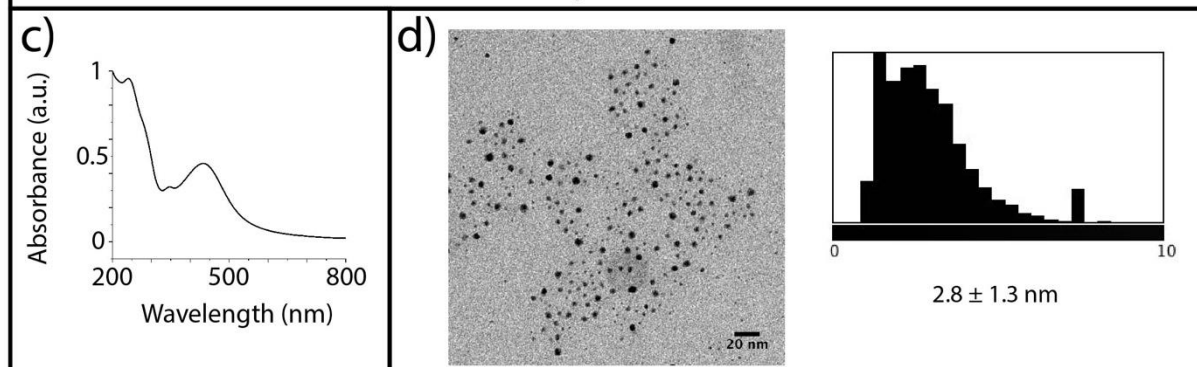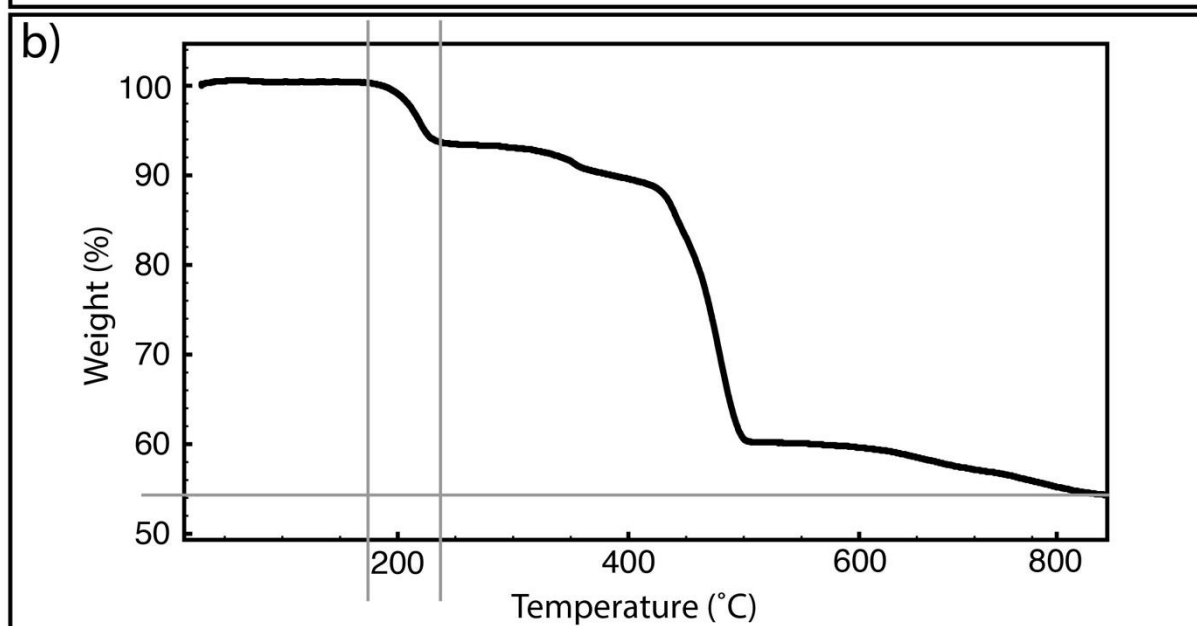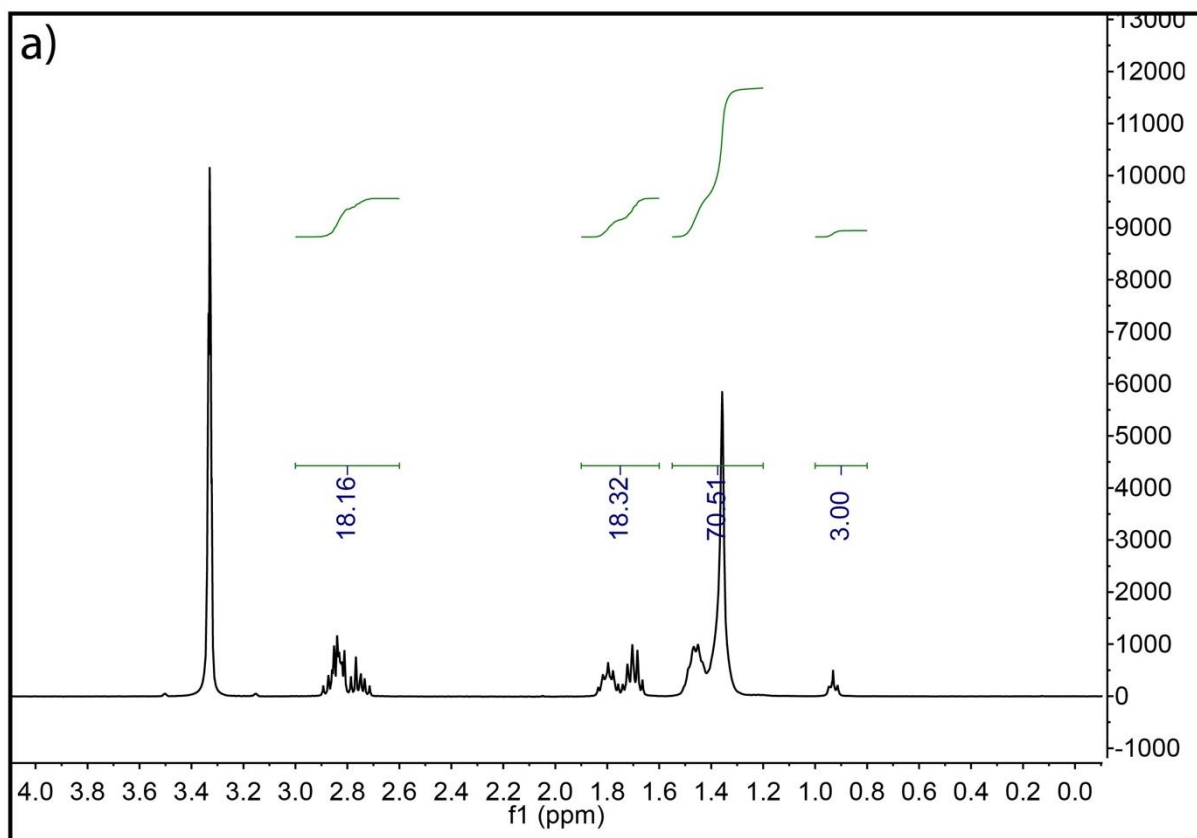
2- To produce amphiphilic nanoparticles phosphonic acid (11-mercaptophosphonic acid, MUP) terminated ligands were used instead of MUS. The synthesis resulted in 3.13 ± 1.4 nm core size with 7% OT on the surface (calculated using ^{1}H-NMR of the etched particles). TGA and calculations revealed 5 ligands per nm^2, similar to 2:1 MUS:OT nanoparticles. However, the solubility of MUP:OT, amphiphilic nanoparticles in physiological buffers is drastically lower than that of MUS:OT nanoparticles. MUP:OT nanoparticles dissolve in basic solutions, as well as water-ethanol mixtures. Therefore, in our experience amphiphilic nanoparticles having sulfonated ligands on the surface are more colloidally robust in physiological buffers compared to other amphiphilic nanoparticles tested. All the characterization results are depicted in Figure 2.

a)

b)

c)

d)

3.13 ± 1.4 nm
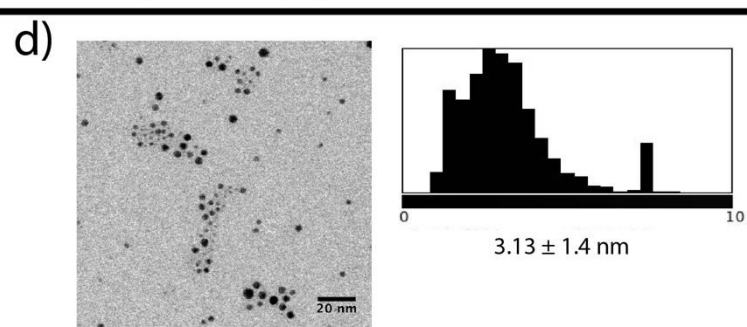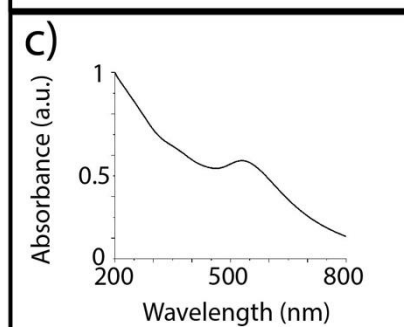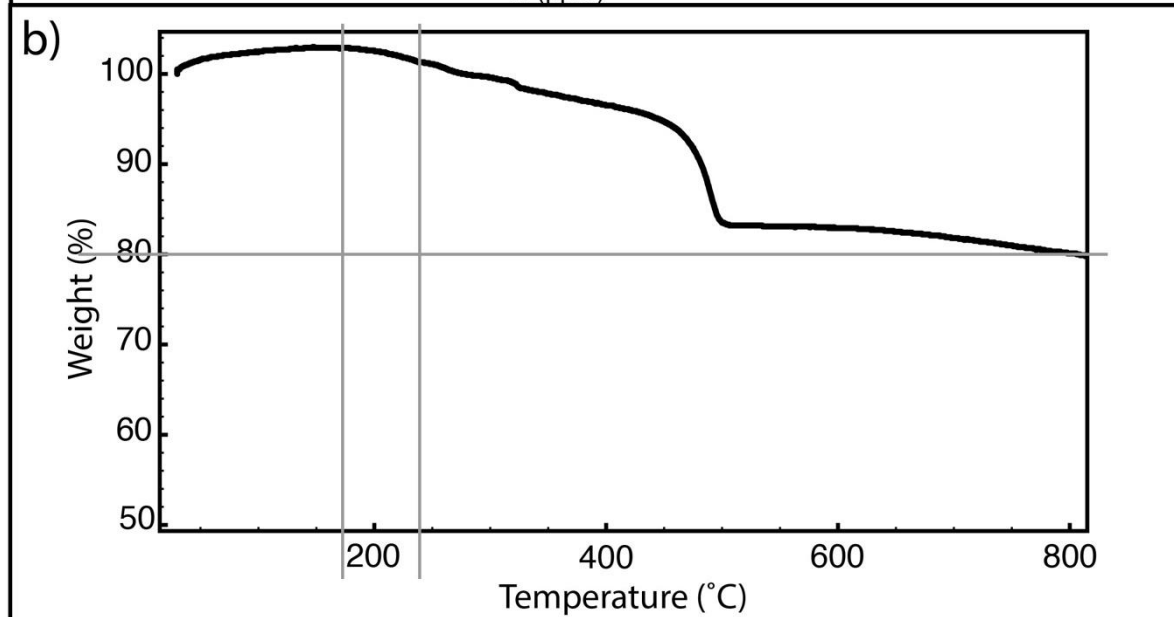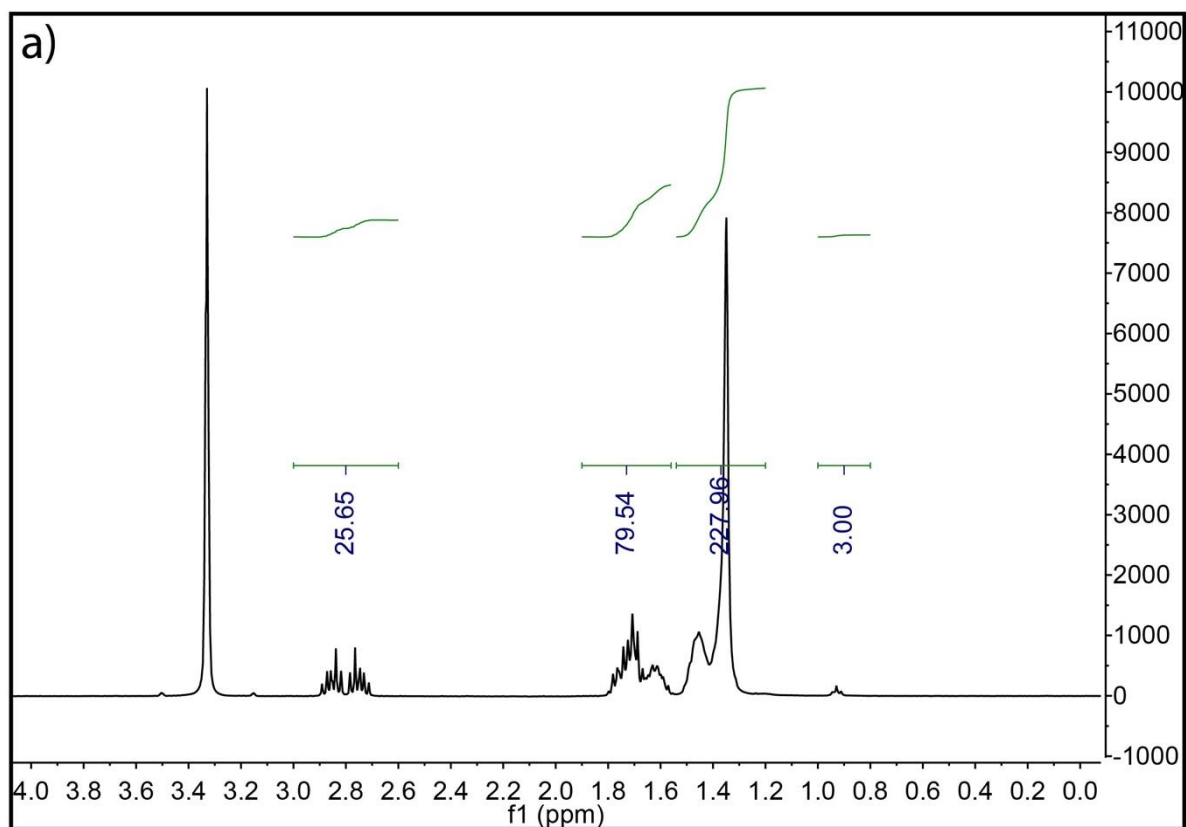
3- Figure 5 is added. The stability of the prepared AuNP is extremely high. No distinct degradation of MUS:OT gold nanoparticles could be seen after 1 year. This synthesis method does not result in nanoparticles larger than the dimensions reported in this protocol.

The chemical names were corrected and a spellcheck was ran throughout the manuscript.

Reviewer 3

The manuscript "Synthesis and Characterization of Amphiphilic Gold Nanoparticles therapy by Guvenet al., underwent review and work was found scientifically sound and well structured. The discussion of the manuscript is also very well written.
This work is dedicated to the one-phase chemical reduction followed by thorough purification to generate these nanoparticles. This approach seems promising. However, the submitted manuscript provokes some questions and comments (see below).
1 The level of English is not good especially in introduction.
2 Generally in abstract references should be avoided.
3 Line 44 what is the meaning of this sentence "in water 9combined with"
4 Line 71, remove 9 from start of next sentence "9 Moreover, these"
5 The author does not refer the current published articles dedicated to the synthesis of traditional methods. Please incorporate these references in revised manuscript,
a Mirza, A.Z., Shamshad, H. Fabrication and characterization of doxorubicin functionalized PSS coated gold nanorod. Arabian Journal of Chemistry (2014), http://dx.doi.org/10.1016/j.arabjc.2014.08.009.
c Eugenia Li Ling Yeo, Joshua U-Jin Cheah, Bing Yi Lim, Patricia Soo Ping Thong, Khee Chee Soo, and James Chen Yong Kah, Protein Corona around Gold Nanorods as a Drug Carrier for Multimodal Cancer Therapy, ACS Biomater. Sci. Eng., 2017, 3 (6), pp 1039-1050.
b Mirza, A.Z., A novel drug delivery system of gold nanorods with doxorubicin and study of drug release by single molecule spectroscopy, Journal of Drug Targeting, Volume 23, Issue 1, 2015, Pages 52-58.
6 Author should mention name of chemical with abbreviation then use abbreviation throughout the manuscript for example "11-mercaptoundecane sulfonic acid (MUS)".
7 Line 166 something missing after chemical name "1.3. 11-mercapto-1-undecanesulfonate (MUS):"?
8 Why author mention protocol in numbering not in paragraph?.
9 ml and µl should be mL and µL throughout the manuscript.
10 There is missing of Figure 5. Size distribution of nanoparticles in manuscript. Please provide image of TEM.
This paper should be accepted after major revision.

1- We have improved the level of the technical English employed throughout the text.
2- References in the abstract were removed.
3- It was a misspelling and it was corrected. Line 79
4- Misspelling was corrected.
5- The references were added. Lines 66 and 67.
6- Abbreviations were mentioned once and used throughout the manuscript.

7- There was not missing part on the sentence. '11-mercaptoundecane-1-undecanesulfonate (MUS)'in line 215 represents the headline of the 3rd and final step of overall MUS ligand synthesis.

8- The format of the JOVE paper requests the protocol with numbering.

9- The units were corrected throughout the manuscript.

10- Figure 5 is provided.