Insights on the Application of the Retro Michael-type Addition on Maleimide-Functionalized Gold Nanoparticles in Biology and Nanomedicine

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Supporting Information
# Table of Contents

1. Materials and Methods ................................................................. 3  
   1.1. $^1$H NMR measurements for the retro Michael-type addition on MPA-Maleimide-AuNPs and hydrolyzed MPA-Maleimide-AuNPs ................................................................. 4  
   1.2. $^1$H NMR measurements for the retro Michael-type addition on MPA-NMM and hydrolyzed MPA-NMM adducts .................................................................................. 6  
   1.3. Fluorescence measurements for the retro Michael-type addition on fluorogenic AuNPs and hydrolyzed fluorogenic AuNPs ......................................................... 7  

2. Synthesis of compounds ................................................................. 9  
   2.1. Synthesis of NMM-MPA Michael addition adduct ............................ 9  
   2.2. Synthesis of hydrolyzed NMM-MPA Michael addition Adduct ........ 9  
   2.3. Synthesis of 2-(4-(tritylthio)phenyl)acetic acid (trityl-protected MPA) ................................................................................................. 10  
   2.4. Synthesis of rhodamine B-trityl-protected MPA ................................ 10  
   2.5. Synthesis of rhodamine B-MPA ....................................................... 11  

3. Synthesis of Maleimide-AuNP conjugates ....................................... 12  
   3.1. Synthesis of MPA-Maleimide-AuNPs adduct .................................... 12  
   3.2. Synthesis of hydrolyzed MPA-Maleimide AuNPs .............................. 12  
   3.3. Synthesis of fluorogenic AuNPs ...................................................... 12  
   3.4. Synthesis of hydrolyzed fluorogenic AuNP ....................................... 13  

4. References ....................................................................................... 19
1. Materials and Methods

The following reagents were used as received. N-methylmaleimide (NMM), 4-mercaptophenylacetic acid (MPA), glutathione (GS), N,N-Diisopropylethylamine (DIPEA), triisopropylsilane (TIPS), tetrachloroauric acid trihydrate (HAuCl₄·3H₂O), sodium borohydride (NaBH₄), deuterated acetonitrile (CD₃CN), and deuterated methanol (CDCl₃) were purchased from Sigma-Aldrich. Deuterated chloroform (CDCl₃), and deuterium oxide (D₂O) were purchased from Cambridge Isotope Laboratories. Sodium dihydrogen-phosphate (NaH₂PO₄), trifluoroacetic acid (TFA), and sodium monohydrogen-phosphate (Na₂HPO₄) were purchased from Caledon. All common solvents, dry methanol (MeOH), sodium hydroxide (NaOH), and hydrochloric acid (HCl) were purchased from Caledon. Glacial acetic acid (99.7%) (AcOH) was purchased from BDH. N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU) was purchased from AK Scientific. Trityl chloride was purchased from Alfa Aesar. Dialysis membranes (MWCO 6000-8000 Da) were purchased from Spectra/Per.

¹H and ¹³C NMR spectra were recorded on a Varian Inova 400 or 600 MHz spectrometer using CDCl₃, CD₃OD or 0.2 M PBS (pH 7.4, 10% D₂O) as a solvent. ¹H NMR spectra are reported as δ in units of parts per million (ppm) relative to residual chloroform (δ 7.26, s), methanol (δ 3.34, s), water (δ 4.79, s), or dichloromethane (δ 5.31, s). Multiplicities are reported as follows: s (singlet), d (doublet), t (triplet), q (quartet), quin (quintuplet), dd (doublet of doublets), m (multiplet), and bs (broad signal). Coupling constants are reported as J values in Hertz (Hz). The number of protons (n) for a given resonance is indicated as nH, and is based on spectral integration values. ¹³C NMR spectra are reported as δ in units of parts per million (ppm) relative to CDCl₃ (δ 77.2), and CD₃OD (δ 49.0). Spectra were analyzed using ACD/SpecManager software v11.01. The spectra were baselined manually and peaks automatically integrated.

UV-Visible spectra have been recorded using a Varian Cary 100 Bio spectrometer and 1 cm quartz cuvettes. 10⁻⁵ M solutions of rhodamine B-MPA were prepared in spectroscopic grade methanol or 0.2 M PBS pH 7.4. The background was automatically subtracted from each spectrum.

Emission spectra were recorded using a Photon Technologies International, Inc. Quanta Master-7/2005 fluorimeter and 1 cm quartz cuvettes. 10⁻⁶ M solutions of rhodamine B-MPA were prepared in spectroscopic grade methanol or 0.2 M PBS pH 7.4.

Infrared spectra were recorded using a Bruker Vector33 spectrometer by making a thin film of sample on a KBr disk. The background was automatically subtracted from each spectrum.
Thermogravimetric analysis (TGA) data were recorded by loading the sample in a 70 µl ceramic crucible and heating from 25 ºC to 750 ºC at rate of 10 ºC min\(^{-1}\). The experiment was run under a nitrogen flow of 70 ml min\(^{-1}\) in a Mettler Toledo TGA/SDTA 851 instrument.

Transmission electron microscopy (TEM) images were recorded from a TEM Philips CM10. The TEM grids (Formvar carbon film on 400 mesh copper grids) were purchased from Electron Microscopy Sciences and prepared by dropcasting a drop of nanoparticles solution directly onto the grid surface. Prior to the measurements the grids were left to dry for overnight.

1.1. \(^1\)H NMR measurements for the retro Michael-type addition on MPA-Maleimide-AuNPs and hydrolyzed MPA-Maleimide-AuNPs

\[^1\]H NMR spectroscopy with W5 water suppression was used to monitor the retro Michael-type addition reaction of the MPA-maleimide adduct on the AuNPs. In general, in an NMR tube, MPA (1eq), maleimide-AuNPs (1 eq. in maleimide) and GS (1eq) were dissolved in 556 µL of a 0.2 M phosphate buffer solutions (PBS) pH 7.4 with 10% D\(_2\)O. The final concentrations of MPA, maleimide and GS were 18 mM each. After the addition of all reagents, the pH of the mixture was checked to ensure that it remained 7.4, within the pH range of maleimide stability. \(^1\)H NMR spectra were recorded periodically to follow the reaction.

Freshly prepared solutions of Maleimide-AuNPs, MPA and GS were made right before performing each experiment to avoid formation of disulfides or hydrolyzed maleimide. For the Maleimide-AuNP solution, 20 mg of Maleimide-AuNPs were transferred into a small vial and dried down into a film. Next, 444.7 µL of the 0.2 M PBS pH 7.4 with 10% D\(_2\)O were added. The solution of maleimide-
AuNPs was sonicated for 5 minutes to ensure maximum dissolution of the nanoparticles. Next, 83.3 µL of a 72 mM solution of MPA in the PBS (0.2 M, pH 7.4, 10% D₂O) was added to the vial containing the Maleimide-AuNPs solution. The Michael addition reaction was carried out for five minutes while sonicating. Once five minutes had passed, the MPA-maleimide-AuNPs adduct was quantitatively transferred into a NMR tube. A ¹H NMR of the MPA-maleimide-AuNPs adduct was recorded. During this step a small amount of MPA attacks the gold core through a direct ligand exchange reaction. This amount can be easily calculated from the ¹H NMR spectrum recorded right after the addition of GS (vide infra) because the kinetic of the ligand exchange reaction between GS and MPA is much faster than that of the retro Michael-type addition reaction. Lastly, 28.0 µL of a 357 mM solution of GS in the PBS (0.2 M, pH 7.4, 10% D₂O) were added to the NMR tube to obtain a final concentration of GS of 18 mM and allow the comparison of our results with those of Baldwin and Kiick.¹ The NMR tube was shaken vigorously, and immediately afterwards a ¹H NMR spectrum was recorded to designate t = 0. The NMR tube was then placed in a 37 °C oil bath signifying the beginning of the kinetics study. The reaction was monitored periodically for 7 days. The experiment was repeated in triplicate.

For quantifying the release of MPA due to the retro reaction over time, all ¹H NMR spectra recorded during the kinetic experiment were corrected by subtracting the amount MPA that was introduced through direct ligand exchange. The amount of MPA that was introduced through the ligand exchange was obtained from the ¹H NMR spectrum recorded at t = 0. This amount was found to be 22%, while 78% was the amount of MPA that was conjugated onto the interfacial maleimides through the Michael-type addition reaction.

The same procedure was applied for the experiment involving the hydrolyzed MPA-maleimide AuNPs adduct. For this experiment, 20 mg of hydrolyzed MPA-maleimide-AuNPs were transferred into a small vial and dried down into a film at the bottom of the vial. Next 527 µL of the PBS were added. The hydrolyzed MPA-maleimide-AuNPs solution was sonicated to ensure maximum dissolution of the nanoparticles. Next the hydrolyzed MPA-maleimide-AuNPs solution was quantitatively transferred into a NMR tube. A ¹H NMR spectrum of the hydrolyzed MPA-maleimide-AuNPs adduct was recorded. Lastly 28.0 µL of a 357 mM solution of GS dissolved in the PBS (0.2 M, pH 7.4, 10% D₂O) was added to the NMR tube to obtain a final concentration of GS of 18 mM. The NMR tube was shaken vigorously, and immediately afterwards a ¹H NMR spectrum was recorded to designate t = 0. The NMR tube was then placed in a 37 °C oil bath signifying the beginning of the kinetics study. The reaction was monitored periodically for 7 days. The experiment was repeated in triplicate.
1.2. $^1$H NMR measurements for the retro Michael-type addition on MPA-NMM and hydrolyzed MPA-NMM adducts

$^1$H NMR spectroscopy with W5 water suppression was used to monitor the retro Michael-type addition reaction of MPA-NMM conjugates. In general, in an NMR tube MPA (1eq), NMM (1 eq) and GS (1eq) were dissolved in 1000 µL of a 0.2 M PBS pH 7.4 with 10% D$_2$O. The final concentrations of MPA, NMM and GS in the 1000 µL of PBS were 18 mM each. After the addition of all reagents the pH of the mixture was checked to ensure that it remained 7.4, within the pH range of maleimide stability.

Freshly prepared solutions of NMM, MPA and GS were made right before performing each experiment to avoid formation of disulfides or hydrolysis of the maleimide. In a NMR tube 500 µL of a 35.7 mM solution of MPA in PBS (0.2 M, pH 7.4, 10% D$_2$O) were added. A $^1$H NMR spectrum was acquired. Next 250 µL of a 72.0 mM solution of NMM in the PBS (0.2 M, pH 7.4, 10% D$_2$O) were added to the NMR tube. The NMR tube was shaken vigorously, and the reagents were allowed to react for five minutes to ensure that the Michael addition could occur. A $^1$H NMR spectrum of the MPA-NMM adduct was recorded. Lastly, 250 µL of a 72.0 mM solution of GS dissolved in the PBS was added to obtain a final concentration of GS of 18 mM. The NMR tube was shaken vigorously, and immediately afterwards a $^1$H NMR spectrum was recorded to designate t = 0. The NMR tube was placed in an oil bath at 37 °C to signify the beginning of the kinetics study. The reaction was monitored for 7 days. The experiment was completed in triplicate.

The same procedure was applied for the experiment involving the hydrolyzed MPA-NMM adduct. For this experiment, 500 µL of freshly prepared 36.0 mM solution of hydrolyzed MPA-NMM adduct in PBS (0.2 M, pH 7.4, 10% D$_2$O) were added to a NMR tube. A $^1$H NMR spectrum was acquired. Next
one equivalent of a 36.0 mM solution of GS dissolved in PBS was added (the final concentration of GS is 18 mM). The NMR tube was shaken vigorously, and immediately afterwards a $^1$H NMR spectrum was recorded to designated $t = 0$. The NMR tube was placed in an oil bath at 37 °C to signify the beginning of the kinetics study. The reaction was monitored for 7 days. The experiment was completed in triplicate.

1.3. Fluorescence measurements for the retro Michael-type addition on fluorogenic AuNPs and hydrolyzed fluorogenic AuNPs
Fluorescence spectroscopy was used to monitor the retro Michael-type addition reaction of the fluorogenic ("unlocked") AuNPs. In general, in an Eppendorf tube fluorogenic AuNPs (1eq) and GS (1 eq. with respect to original maleimide) were dissolved in 500 µL of a 0.2 M PBS pH 7.4. The final concentrations of rhodamine B-MPA-maleimide adduct and GS in the 500 µL of PBS were 10 mM each. This concentration was selected because represents the intracellular concentration of GS. After the addition of all reagents, the pH of the mixture was checked to ensure that it remained 7.4, within the pH range of maleimide stability. Fluorescence emission (λ_{exc} = 556.6 nm) and excitation spectra (λ_{em} = 591.5 nm) were recorded periodically to follow the reaction.

Freshly prepared solutions of fluorogenic AuNPs and GS were made right before performing each experiment to avoid formation of disulfides or hydrolyzed rhodamine B-MPA-maleimide Michael adduct. For the fluorogenic AuNP solution, 9 mg of fluorogenic AuNPs were transferred into a small vial and dried down into a film. Next, 460 µL of the 0.2 M PBS pH 7.4 were added. The AuNPs were sonicated for 5 minutes to ensure maximum dissolution of the nanoparticles. The solution was quantitatively transferred into an Eppendorf tube. 10 µL of this solution were diluted to 10 mL to obtain a solution 1•10^{-5} M with respect to the original maleimide functionalities on the nanoparticles. A UV-Vis spectrum, and subsequently fluorescence emission and excitation spectra were recorded. Subsequently, to the original solution in the Eppendorf tube 50 µL of a solution 0.1 M of GS in 0.2 M PBS pH 7.4 were added. The Eppendorf tube was shaken vigorously using a vortex, and immediately afterwards a sample of 10 µL of the reaction mixture was taken, and diluted to 10 mL. A UV-Vis spectrum was taken to make sure that the solution was optically matched with the previous sample absorption spectrum, and subsequently the fluorescence emission end excitation spectra were recorded to designate t = 0. The Eppendorf tube was then placed in a 37 °C oil bath signifying the beginning of the kinetic study. The reaction was monitored periodically for 5 days. The experiment was repeated in triplicate.

The same procedure was applied for the experiment involving the hydrolyzed ("locked") fluorogenic AuNPs adduct. For this experiment, 4.5 mg of hydrolyzed fluorogenic AuNPs were transferred into a small vial and dried down into a film at the bottom of the vial. Next, 230 µL of the 0.2 M PBS pH 7.4 were added. The hydrolyzed MPA-maleimide-AuNPs solution was sonicated to ensure maximum dissolution of the nanoparticles. The solution was quantitatively transferred into an Eppendorf tube. 5 µL of this solution were diluted to 5 mL to obtain a solution 1•10^{-5} M with respect to the original maleimide functionalities on the nanoparticles. A UV-Vis spectrum, and subsequently the fluorescence emission and excitation spectra were recorded. Subsequently, to the original solution in the Eppendorf tube 25 µL of a solution 0.1 M of GS in 0.2 M PBS pH 7.4 were added. The Eppendorf tube was
shaken vigorously using a vortex, and immediately afterwards a sample of 5 µL of the reaction mixture was taken, and diluted to 5 mL. A UV-Vis spectrum was taken to make sure that the solution was optically matched with the previous sample’s absorption spectrum, and subsequently fluorescence emission and excitation spectra were recorded to designate t = 0. The Eppendorf tube was then placed in a 37 °C oil bath signifying the beginning of the kinetic study. The reaction was monitored periodically for 5 days. The experiment was repeated in triplicate.

2. Synthesis of compounds

2.1. Synthesis of NMM-MPA Michael addition adduct

\[
\text{N-methylmaleimide (200 mg, 1.80 mmol) was dissolved in the minimum amount of methanol. Next, 4-mercaptophenylacetic acid (300 mg, 1.80 mmol) was added. The solution was stirred at room temperature for 1 hour. Afterwards the solvent was removed in vacuo. The Michael addition adduct was purified by dissolving it in the minimum amount of CH}_2\text{Cl}_2 \text{ followed by the addition of a large volume of hexanes. A white suspension formed upon addition of hexanes and was pipetted off. This process was repeated until the white precipitate was no longer produced. The milky solution was dried in vacuo to a white powder with a yield of 93\%.} \]

\[\text{\textsuperscript{1}H-NMR (CDCl}_3, 400 MHz): \delta (ppm) 7.50 (d, 2H, J = 8 Hz), 7.28 (d, 2H, J = 8 Hz), 4.03 (dd, 1H, J = 8 Hz, 4 Hz), 3.67 (s, 2H), 3.16 (dd, 1H J = 16 Hz, 8 Hz), 2.92 (s, 3H), 2.71 (dd, 1H, J = 16 Hz, 4 Hz).} \]

\[\text{\textsuperscript{13}C NMR (CDCl}_3, 100 MHz): \delta (ppm) 176.0, 175.6, 174.5, 134.59, 134.35, 130.4, 129.7, 44.1, 40.4, 36.3, 25.1.} \]

\[\text{FT-IR (KBr disk): } \nu (\text{cm}^{-1}) 3453, 3160, 3049, 2930, 1776, 1700, 1557, 1493, 1436, 1409, 1384, 1283, 1193, 1119, 1058, 1017, 953, 900, 809, 770, 734. \]

\[\text{HRMS (EI) [M] (C}_13\text{H}_12\text{NO}_4\text{S) calc: 279.0565, found: 279.0564.} \]

2.2. Synthesis of hydrolyzed NMM-MPA Michael addition Adduct

\[
\text{NMM-MPA adduct (100 mg, 3.50 mmol) was dispersed in 5 mL of distilled water. Next, one pellet of NaOH (0.0986 g) was added to the solution causing the NMM-MPA adduct to become completely soluble in water. The reaction was allowed to proceed for 30 minutes. The solution was acidified by the addition of an aqueous solution of HCl 1M to a pH \sim 2. The hydrolyzed adduct was extracted by washing it three times with ethyl acetate. The product was dried with Na}_2\text{SO}_4 \text{ and the solvent was removed in vacuo. Next, the dried product was redissolved in water. The solution}
\]
was filtrated and the filtrate was freeze-dried to yield a hygroscopic yellow solid with a 90% yield. The hydroyzed product was obtained in two isomers in a 1:4 ratio as determined by \(^1\)H NMR spectroscopy. \(^1\)H NMR (CD\(_3\)OD, 400 MHz): \(\delta\) (ppm) 7.45 (m, 2H), 7.26 (m, 2H), 4.04-3.92 (m, 1H), 3.60 (m, 2H), 2.95-2.55 (m, 5H). \(^1\)C NMR (CDCl\(_3\), 100 MHz): \(\delta\) (ppm) 175.3, 174.9, 173.65, 173.03, 137.0, 136.8, 134.9, 131.29, 131.21, 41.6, 39.0. 37.6, 26.69, 26.45. FT-IR (KBr disk): \(\nu\) (cm\(^{-1}\)) 3342, 3104, 2943, 2625, 1716, 1628, 1564, 1494, 1410, 1236, 1165, 1090, 1018, 909, 854, 807, 734.

2.3. Synthesis of 2-(4-(tritylthio)phenyl)acetic acid (trityl-protected MPA)

![Chemical structure](image)

To a solution of mercaptophenylacetic acid (MPA) (206 mg, 1.23 mmol, 1.0 eq.) in dry CH\(_2\)Cl\(_2\) (15 mL) under inert atmosphere, trityl chloride (423 mg, 1.52 mmol, 1.2 eq.) in dry CH\(_2\)Cl\(_2\) (15 mL) was added with stirring. The solution was left to react for 2 hours. Subsequently, the solvent was removed in vacuo. The product was purified by column chromatography using 1:4 acetone:CH\(_2\)Cl\(_2\) as the eluent. The product was isolated in 84% yield as a pale yellow solid (0.424 g, 1.03 mmol). \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta\) (ppm) 7.42-7.41 (m, 6 H) 7.28-7.18 (m, 9 H) 6.92 (m, 4 H), 3.51 (s, 2H). \(^1\)C NMR (100 MHz, CDCl\(_3\)): \(\delta\) (ppm) 117.1, 144.4, 134.6, 133.6, 132.7, 130.0, 129.1, 127.7, 126.7, 70.8, 40.5. HRMS (EI) [M + Na] (C\(_{27}\)H\(_{22}\)NaO\(_2\)S): calc. 433.1233, found 433.1239.

2.4. Synthesis of rhodamine B-trityl-protected MPA

The rhodamine B-piperazine starting material was synthesized according to our previously established procedure.\(^2\) To an ice-cold solution of trityl-protected MPA from the previous step (0.142 mg, 0.345 mmol, 1.3 eq.) in dry CH\(_3\)CN (10 mL) under inert atmosphere, an ice-cold solution of HBTU (207 mg, 0.546 mmol, 2.0 eq.) and DIPEA (0.10 mL, 74 mg, 0.57 mmol, 2.1 eq.) in dry CH\(_3\)CN (10 mL) was added with stirring. The reaction was let to proceed for 30 minutes at 0 °C. After 30 minutes a solution of rhodamine B-piperazine (205 mg, 0.278 mmol, 1.0 eq.) in dry CH\(_3\)CN (10 mL) was added to the reaction mixture and the solution was stirred for an additional 30 minutes at 0 °C. The reaction mixture was then quenched with a saturated solution of sodium bicarbonate and extracted with ethyl acetate. The organic phase was washed with water and dried over magnesium sulfate. The solvent was removed in vacuo and the crude product was purified by column chromatography using 1:4 acetone:CH\(_2\)Cl\(_2\) as the eluent. The product was isolated in 70% yield as a pale yellow solid (0.286 g, 0.65 mmol). \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta\) (ppm) 7.70-7.60 (m, 10 H) 7.40-7.30 (m, 15 H) 7.00-6.90 (m, 5 H) 6.80-6.70 (m, 5 H) 3.50 (s, 2H). \(^1\)C NMR (100 MHz, CDCl\(_3\)): \(\delta\) (ppm) 154.0, 143.0, 133.0, 132.0, 131.0, 130.0, 129.0, 128.0, 127.0, 126.0, 125.0, 124.0, 123.0, 122.0, 121.0, 119.0, 118.0, 117.0, 116.0, 115.0, 114.0, 113.0, 112.0, 111.0, 110.0, 109.0, 108.0, 107.0, 106.0, 105.0, 104.0, 103.0, 102.0, 101.0, 100.0, 99.0, 98.0, 97.0, 96.0, 95.0, 94.0, 93.0, 92.0, 91.0, 90.0, 89.0, 88.0, 87.0, 86.0, 85.0, 84.0, 83.0, 82.0, 81.0, 80.0, 79.0, 78.0, 77.0, 76.0, 75.0, 74.0, 73.0, 72.0, 71.0, 70.0, 69.0, 68.0, 67.0, 66.0, 65.0, 64.0, 63.0, 62.0, 61.0, 60.0, 59.0, 58.0, 57.0, 56.0, 55.0, 54.0, 53.0, 52.0, 51.0, 50.0, 49.0, 48.0, 47.0, 46.0, 45.0, 44.0, 43.0, 42.0, 41.0, 40.0, 39.0, 38.0, 37.0, 36.0, 35.0, 34.0, 33.0, 32.0, 31.0, 30.0, 29.0, 28.0, 27.0, 26.0, 25.0, 24.0, 23.0, 22.0, 21.0, 20.0, 19.0, 18.0, 17.0, 16.0, 15.0, 14.0, 13.0, 12.0, 11.0, 10.0, 9.0, 8.0, 7.0, 6.0, 5.0, 4.0, 3.0, 2.0, 1.0, 0.0. HRMS (EI) [M + Na] (C\(_{77}\)H\(_{82}\)NaO\(_2\)S): calc. 433.1233, found 433.1239.
reaction mixture. The reaction temperature was brought up to room temperature, and the reaction mixture was let to react for two hours. The solvent was removed in vacuo. The residual solid was washed with hexanes and redissolved in CH$_3$CN. This washing procedure was repeated 3 times. The crude product was then purified by column chromatography using 1:4 acetone:CH$_2$Cl$_2$ as the eluent. The product was isolated as a purple solid in 83.7% yield (275 mg, 0.271 mmol). $^1$H NMR (400 MHz, CD$_2$Cl$_2$): $\delta$ (ppm) 7.69-6.74 (m, 29 H), 3.64-3.17 (m, 18 H) 3.30 (m, 8 H), 1.29 (t, $J = 8$ Hz, 12 H). $^{13}$C NMR (100 MHz, CD$_2$Cl$_2$): $\delta$ (ppm) 171.4, 167.8, 158.2, 156.1, 144.95, 144.86, 144.82, 135.64, 135.28, 135.11, 134.8, 132.4, 130.79, 130.35, 129.95, 129.57, 129.54, 129.51, 129.36, 128.35, 128.19, 128.11, 127.26, 127.16, 114.2, 96.6, 71.2, 66.1, 61.2, 54.4, 46.5, 41.2, 15.6, 14.4, 12.8. HRMS (EI) [M] (C$_{59}$H$_{59}$N$_4$O$_3$S): calc. 903.4302, found 903.431.

2.5. Synthesis of rhodamine B-MPA

To a solution of rhodamine B-trityl-protected MPA (230 mg, 0.226 mmol, 1.0 eq.) from the previous synthesis in dry CH$_2$Cl$_2$ (7 mL) under inert atmosphere and in the dark, TIPS (0.230 mL, 178 mg, 1.12 mmol, 5 eq.) and trifluoroacetic acid (0.350 mL, 522 mg, 4.57 mmol, 20 eq.) were added. The reaction mixture was let to react for 2 hours, and the reaction was monitored by TLC. The solvent was removed, the crude was washed with hexanes and redissolved in CH$_2$Cl$_2$. This washing procedure was repeated three times. The crude purple solid was finally purified by column chromatography, using 3:1 CH$_2$Cl$_2$: ethanol as the eluent. Rhodamine B-MPA was isolated in 60% yield as a purple solid (102 mg, 0.131 mmol). Rhodamine B-MPA tends to easily oxidize and form disulfides. The compound was stored under argon, in the dark, and in the freezer. $^1$H NMR (400 MHz, CD$_2$Cl$_2$) (see Figure SI5): $\delta$ (ppm) 7.69-6.76 (m, 14 H) 3.66-3.57 (m, 10 H) 3.40-3.26 (m, 8 H), 1.31 (t, $J = 8$Hz, 12 H). $^{13}$C NMR (100 MHz, CD$_2$Cl$_2$): $\delta$ (ppm) 158.3, 156.3, 144.5, 132.6, 130.49, 130.27, 130.07, 129.9, 128.83, 128.10, 126.8, 114.62, 114.31, 96.8, 57.4, 46.7, 40.4, 12.9. HRMS (EI) [M] (C$_{40}$H$_{45}$N$_4$O$_3$S): calc. 661.3207, found 661.3227. FT-IR (KBr disk): $\nu$ (cm$^{-1}$) 3055, 2974, 2924, 2864, 2713, 1687, 1637, 1589, 1520, 1469, 1416, 1340, 1275, 1249, 1181, 1128, 1074, 1008, 972, 923, 820, 728 cm$^{-1}$. UV-vis (methanol, $\lambda_{\text{max}}$, $\varepsilon$): 565 nm, 0.3589 M$^{-1}$ cm$^{-1}$; 530 nm, 0.1763 M$^{-1}$ cm$^{-1}$. 11
3. Synthesis of Maleimide-AuNP conjugates

3.1. Synthesis of MPA-Maleimide-AuNPs adduct
The MPA-maleimide AuNPs adduct was synthesized by reacting 1 eq. of MPA to 1 eq. of maleimide groups on the AuNPs surface in a 0.2 M PBS pH 7.4. An excess of MPA was avoided because of the possibility of place exchange reaction between MPA and the AuNP’s ethylene glycol-based thiolated ligands. If excess MPA is used, this leads to AuNPs aggregation. In a typical synthesis 20 mg of Maleimide-AuNPs were transferred into a small vial and dried down into a film. Next 444.7 µL of the 0.2 M PBS pH 7.4 were added. The maleimide-AuNPs were sonicated to ensure maximum dissolution. Next 83.3 µL of a 72 mM solution of MPA in the 0.2 M PBS pH 7.4 were added to the Maleimide-AuNPs solution and allowed to react for five minutes while using sonication. The AuNPs became even more soluble in water upon addition of MPA. The MPA-Maleimide-AuNPs were then purified by overnight dialysis in nanopure water. See $^1$H NMR spectrum in Figure SI1.

FT-IR (KBr disk): $\nu$ (cm$^{-1}$) 3255, 2924, 2855, 1705, 1486, 1457, 1401, 1348, 1222, 1193, 1132, 1109, 1080, 1007, 874, 799, 768.

3.2. Synthesis of hydrolyzed MPA-Maleimide AuNPs
The MPA-Maleimide-AuNPs (20 mg) were dissolved in 5 mL of 0.5 M NaOH solution and stirred vigorously for one hour. The particles were then purified by overnight dialysis in water. See $^1$H NMR spectrum in Figure SI4.

FT-IR (KBr disk): $\nu$ (cm$^{-1}$) 3257, 2931, 2877, 1650, 1604, 1401, 1351, 1300, 1250, 1196, 1107, 1038, 926, 850.

3.3. Synthesis of fluorogenic AuNPs
To a solution of Maleimide-AuNP (17.5 mg) in 500 µL of CD$_3$OD / 10% CD$_3$CN and in the dark, 43.8 µL of a solution 0.05 M in CD$_3$OD of rhodamine-B-MPA and 43.8 µL of a solution 0.05 M in CD$_3$OD of MPA were added. The reaction was monitored by $^1$H NMR spectroscopy by following the disappearance of the maleimide’s alkene protons. When the reaction was completed, the solvent was removed and the fluorogenic AuNPs were purified by dialysis in methanol for 2 days. The solvent was changed 5 times overall. After two days the solvent was removed, yielding the fluorogenic AuNPs as a purple tinged black solid. See $^1$H NMR spectrum in Figure SI6.

FT-IR (KBr disk): $\nu$ (cm$^{-1}$) 2859, 1774, 1703, 1635, 1587, 1527, 1464, 1421, 1338, 1274, 1246, 1180, 1102, 1007, 922, 836.
3.4. Synthesis of hydrolyzed fluorogenic AuNP

The fluorogenic AuNP (6.3 mg) were dissolved in 0.5 M aqueous NaOH (1.57 mL) and stirred for 1 hour. The particles were then purified by overnight dialysis in water, yielding the hydrolyzed fluorogenic AuNPs as a purple tinged black solid.  

FT-IR (KBr disk): \(\nu (\text{cm}^{-1})\) 3259, 2917, 2869, 1701, 1643, 1588, 1529, 1452, 1411, 1348, 1276, 1248, 1180, 1105, 1034, 925, 839, 802, 721.

Figure S11: \(^1\)H NMR spectrum of MPA-Maleimide-AuNPs in 0.2 M PBS (pH 7.4, 10% D\(_2\)O). Spectrum recorded with W5 water suppression and referenced against residual CH\(_2\)Cl\(_2\) (5.31ppm).
Figure S12: Typical thermogravimogram of Maleimide-AuNP.

Figure S13: $^1$H NMR spectrum of the retro Michael-type addition reaction at the MPA-Maleimide-AuNP’s interface. Spectrum recorded after 160h of reaction in 0.2 M PBS (pH 7.4, 10% D$_2$O) with W5 water suppression, and referenced against residual CH$_2$Cl$_2$ (5.31ppm).
Figure SI4: $^1$H NMR spectrum of hydrolyzed MPA-maleimide-AuNPs adduct in 0.2 M PBS (pH 7.4, 10% D$_2$O). Spectrum recorded with W5 water suppression and referenced against residual CH$_2$Cl$_2$ (5.31ppm).

Figure SI5: $^1$H NMR spectrum of rhodamine B-MPA recorded in CD$_2$Cl$_2$. Spectrum referenced against residual CH$_2$Cl$_2$. 
Figure S16: $^1$H NMR spectrum of fluorogenic AuNPs recorded in CD$_3$OD. Spectrum referenced against residual CH$_3$OH.

Figure S17. TEM images of A) Maleimide-AuNP; B) MPA-Maleimide-AuNP; C) Hydrolyzed MPA-Maleimide-AuNP; D) MPA-Maleimide-AuNP after retro Michael addition reaction and exchange with glutathione; E) Fluorogenic AuNP.
Figure S18. $^1$H COSY NMR spectrum recorded with W5 water suppression of retro Michael-type addition on MPA-Maleimide-AuNP. Spectrum acquired 8h from the addition of glutathione. Spectrum recorded in 0.2 M PBS (pH 7.4, 10%D$_2$O).
Figure SI9. $^1$H COSY NMR spectrum recorded with W5 water suppression of retro Michael-type addition on MPA-Maleimide-AuNP. Spectrum acquired 48h from the addition of glutathione. Spectrum recorded in 0.2 M PBS (pH 7.4, 10%D$_2$O).
4. References

