Langmuir

Article

pubs.acs.org/Langmuir

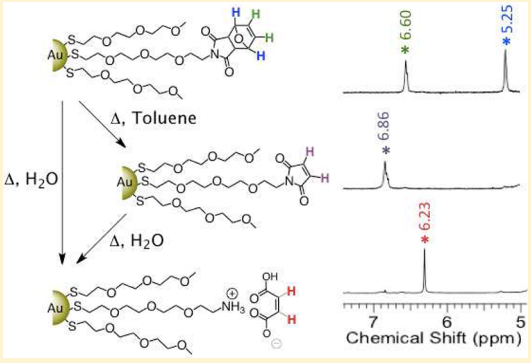
Improved Methodology for the Preparation of Water-Soluble Maleimide-Functionalized Small Gold Nanoparticles

Pierangelo Gobbo and Mark S. Workentin*

Department of Chemistry and the Centre for Materials and Biomaterials Research, Western University Canada, London, Ontario N6A 5B7, Canada

Supporting Information

ABSTRACT: Improved methodology to prepare maleimide-functionalized, water-soluble, small (<3 nm) gold nanoparticles using a retro-Diels–Alder strategy that we developed for similar organic-soluble AuNP's is described. Importantly, our results suggest that a recent paper by Zhu, Waengler, Lennox, and Schirrmacher describing a similar strategy gave results inconsistent with the formation of the titled maleimide-modified AuNP (Zhu, J.; Waengler, C.; Lennox, R. B.; Schirrmacher, R. *Langmuir* **2012**, *28*, 5508) as the major product, but consistent with the major product being an adduct derived from the hydrolysis of maleimide formed under the conditions used for the required deprotection of the maleimide. Our methodology provides an efficient and accessible route to pure maleimide-modified small AuNP's that circumvents the formation of the hydrolysis product. The maleimide-modified small AuNP's are versatile because they are soluble in water and in a wide range of organic solvents and their reactivity can now be properly exploited as a reactive moiety in Michael addition for bioconjugation studies in aqueous solution.

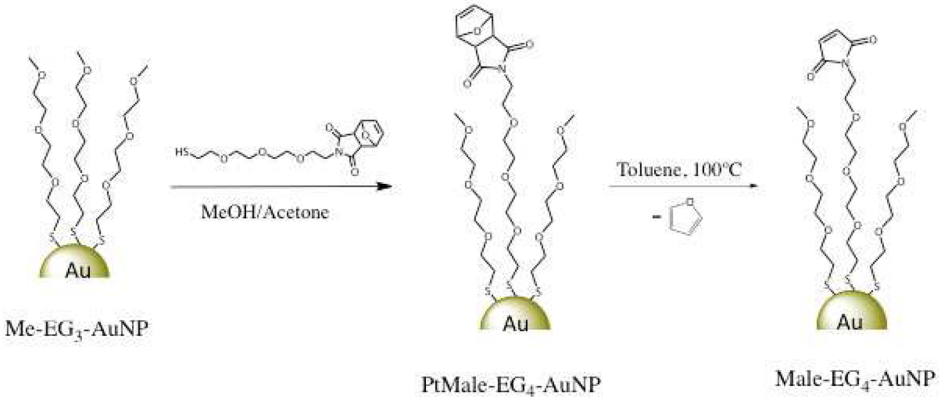


INTRODUCTION

Maleimide functionalities at the interface of small hydrosoluble nanoparticles are of interest in biological applications because of the ability of the maleimide moiety to react through Michael addition with nucleophiles, especially amine and thiols. This general methodology is extensively exploited to label peptides, proteins, DNA strands, and cells.[1,2] Thus, having a maleimide group at the interface of a AuNP would allow for the exploitation of this type of reactivity in the use of the nanoparticle in applications such as drug or substrate delivery or as an optical marker for diagnostics in biological systems.[3,4] Such water-soluble AuNP's are a desirable platform because of their chemical stability, the biocompatibility of the gold core, and the ability to tune the functionality at the interface through the reactivity of the maleimide. Indeed, interfacial maleimide can react with amine or thiol functionalities present on liposomes or cell membranes, allowing the nanoparticle to be immobilized on the biosystem while preserving all of the optothermal properties of the original nanoparticle.[5] Despite the numerous potential biological applications that these water-soluble maleimide AuNP's can have, until recently the only hydrosoluble maleimide-functionalized nanoparticles synthesized were larger than 15 nm and their synthesis involved a difficult place-exchange reaction from citrate-protected or from CTAB-protected (cetyltrimethyl ammonium bromine) gold nanoparticles with very bulky and complex polyethylene glycol-based ligands.[5,6] Monomaleimide-functionalized gold nanoparticles derived from phosphine-protected gold cores have been reported but are not very stable and thus are of limited utility.[7] Recently, Zhu, Waengler, Lennox, and Schirrmacher described an approach to preparing stable water-soluble maleimide-tethered gold nanoparticles with an average core size of ca. 3 nm.[8] The goal was to synthesize small, stable maleimide water-soluble AuNP's through a route involving less-elaborate ligands that are easier and faster to synthesize and are based on tri- or tetra-ethylene glycol. Because of the smaller size of the Au core and the simplicity of the ligands, these nanoparticles were expected to be easier to make and characterize, allowing the effective number of functionalities present at the nanoparticle interface to be calculated to provide the ability for more careful control of the number of potential bioconjugation sites. Their approach utilized a strategy that our group developed for the preparation of small organic-soluble maleimide AuNP's in the same size regime,[9] which itself was based on approaches used to install maleimide on other types of materials.[10−12] In fact, the strategy has long been used as a click-type reaction in macromolecular synthesis.[13] The strategy involves incorporating the maleimide moiety onto a AuNP as its furan Diels–Alder adduct and then liberating the free maleimide when desired by taking advantage of the propensity of this adduct to undergo an efficient retro-Diels–Alder reaction with the loss of furan at elevated temperatures (Scheme 1). This strategy must be employed because AuNP's of these types and sizes are prepared either by direct synthesis

Received: May 28, 2012
Revised: July 16, 2012
Published: August 10, 2012

Scheme 1. Synthesis of Maleimide-Functionalized Gold Nanoparticles



or by a place-exchange reaction that requires the thiol derivative of the functionality that you wish to install on the AuNP. In either method, a maleimide-functionalized thiol cannot be used directly because of its (desired) susceptibility to Michael additions and also because the ligand would simply react with itself. To avoid this and other complications, in our original report we prepared the dodecanethiol-derivatized maleimide-furan Diels–Alder adduct and introduced this ligand by a place exchange onto dodecane thiol-protected AuNP's ca. 2.2 nm in size. When desired, the free maleimide-AuNP's could be liberated by heating to 100 °C in toluene, which resulted in the loss of the furan that could then be removed to yield organic-soluble maleimide-modified AuNP's with only a small increase in the core size.[9] We subsequently utilized maleimide-AuNP's to prepare AuNP networks through a Diels–Alder strategy[9] and use it as a reaction template to prepare a wide variety of organic-soluble AuNP's using Diels–Alder and 1,3-dipolar cycloaddition reactions and Michael additions.[14−16]

This strategy was utilized by Lennox and Schirrmacher to prepare water-soluble maleimide-AuNP's with the difference being that both the ligand in the starting (base) AuNP and the tether used for the furan-protected maleimide–thiol used in the place exchange were based on PEG ligands to make them water-soluble. This represents an important breakthrough because many applications using AuNP, especially those for biological systems, require water solubility and the ones we originally prepared were of limited utility because they were soluble only in organic solvents. Having a water-soluble maleimide-modified AuNP is especially relevant because of the wide use of Michael addition reactions of maleimide in bioconjugation-type studies. In this regard, the contribution of Lennox, Schirrmacher, and co-workers was important. However, because of our experience in this area, when their work appeared we were perplexed by aspects of the characterization of the maleimide-AuNP that differed from that previously published for the organic-soluble version and other derivatives. In particular, the maleimide-AuNP (after the removal of furan) was reported to have its olefinic protons 0.5 ppm upfield from the olefinic protons in the furan-maleimide adduct, whereas in all other studies and indeed for model maleimide compounds these protons are downfield from the olefinic protons of the furan-maleimide adduct. This is a dramatic solvent effect and perhaps suggests that what they prepared was not the maleimide-AuNP. We were surprised by the apparent low reactivity towards Michael addition given the high apparent loading of maleimide at the interface because unlike the Michael addition reactions we reported for maleimide-AuNP in organic solvents that require high pressure to proceed efficiently, this type of reaction should be rapid and efficient in aqueous and protic solvents.

Herein, we report an efficient method for preparing small, 2.5-nm-core-size water-soluble maleimide-AuNP using a different synthesis approach but still utilizing the furan-maleimide adduct to prepare the AuNP's and liberating the maleimide-AuNP through the retro-Diels–Alder reaction. Characterization confirms the formation of the target maleimide-AuNP, particularly the ^{1}H NMR spectroscopy that verifies the olefinic protons in the expected chemical shift region. Furthermore, we show that when the deprotection of the furan-maleimide AuNP adduct is carried out in the presence of water the initially formed interfacial maleimide moieties are hydrolyzed at these temperatures to yield an interfacial polyammonium/maleic acid salt adduct. The method we describe outlines how to prepare small (<3 nm) maleimide-AuNP's that are soluble in both water and a host of other organic solvents. The latter allows for their preparation under conditions that avoid water and the resulting secondary hydrolysis reaction of the maleimide moiety. The versatile maleimide-AuNP's we describe are easy to prepare and suitable for bioconjugation studies.

■ RESULTS AND DISCUSSION

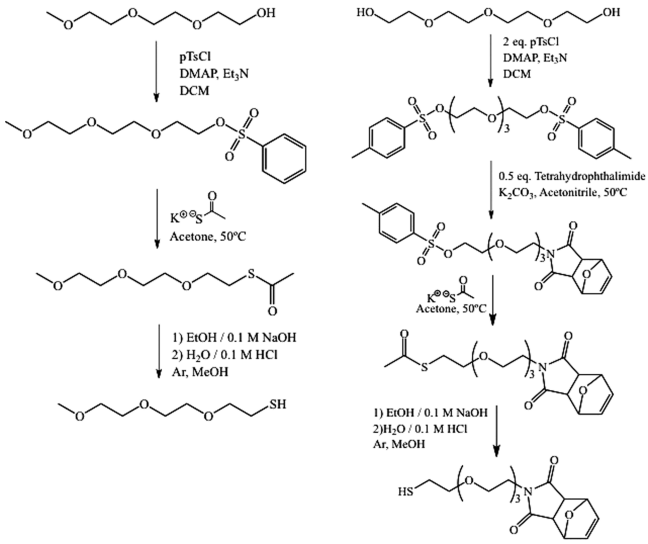
Our approach to preparing maleimide-AuNP's is shown in Scheme 1 and follows the strategy that we reported for organic-soluble maleimide-AuNP's of the same size, with the difference being that the thiolate ligands are PEGylated. This approach first involved the synthesis of methyl-terminated triethylene glycol thiol (Me-EG_3-SH) monolayer-protected AuNP's (Me-EG_3-AuNP's). The methyl-terminated EG_3 ligands were selected as the base ligand for the nanoparticles because unlike hydroxyl-terminated (HO-EG_3-AuNP) species they are soluble in both water and a wider selection of organic solvents, making the resulting maleimide gold nanoparticles more versatile. Me-EG_3-AuNP was then subjected to place-exchange reaction conditions in the presence of the Diels–Alder furan-protected maleimide tetraethylene glycol thiol (Pt-maleimide-EG_4-SH). This reaction was carried out in a mixture of dry methanol and acetone as the solvent. It is important to note that during this step the maleimide must be protected; otherwise, it will undergo a Michael addition with the thiols present in solution. The final step in the preparation of the desired maleimide-AuNP was the deprotection of the maleimide at the AuNP

dx.doi.org/10.1021/la302168g | Langmuir 2012, 28, 12357−12363

interface through the retro-Diels–Alder reaction at 100 °C in toluene (Scheme 1).

The required Me-EG_3-SH and Pt-maleimide-EG_4-SH ligands were synthesized starting from readily available triethylene glycol monomethyl ether (Me-EG_3-OH) and tetraethylene glycol (HO-EG_4-OH), respectively (Scheme 2). The maleimide

Scheme 2. Synthesis Paths for the Preparation of Me-EG_3-SH (Left) and Pt-maleimide-EG_4-SH (Right)



ligand was synthesized with one more ethylene glycol unit than in the base ligand to lower the steric hindrance on the maleimide functionality and make it easier to react through Michael addition or other cycloaddition reactions.[14−16] To synthesize Me-EG_3-SH, Me-EG_3-OH was tosylated to transform the hydroxyl group into a good leaving group. The tosylated Me-EG_3-OH was then converted to its corresponding thioacetate via an S_N2 reaction. The thiol was obtained through basic hydrolysis of the thioacetate functionality (Scheme 2). The same strategy was employed to synthesize Pt-maleimide-EG_4-SH. In this case, both hydroxyl groups of HO-EG_4-OH were tosylated, then one of the tosylated extremities of the molecule was reacted with furan-protected maleimide (3,6-endoxo-Δ^4-tetrahydrophthalimide), and then the other tosylated extremity was converted to the thioacetate and subsequently hydrolyzed under basic conditions to generate the desired thiol, Pt-maleimide-EG_4-SH. A detailed synthesis procedure and a characterization of the ligands are reported in the Supporting Information.

Me-EG_3-AuNP was synthesized using a modified one-phase synthesis method.[17] Briefly, the gold salt, $HAuCl_4 \cdot 3H_2O$, was dissolved in a mixture of methanol and glacial acetic acid. Me-EG_3-SH was dissolved in this solution, and finally a fresh solution of $NaBH_4$ was slowly added. The mixture was stirred overnight at room temperature. Purification of the resulting nanoparticles involved extracting them from the aqueous phase using toluene. Successively, unreacted Me-EG_3-SH was washed away from a film of nanoparticles inside a round-bottomed flask using cyclohexane, in which the AuNP's are not soluble. Subsequently, the AuNP's were purified by dialysis. Through control of the gold–thiol ratio, it is possible to control the size of Me-EG_3-AuNP. In this work, a ratio of 1:3 gold/thiol was employed to yield 2.0 ± 0.3 nm Me-EG_3-AuNP's. These AuNP's were characterized by UV–vis spectroscopy and transmission electron microscopy (TEM) images (Supporting Information). ^{1}H NMR spectroscopy showed the expected broadened peaks at 3.34, 3.58, and 3.66 ppm, corresponding to the Me-EG_3-SH ligands (Figure 1, spectrum i). Thermogravi-

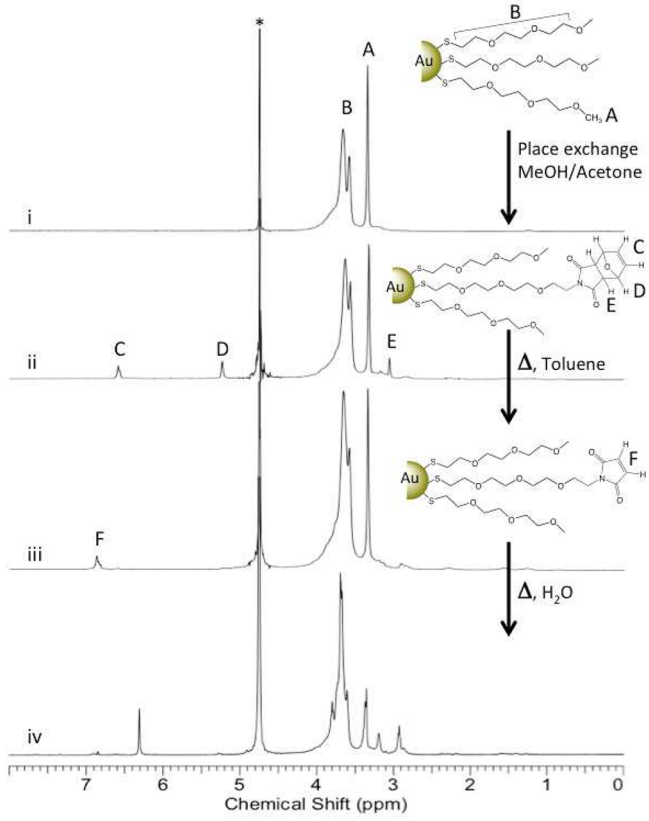


Figure 1. ^{1}H NMR spectra (recorded in D_2O) of (i) Me-EG_3-AuNP, (ii) Pt-maleimide-EG_4-AuNP, (iii) maleimide-EG_4-AuNP, and (iv) the hydrolysis product of maleimide-EG_4-AuNP. * indicates residual H_2O.

metric analysis (TGA) revealed that 38.3% of the AuNP's are composed of the PEGylated ligands, corresponding to 2.13 μmol of Me-EG_3-SH per milligram of AuNP (Figure 2).

These nanoparticles, having PEG ligands with terminal methylether units at the solution interface, have the greater advantage of being soluble in both organic and water solutions. In particular, these Me-EG_3-AuNP's are soluble in water,

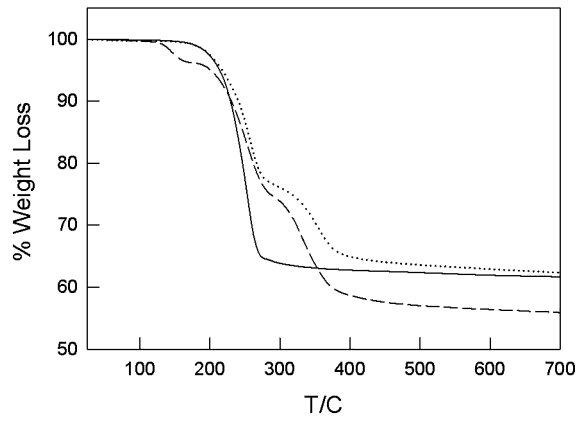


Figure 2. TGA of Me-EG_3-AuNP (—), Pt-maleimide-EG_4-AuNP (---), and maleimide-EG_4-AuNP (···).

dx.doi.org/10.1021/la302168g | Langmuir 2012, 28, 12357−12363

methanol, ethanol, tetrahydrofuran, dichloromethane, chloroform, toluene, ethyl acetate, acetone, dimethylformamide, and acetonitrile. They can be repeatedly dried and redissolved in these solvents with little to no degradation or aggregation (none was apparent).

Pt-maleimide-EG_4-AuNP was synthesized using a place-exchange reaction. In a typical synthesis, 100 mg of Me-EG_3-AuNP is mixed with 37.1 mg of Pt-maleimide-EG_4-SH for 15 min in 20 mL of dry methanol and 2.5 mL of acetone. The solvent was then evaporated under vacuum, and the resulting film was washed with cyclohexane (in which it is not soluble) to get rid of excess Pt-maleimide-EG_4-SH and exchanged Me-EG_3-SH. Pt-maleimide-EG_4-AuNP's were then purified further by dialysis to remove any unbound ligands. The furan-protected nanoparticles, Pt-maleimide-EG_4-AuNP's, were characterized by ^{1}H NMR and UV−vis spectroscopy, TGA, and TEM. The nanoparticles synthesized under these conditions are found to maintain their size of 2.0 ± 0.3 nm according to TEM images (Supporting Information). TGA confirmed the presence of the protected maleimide ligand (Figure 2). The first weight loss accounting for 3.9% takes place between 100 and 170 °C and is related to the loss of furan. This feature in the TGA is diagnostic of the incorporation of the protected maleimide, confirms the reversibility of the Diels−Alder reaction at around 100 °C, and can be used to determine the amount of maleimide ligand incorporated onto the AuNP's. Using the percentage mass loss of furan (3.9%) as an indication of the amount of furan-protected maleimide incorporated onto the AuNP's allows us to estimate that 30% of ligands on the Pt-maleimide-EG_4-AuNP's were the required furan-protected maleimide ligands. This loss of furan is then followed by the loss of triethylene glycol monomethyl ether ligands and the deprotected maleimide ligand. These data can be compared with previous work carried out in our laboratories on small maleimide organic-soluble nanoparticles.[9]

^{1}H NMR spectroscopy of the Pt-maleimide-EG_4-AuNP's (recorded in D_2O as the solvent, with the residual H_2O signal used as a reference) shows the appearance of the three broadened peaks related to the furan-protected maleimide functional group (Figure 1, spectrum ii). The peak at 6.60 ppm is related to the two alkene protons, the peak at 5.25 ppm is related to the protons adjacent to the bridging oxygen of the Diels−Alder adduct, and the peak at 3.07 ppm is related to the two protons closer to the carbonyl groups.[9] The relative integration of the $-CH_3$ peak due to the Me-EG_3S ligands with those of furan-protected maleimide confirms the 30% incorporation of the desired ligand onto the particle. Furthermore, the solubility of these nanoparticles is unchanged with respect to that of the Me-EG_3-AuNP's.

The choice of the solvent for the place-exchange reaction was found to be of crucial importance to the final synthesis of maleimide-AuNP's. Because the place-exchange reaction takes place on the time scale of minutes, a solvent with a low vapor pressure is required for the reaction. The use of the methanol−acetone mixture as the solvent for the place-exchange reaction guarantees better control over the reaction time because it can be removed faster than water. Better control over the reaction time allows better control of the number of protected maleimide ligands that are introduced onto AuNP's. The quantity of the furan-protected maleimide ligand strongly influences the nanoparticle solubility. For example, when the percentage of furan-protected maleimide−thiol was over 40%, the resulting AuNP's were no longer soluble in toluene even though they are still readily redissolvable in water. The solubility in toluene is of crucial importance because toluene is a solvent suitable for the deprotection of maleimide without interference from competing reactions of maleimide (vide infra).

To carry out the retro-Diels−Alder reaction to liberate the desired maleimide moiety at the interface, Pt-maleimide-EG_4-AuNP's were dissolved in toluene and the solution was stirred overnight at 100 °C. The solvent was then evaporated under vacuum, and the AuNP film was washed with cyclohexane as described previously. Maleimide-EG_4-AuNP was characterized by ^{1}H NMR and UV−vis spectroscopy, TGA, and TEM. TEM images and UV−vis spectroscopy revealed a slight increase in the nanoparticle size to 2.5 ± 0.3 nm, similar to what we observed during the preparation of our original organic-soluble maleimide-AuNP's.[9] TGA (Figure 2) of the resulting maleimide-EG_4-AuNP's did not show the first weight loss between 100 and 170 °C present in the Pt-maleimide-EG_4-AuNP's, confirming that this was most likely due to the loss of furan in the latter. According to the mass loss measured, on 1 mg of maleimide-EG_4-AuNP's there are 1.30 μmol of Me-EG_3-SH and 0.50 μmol of Pt-maleimide-EG_4-SH, corresponding to a 28% maleimide ligand. TGA showed the presence of two ligands in essentially the same ratio as was obtained with the Pt-maleimide-EG_4-AuNP's.

^{1}H NMR spectroscopy of the product (Figure 1, spectrum iii) showed the disappearance (absence) of the peaks related to the furan Diels−Alder adduct (6.60, 5.25, and 3.07 ppm) and a new signal downfield at 6.86 ppm, as expected for olefin peaks of the maleimide moiety. This result completely agrees with our previous characterization of maleimide organic-soluble nanoparticles.[9] The simplified formula for the maleimide-EG_4-AuNP's can be calculated from the TGA and TEM measurements, assuming a spherical shape of the gold core.[8] Maleimide-EG_4-AuNP's were found to have the simplified formula $Au_{483}(Me\text{-}EG_3\text{-}S)_{198}(maleimide\text{-}EG_4\text{-}S)_{77}$.

As stated above, the solubility of the Pt-maleimide-EG_4-AuNP's in toluene is a key component in the synthesis of maleimide-EG_4-AuNP's. Some attempts to deprotect the Pt-maleimide-EG_4-AuNP's were carried out by employing acetonitrile, acetone, and water as reaction solvents, and all of them resulted in a nanoparticle size growth characterized by a change in the solution color from dark brown (typical of a nanoparticle size between 2 and 3 nm) to ruby (typical of nanoparticles with diameter of between 4 and 5 nm).

Performing the retro-Diels-Alder deprotection in water gave very different results. After the reaction of Pt-maleimide-EG_4-AuNP in H_2O at 100 °C overnight, there was no peak due to the expected maleimide olefin protons at 6.86 ppm but a signal appeared at 6.23 ppm, upfield with respect to the peak at 6.60 ppm of the alkene protons of the protected maleimide ligand. The appearance of this upfield signal is also accompanied by significant changes in the region between 3 and 4 ppm. The peak at 3.07 ppm disappears, and three broad peaks (one at 3.15 ppm, one at 2.89 ppm, and one at 3.77 ppm) appear. This is also consistent with that observed in the study by Lennox and Schirrmacher.[8] Because the peak they assigned to maleimide was significantly upfield from where it was expected, we suspected that it was due to a reaction of the liberated maleimide with H_2O under the same reaction conditions.

To explore this possibility, we performed a number of control reactions. First, we took our fully characterized maleimide-EG_4-AuNP, prepared by deprotection in toluene,

and heated this sample in water to 100 °C (similar to the deprotection conditions employed by Lennox, Schirrmacher, and co-workers). ^{1}H NMR spectroscopy shows the loss of the signal of the olefinic protons in maleimide from 6.86 ppm, a new peak growing in at 6.23 ppm, and the appearance of the same peaks in the region between 3 and 4 ppm as observed after the direct deprotection of Pt-maleimide-EG$_4$-AuNP's in water. The spectrum is shown in Figure 1(iv). This latter spectrum suggests that this product arises from the hydrolysis of maleimide by water under these conditions. But what could be the hydrolysis product that would give rise to such a simple singlet in the ^{1}H NMR spectrum at ca. 6.2 ppm and still be associated with the AuNP? To address this issue, we carried out a number of model reactions.

For simplicity, *N*-methylmaleimide was chosen as the first model compound. Its ^{1}H NMR spectrum was recorded in D_2O, and it revealed the expected signals at 2.94 ppm (CH_3–N) and 6.80 ppm (olefinic protons of the maleimide). This sample was then heated in nanopure water at 100 °C in a round-bottomed flask for 48 h. After reaction, the pH of the solution had changed from neutral to acidic. The solvent was then evaporated, and the reaction product was characterized by ^{1}H and ^{13}C NMR spectroscopy and mass spectrometry. As shown in Figure 3, ^{1}H NMR spectroscopy reveals the disappearance of the peaks of *N*-methylmaleimide at 2.94 and 6.80 ppm, related respectively to the methyl protons and to the olefinic protons. The main products of the reaction are represented by the peaks at 2.55 and 6.30 ppm, which are the same as those observed after heating the maleimide-EG$_4$-AuNP's in water. On the basis of the reactants (*N*-methylmaleimide and water) and the resulting ^{1}H NMR analysis, we suspected that the *N*-methyl maleimide was hydrolyzed to methylamine (as methylammonium, $CH_3NH_3^+$) and maleic acid (as hydrogen maleate, $HOC(O)=C(O)O^-$) under these conditions. Indeed, the large amount of water (the solvent) and the high temperature favor the hydrolysis of *N*-methylmaleimide. To validate the hypothesis of forming methylamine and maleic acid, small amounts of authentic *N*-methylamine and subsequently authentic maleic acid were added to the NMR tube containing the products of hydrolysis of the model compound. ^{1}H and ^{13}C NMR spectra recorded after every addition show an increase in the corresponding peak intensities upon addition of the pure reagent and, importantly, no new signals were recorded (Figures SI 12 and SI 13). Note that because of the pH change after the addition of excess base and then excess acid, the peaks, especially those corresponding to the maleic acid, shift slightly depending on the extent of salt formation but remain in the 6.2–6.35 ppm range. Mass spectrometry also revealed the presence of the molecular peaks of methylamine and maleic acid. Finally, the Kaiser test for the detection of primary amine was conducted for the reaction mixture and for a control solution of *N*-methylmaleimide in water.[18] The reaction mixture becomes dark blue (positive test), indicating the presence of a primary amine, and the *N*-methyl maleimide control solution becomes yellow (negative test) (Figure SI 11).

To understand better the process that leads to the hydrolysis reaction on the AuNP's and to explain what might have occurred in the earlier study, the protected maleimide tetraethylene glycol thioacetate (Pt-maleimide-EG$_4$-SAc) was employed as a second model compound. A few milligrams of Pt-maleimide-EG$_4$-SAc were inserted into a J. Young NMR tube and dissolved in D_2O, and the temperature was increased to 100 °C. ^{1}H NMR spectra were recorded after various time intervals (Figure SI 14 in the Supporting Information). In addition to the expected hydrolysis of the thioacetate moiety (indicated by the signal due to acetic acid at 2.06 ppm), the signals due to the protected maleimide were also monitored as a function of time during heating in D_2O. This study shows the progressive deprotection of the maleimide functionality, indicated by the appearance of a signal at 6.85 ppm that is due to maleimide and then its progressive hydrolysis. After 8 h, the intensity of the peaks of protected maleimide (3.11, 5.29, and 6.60 ppm) markedly decreases, and this decrease is accompanied by the appearance of the furan peaks (6.47 ppm and 7.53 ppm), the peak related to the alkene protons of maleimide (6.85 ppm), the supposed maleic acid/maleate peak (6.31 ppm), and the peak of the protons alpha to the amine of the resulting amine(ammonium) tetraethylene glycol (^+H_3N-EG$_4$-S-D) (3.2 ppm). After 24 h, the amount of starting material is further decreased but the product of the hydrolysis of maleimide (indicated by the peak at 6.31 ppm) becomes the major product. After 48 h, the amounts of protected and deprotected maleimide are negligible and the major product is represented by maleic acid/maleate and ^+H_3N-EG$_4$-SD.

The same experiment was performed using our maleimide-EG$_4$-AuNP, and it showed the same changes as described for model ligand Pt-maleimide-EG$_4$-SAc (Figure SI 15) with the

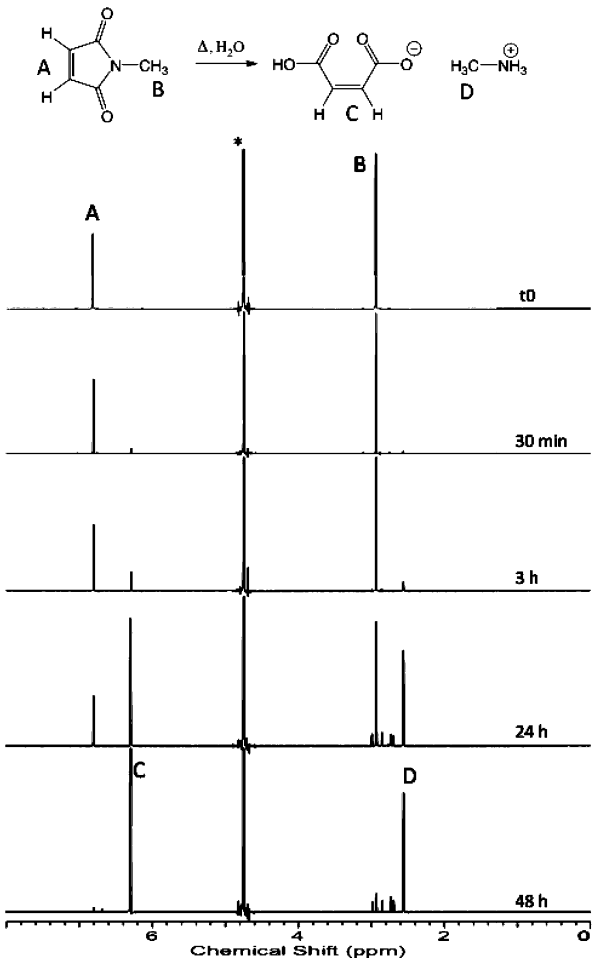


Figure 3. ^{1}H NMR spectrum recorded in D_2O showing the kinetics of hydrolysis of *N*-methylmaleimide. Peaks A and B are relative to the reactant, and peaks C and D are relative to the main products. * is the H_2O signal.

dx.doi.org/10.1021/la302168g | *Langmuir* 2012, 28, 12357–12363

loss of the maleimide signals at 6.86 ppm and the appearance of the signal at 6.23 ppm. At the end of this experiment and to confirm that the signal at 6.23 ppm was due to the maleic acid product from the hydrolysis of maleimide, a small amount of authentic maleic acid was added to the fully hydrolyzed AuNP's and an increase in the peak at 6.23 ppm was recorded (Figure SI 16). The addition of authentic *N*-methylmaleimide shows the expected olefinic signal at 6.86 ppm. In total, these experiments demonstrate that on heating in water to 100 °C the protected maleimide undergoes the expected retro-Diels–Alder reaction to form the desired maleimide, but this reacts under conditions that yield the corresponding R-NH_3^+ and maleate by the hydrolysis of maleimide.

These results highlight the importance of the choice of solvent for the maleimide deprotection reaction. Water, even though it is a green solvent and has a high boiling point, was found to react irreversibly at high temperatures with the maleimide functionalities present at the nanoparticle interface. Hydrolysis causes the release of maleic acid and the formation of amine functionalities at AuNP's, which can couple as their salt. Our approach allows for the deprotection to be carried out in toluene, circumventing the hydrolysis of the desired maleimide functionality at the interface.

To demonstrate that maleimide-EG_4-AuNP's react via the Michael addition reaction for the possible application as a bioconjugation template, the maleimide-EG_4-AuNP's were functionalized with cysteine. In this experiment, 50 mg of maleimide-EG_4-AuNP's was mixed with 0.8 mg of L-cysteine for 1 h in nanopure water. The sample was then purified by dialysis to remove any unreacted nucleophile and to leave only the cysteine-functionalized AuNP's. The ^{1}H NMR spectrum showed the complete disappearance of the peak at 6.86 ppm due to maleimide and the appearance of broadened peaks at 3.17 and 3.25 ppm, expected for the Michael addition product, which partially overlapped with the peak at 3.32 (Figure SI 17).

CONCLUSIONS

In this article, we described the first synthesis method leading to stable maleimide, water-soluble, small (<3 nm) gold nanoparticles through a retro Diels–Alder strategy. The amount of protected maleimide ligand at the interface accounted for 30% of the total ligands. Attempts to incorporate more led to solubility issues, with the ligand becoming insoluble in toluene, and thus the maleimide deprotection reaction cannot be carried out. Indeed, toluene was found to be the solvent suitable for conducting the retro-Diels–Alder reaction to lead to the desired water-soluble maleimide-AuNP's. The use of water in the deprotection reaction cannot lead to pure maleimide-functionalized AuNP's because under the conditions needed for deprotection the hydrolysis of maleimide occurs efficiently, giving a polyammonium-functionalized AuNP associated with the maleate anion. Maleimide-AuNP's derived from the improved method presented here are stable, soluble in aqueous solution as well as a selection of organic solvents, and reactive toward Michael addition, thus broadening the application and versatility of their use in biochemistry and biology. We are currently utilizing the water-soluble maleimide-EG_4-AuNP's prepared using our strategy for bioconjugation studies.

ASSOCIATED CONTENT

Supporting Information

General experimental details, details of the synthesis and characterizations of ligands, gold nanoparticles, model reactions, and zeta potential measurements. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: mworkent@uwo.ca.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work is supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) under the Discovery Grants program and Western University Canada. We thank Professor J. P. Guthrie for helpful discussions and Professor E. R. Gillies for access to her nanoparticle zeta potential instrumentation.

REFERENCES

(1) Hermanson, G. T. *Bioconjugate Techniques*, 2nd ed.; Academic Press: San Diego, 2008.

(2) Kim, Y.; Ho, S. O.; Gassman, N. R.; Korlann, Y.; Landorf, E. V; Collart, F. R.; Weiss, S. Efficient site-specific labeling of proteins via cysteines. *Bioconjugate Chem.* **2008**, *19*, 786–791.

(3) Jain, S.; Hirst, D. G.; O'Sullivan, J. M. Gold nanoparticles as novel agents for cancer therapy. *Br. J. Radiol.* **2012**, *85*, 101–113.

(4) Manju, S.; Sreenivasan, K. Gold nanoparticles generated and stabilized by water soluble curcumin–polymer conjugate: blood compatibility evaluation and targeted drug delivery onto cancer cells. *J. Colloid Interface Sci.* **2012**, *368*, 144–151.

(5) Ba, H.; Rodriguez-Fernández, J.; Stefani, F. D.; Feldman, J. Immobilization of gold nanoparticles on living cell membranes upon controlled lipid binding. *Nano Lett.* **2010**, *10*, 3006–3012.

(6) Oh, E.; Susumu, K.; Blanco-Canosa, J. B.; Medintz, I. L.; Dawson, P. E.; Mattoussi, H. Preparation of stable maleimide-functionalized Au nanoparticles and their use in counting surface ligands. *Small* **2010**, *6*, 1273–1278.

(7) Gregori, L.; Hainfeld, J. F.; Simon, M. N.; Goldgaber, D. Binding of amyloid beta protein to the 20 S proteasome. *J. Biol. Chem.* **1997**, *272*, 58–62.

(8) Zhu, J; Waengler, C.; Lennox, R. B.; Schirrmacher, R. Preparation of water-soluble maleimide-functionalized 3 nm gold nanoparticles: a new bioconjugation template. *Langmuir* **2012**, *28*, 5508–5512.

(9) Zhu, J.; Kell, A. J.; Workentin, M. S. A retro-Diels Alder reaction as an alternative route to uncovering maleimide-modified surfaces on monolayer protected nanoparticles. *Org. Lett.* **2006**, *8*, 4993–4996.

(10) Yousaf, M. N.; Mrksich, M. Diels Alder reaction for the selective immobilization of protein to electroactive self-assembled monolayers. *J. Am. Chem. Soc.* **1999**, *121*, 4286–4287.

(11) Mantovani, G.; Lecolley, F.; Tao, L.; Haddleton, D. M.; Clerx, J.; Cornelissen, J. J. L. M.; Velonia, K. Design and synthesis of *N*-maleimido-functionalized hydrophilic polymers via copper-mediated living radical polymerization: A suitable alternative to PEGylation chemistry. *J. Am. Chem. Soc.* **2005**, *127*, 2966–2973.

(12) Toncelli, C.; De Reus, D. C.; Picchioni, F.; Broekhuis, A. A. Properties of reversible Diels-Alder furan/maleimide polymer networks as function of crosslink density. *Macromol. Chem. Phys.* **2012**, *213*, 157–165.

(13) Gandini A. The furan/maleimide Diels–Alder reaction: a versatile click–unclick tool in macromolecular synthesis. *Prog. Polym. Sci.*, published online April 28, 2012, http://dx.doi.org/10.1016/j.progpolymsci.2012.04.002.

(14) Zhu, J; Ganton, M. D.; Kerr, M. A.; Workentin, M. S. Chemical modification of monolayer protected gold-nanoparticels using hyperbaric conditions. *J. Am. Chem. Soc.* **2007**, *129*, 4904−4905.
(15) Zhu, J.; Lines, B. M.; Ganton, M. D.; Kerr, M. A.; Workentin, M. S. Efficient synthesis of isoxazolidine-tethered monolayer-protected gold nanoparticles (MPGNs) via 1,3-dipolar cycloadditions under high-pressure conditions. *J. Org. Chem.* **2008**, *73*, 1099−1105.
(16) Hartlen, K. D.; Ismaili, H.; Zhu, J.; Workentin, M. S. Michael addition reactions for the modification of gold nanoparticles facilitated by hyperbaric conditions. *Langmuir* **2012**, *28*, 864−871.
(17) Zheng, M.; Li, Z.; Huang, X. Ethylene glycol monolayer protected nanoparticles: synthesis, characterization, and interactions with biological molecules. *Langmuir* **2004**, *20*, 4226−4235.
(18) Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides. *Anal. Biochem.* **1970**, *34*, 595−598.