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Coating gold nanoparticles with peptide molecules via a peptide elongation approach

Jinda Fan, Shaowei Chen, Yong Gao *

Department of Chemistry and Biochemistry, Southern Illinois University, Carbondale, IL 62901-4409, USA

Dedicated to Dr. James W. Neckers on the occasion of his 100th birthday.

Abstract

Coating gold nanoparticle surfaces with peptide molecules will expand the application potentialities of these nanomaterials in biomedical sciences. Our studies investigated some details of covering gold nanoclusters with peptide molecules via a peptide elongation approach. This route can minimize the loss of biological activities of peptide molecules by avoiding the use of sensitive side chains for anchoring peptides onto the Au surfaces. Our work demonstrates that the chiral integrity of each amino acid is maintained and the high coupling efficiency could be achieved in the peptide elongation route for coating gold nanoparticles.

Keywords: Peptide elongation; Gold nanoparticles; Peptide molecules

1. Introduction

At the nanometer scale, the chemistry and physics of matter bridge between those of bulky materials and their constituent atoms due to confinement and quantum size effects [1]. These nanomaterials offer prospects of novel, and even size-dependent properties [2–6] that lead to application potentialities in optical devices, microelectronics, catalysts, chemical recognitions and biomedical detections. Alkanethiolate/Au monolayer-protected clusters (MPCs) are of particular interest for biomedical applications owing to their stability, tunable solubility in aqueous solutions, and relative ease of characterization [7]. Biologically active molecules such as peptides and proteins are usually attached to nanoparticles to improve their bio-specificity and expand application potentialities of these types of systems in biological and medical sciences [4,8–11].

Two general routes have been established for coating gold nanoparticles with peptide molecules. The first approach is to react a function group (–SH or $-NH_2$) of a side chain of peptide molecules that are pre-formed via solid-phase peptide synthesis with the particle surfaces, which, however, often results in the loss of activity of biomolecules due to the high sensitivity of these side chain functional groups [12]. The second strategy is to introduce peptide molecules by elongating peptide

* Corresponding author. Tel.: +1-618-453-4904; fax: +1-618-453-6408

E-mail address: ygao@chem.siu.edu (Y. Gao).

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chains via their C-termini on the gold surfaces using the peptide synthesis protocols [13]. This route offers several advantages over the aforementioned side chain anchorage method: (a) it avoids the use of peptide side chains for the synthesis of peptide-nanoparticle complexes and thus minimizes the inactivation of peptide molecules; (b) peptides can be introduced directly onto the gold surfaces, avoiding extra steps of solid-phase peptide synthesis; and (c) the peptide N-termini could be deprotected and serve as a platform for the further functionalization of gold nanoparticle surfaces, expanding application potentialities of these nanomaterials in medical/biological sciences. However, a great deal of the details of peptide elongation on the gold nanoparticle surfaces is not clear at this stage. For example, it is unknown that if the chiral integrity of the α-carbon center of amino acids has been maintained during peptide elongation, and if significant amounts of incomplete peptide chains are generated on the Au surfaces due to potentially low coupling efficiency and the early termination of peptide elongation. The racemization of the chiral centers of peptide molecules could cause the loss of biological activity of the peptide–gold nanoparticle complexes, and the accumulation of incomplete peptide molecules on the Au surfaces will lead to the decreased specificity of the gold nanoparticles towards biological entities [14].

In this paper, we would like to report our preliminary work on investigating the details of peptide elongation for coating peptide molecules onto the Au nanoparticle surfaces. Our synthetic studies here examined the amino acid coupling efficiency versus Au MPC structures, and investigated the chiral integrity of incorporated amino acids. The HPLC and spectroscopic analyses were also used to determine the purity of a pentapeptide synthesized on the gold surfaces.

2. Experimental section

2.1. Materials

N-Boc protected amino acids were purchased from Acros Organics (Somerville, NJ) and used as received without further purification. The PyBop and HOBt coupling reagents and all other chemicals were obtained from Sigma–Aldrich (St. Louis, MO). Water was obtained from a Milli-Q reagent water system purchased from Millipore Corporation (Milford, MA), and organic solvents were pre-dried.

2.2. Preparation of Au MPCs

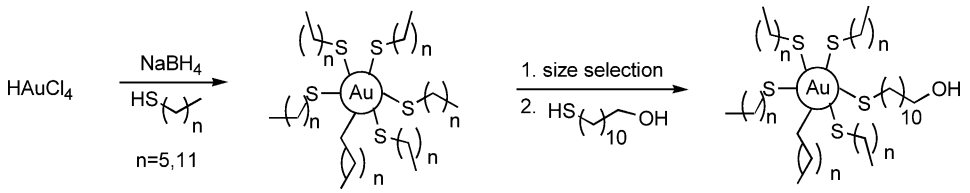
To a stirred solution of tetraoctylammonium bromide (1.5 g, 2.5 equiv.) in 80 ml of toluene was added 0.31 g of $HAuCl_4 \cdot 3H_2O$ (1 equiv.) in 25 ml of water. After about 2 h, the organic phase was isolated and alkanethiolate (1-dodecanethiol or 1-hexanethiol) was added, and the resulting solution was stirred at room temperature for 10 min. Then, $NaBH_4$ (10 equiv.) in 25 ml of water was added over a period of 20 min. After 4 h, the resulting dark organic phase was collected, and the solvent was removed in vacuo. The black residues (Au MPC protected with either 1-dodecanethiol or 1-hexanethiol) were suspended in 80 ml of ethanol, and sequentially washed with 30 ml of ethanol and 150 ml of acetone.

The size selection was achieved and fractioned via incremental precipitation of larger MPCs from a toluene solution by adding a more polar solvent acetone. The core dimensions were then examined by TEM measurements.

The introduction of 11-mercapto-1-undecanol onto the surfaces of gold MPCs was done by adding the desired amount of 11-mercapto-1-undecanol to a stirred solution of gold MPC (100 mg) in methylene chloride (30 ml) at room temperature. After 12 h, 80 ml of acetonitrile was added into the solution and the black residues were collected after centrifugation ($15\,000 \times g$, 30 min). The residues were re-suspended in 80 ml of acetonitrile and washed with 100 ml of acetone.

2.3. Peptide elongation on Au MPCs using Boc strategy

In a typical reaction, the coupling process was carried out in dry methylene chloride solutions (100 ml) with HOBt (17.6 mg, 0.13 mmol), DIPEA



Scheme 1. Synthesis of Au MPCs. The Au nanoparticles protected with 1-dodecanethiol or 1-hexanethiol were fabricated via a reduction reaction. A major fraction with a uniform core dimension of 2.8 nm was used for the introduction of 11-mercapto-1-undecanol in the place-exchange reactions after size selection processes.

(33.6 mg, 0.26 mmol) and PyBOP (67.6 mg, 0.13 mmol) in the presence of the gold MPC (26 μmol) and excessive *N*-Boc-L-amino acid (40.4 mg, 0.13 mmol) at room temperature. The reaction progress was closely monitored with ^{1}H NMR spectroscopy and the reactions were terminated after 2 h via removal of methylene chloride in vacuo. The residues were treated with acetonitrile (100 ml) (Au MPCs generally have very limited solubility in polar organic solvents like acetonitrile and methanol) and the gold MPC was separated through filtration.

The *N*-Boc protecting group was subsequently removed by treating the aforementioned gold MPC in a solution (50 ml) of methylene chloride with TFA (50%) at room temperature. After about 20 min, the methylene chloride and TFA were removed in vacuo to give residues that were used for anchoring the next amino acid directly. The coupling of the next *N*-Boc-amino acids was following similar procedures discussed above.

2.4. Examining the chiral integrity of the α-carbon chiral center by ^{19}F NMR spectroscopy

The aforementioned Au MPC–amino acid deprotected with TFA was dissolved in methylene chloride solution, and treated with *S*-Mosher's chloride (1.2 equiv.) and pyridine (1.2 equiv.) in methylene chloride solution at room temperature. After 2 h, the solution was washed with 1 M HCl aqueous solutions and 0.5 M $NaHCO_3$ solution sequentially, and dried over anhydrous Na_2SO_4. The solvent was then removed in vacuo and the resulting residues were examined by ^{19}F NMR analysis.

2.5. Cleaving peptide FAAAA from the Au MPCs for purity analysis

To a stirred solution of Au MPC-Phe-Ala-Ala-Ala-Ala in methylene chloride (20 ml) was treated with I_2 (50 equiv.) in aqueous solutions. After about 12 h, the organic phase was separated from the aqueous phase, dried over anhydrous Na_2SO_4. The organic phase was then treated with NaOH solution (10 equiv.). After 24 h, the organic phase was separated from the aqueous phase again and dried over anhydrous Na_2SO_4, and the solvent was removed in vacuo. The resulting residues were further treated with 50% TFA in methylene chloride (20 ml), and white powders were collected after removal of solvents in vacuo. The residues were then subjected to HPLC analysis using Delta Pak C_{18} reversed-phase column with UV detection at 240 nm. Linear gradients of 10% CH_3CN and 90% H_2O at a 1.0 ml/min flow rate were employed. The residues were also examined by ^{1}H NMR and Mass spectrometry.

2.6. Instrumentation

^{1}H NMR data were obtained on a Varain VXR-300 system with an Oxford wide-bore magnet and the chemical shifts were reported in parts per million (ppm) downfield relative to tetramethylsilane using the residual proton resonance of solvents as the references: $CDCl_3$ δ 7.25; CD_2Cl_2 δ 5.32. TEM measurements were accomplished with a Hitachi H7100FA operating at an accelerating voltage of 75 or 100 kV. Au nanoparticle specimens were deposited onto Formvar- and silicon-coated, 200 mesh copper grids (Structure Probe, Inc.) and the edge of the grid touched to a filter paper to wick away most of the solvent.

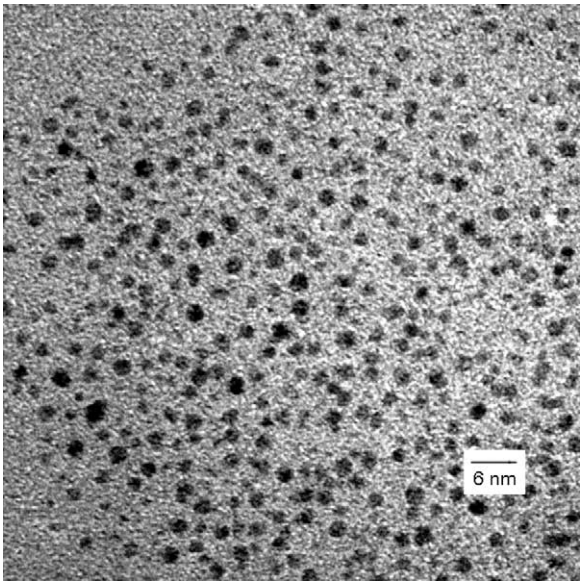


Fig. 1. TEM image of the Au MPC with an average core size of 2.8 nm. The Au MPC is protected with 1-dodecanethiol.

Images were recorded either on conventional photographic films or captured using a Gatan 789 digital camera. Magnification was calibrated using a MAG*I*CAL high resolution magnification standard accurated to $1 \times 10E6$ X. Thermoanalysis was carried out on Mettler Toledo TGA/SDTA 851[e] from 28 to 800 °C at a rate of 10°/min.

3. Results and discussion

The gold MPCs used in our studies were chemically synthesized from hydrogen tetrachloroaurate (III) via a reduction reaction in the presence of alkanethiolate (1-dodecanethiol or 1-hexanethiol) serving as protecting ligands to stabilize the Au cores (Scheme 1). The resulting Au MPCs was selected and fractioned based on their core dimensions via incremental precipitation of larger MPCs from a toluene solution by adding a more polar solvent acetone. A major fraction was obtained and found to have a very narrow core size distribution with an average core dimension of about 2.8 nm that was determined by TEM measurements (Fig. 1), and this fraction was selected for the studies of peptide elongation. 11-Mercapto-1-undecanol ligands were then coated onto the gold nanoparticle surfaces via a place-exchange reaction (Scheme 1). The ratios of 1-dodecanethiol and 11-mercapto-1-undecanol bound to the Au nanoparticle surfaces could be manipulated by varying the concentration of 11-mercapto-1-undecanol in the place-exchange reaction. Thermogravimetric analysis (TGA) was then used to determine the composition of the resulting gold MPCs, and a TGA pattern of the aforementioned major fraction (Fig. 2) shows that the weight percentage of combined organic ligands

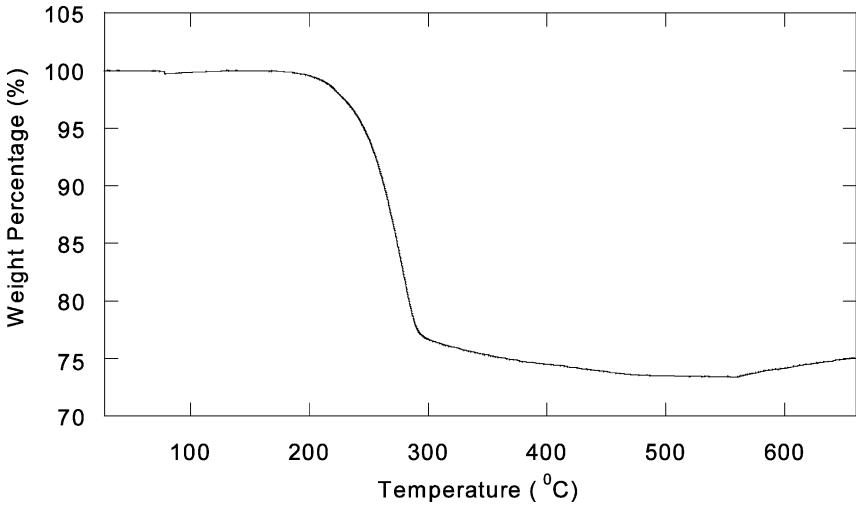


Fig. 2. TGA analysis of the Au MPC protected with 1-dodecanethiol and 11-mercapto-1-undecanol. From 180 to 500 °C, the Au MPC lost 26.7% percent of its weight.

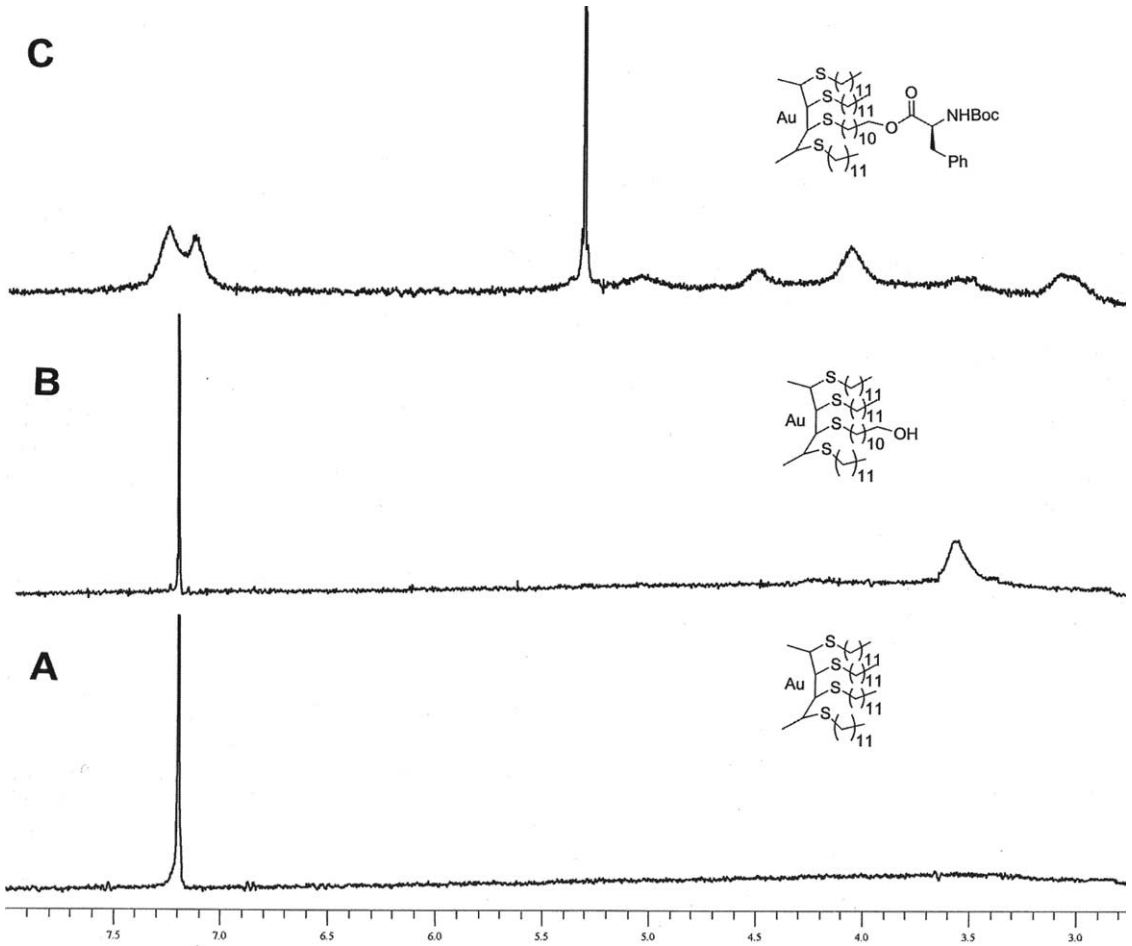
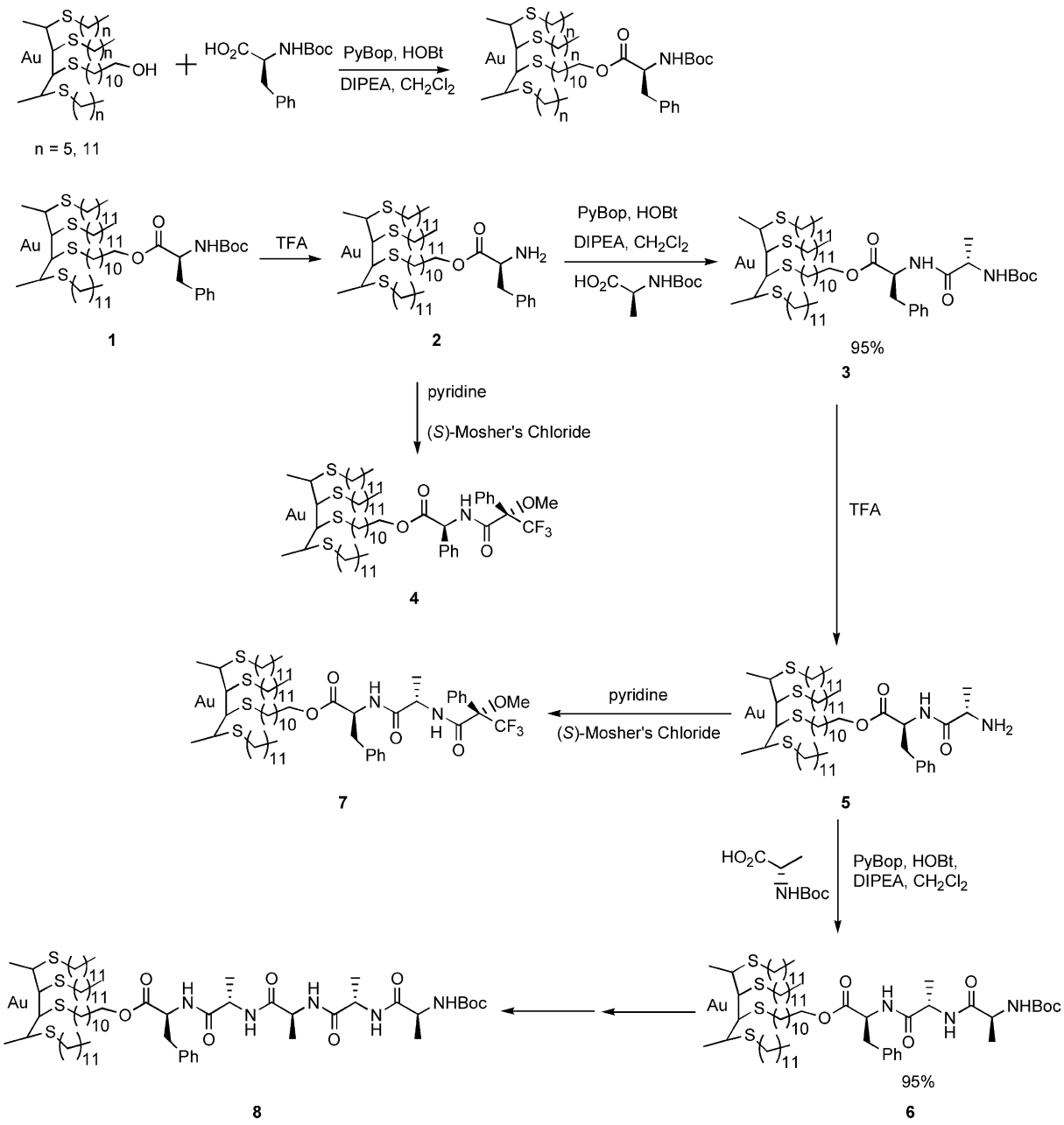


Fig. 3. ^{1}H NMR spectra of the Au MPC protected with 1-dodecanethiol in $CDCl_3$ (A); the Au MPC after place-exchange in $CDCl_3$ (B); and the Au MPC with *N*-Boc-Phe in CD_2Cl_2 (C). The ratio of 1-dodecanethiol and 11-mercapto-1-undecanol in (B) can be determined by integration analysis of the regions of δ 3.42–3.58 (HOCH_2 of 11-mercapto-1-undecanol) and δ 0.61–2.50 (not shown).

(1-dodecanethiol and 11-mercapto-1-undecanol) in the clusters is about 26.7%. The ratio of 1-dodecanethiol and 11-mercapto-1-undecanol bound to the Au surfaces could be determined by ^{1}H NMR analysis. The ^{1}H NMR spectrum of Au MPC protected with 1-dodecanethiol is shown in Fig. 3A, and the one surrounded with 1-dodecanethiol and 11-mercapto-1-undecanol after the place-exchange reaction is in Fig. 3B.

The coupling of the first amino acid *N*-Boc-phenylalanine onto the Au MPCs was achieved by using PyBOP and HOBt as coupling reagents in methylene chloride under homogeneous conditions (Scheme 2) [15]. The –OH groups of 11-mercapto-1-undecanol serve as a linkage point for anchoring the first amino acid, and the loading capacity of the Au MPC could be manipulated by changing the amount of 11-mercapto-1-undecanol on Au surfaces, which could be controlled by adjusting the concentration of 11-mercapto-1-undecanol in the aforementioned place-exchange reaction. The gold MPCs possessing a core size of 2.8 nm that were selected for peptide elongation studies have high solubility in nonpolar solvent methylene chloride, but they are practically insoluble in polar solvents like acetone. The isolation



Scheme 2. Coating Au MPCs with peptides via the peptide elongation approach. A pentapeptide was synthesized on the Au nanoparticle surfaces. The Mosher's amides were utilized for examining the e.e. of each incorporated amino acid.

of the product Au MPC-*N*-Boc-Phe was simply achieved by removing methylene chloride in vacuo and washing residues with polar solvents acetone and acetonitrile to remove unreacted coupling agents. The coupling efficiency was determined based on the weights of recovered gold residues and ^{1}H NMR analysis of the Au MPC–peptide complexes. The ^{1}H NMR spectrum of the con-

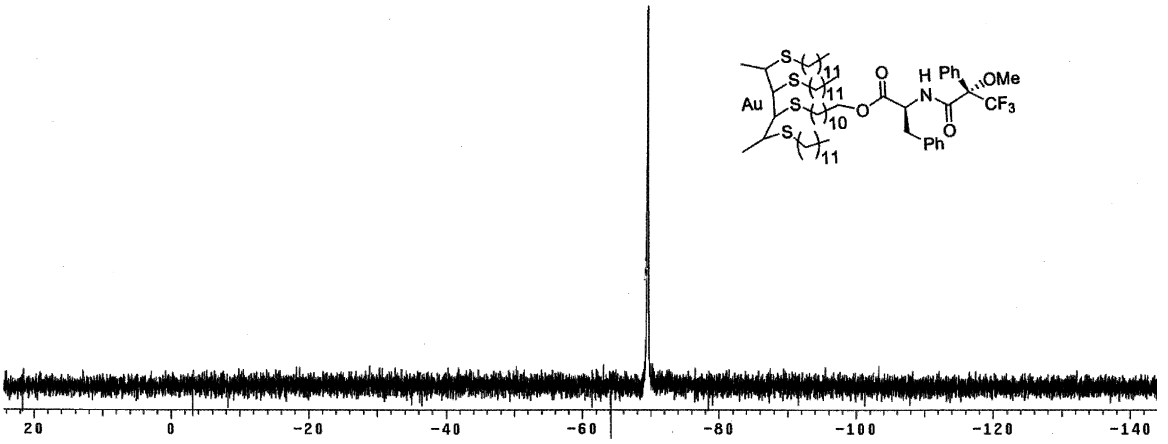


Fig. 4. ^{19}F NMR analysis of the Mosher's amide of phenylalanine on the Au surfaces. The single peak at δ −70 in this spectrum suggests that no racemization occurs during elongation and deprotection steps.

jugate of *N*-Boc-Phe and the gold MPC protected with 1-dodecanethiol and 11-mercapto-1-undecanol is shown in Fig. 3C.

Interestingly, the chain length of alkanethiolate ligands on the gold surfaces was found to affect the coupling efficiency for linking *N*-Boc-Phe and Au MPC molecules (Scheme 2). The Au MPC protected with 1-dodecanethiol (C-12, $n = 11$, Scheme 2) and 11-mercapto-1-undecanol gave 95% yield for coupling *N*-Boc-Phe, but under similar conditions, the Au MPC surrounded with 1-hexanethiol (C-6, $n = 5$, Scheme 2) and 11-mercapto-1-undecanol led to a coupling yield of only 45%. The lower coupling yield of the Au MPC protected with the C-6 alkanethiolate is due to the fact that the Au MPC with shorter protecting ligands intends to aggregate, leading to the greater loss of the peptide–gold complexes and thus the lower isolation yield of the product. On the other hand, the percentage of 11-mercapto-1-undecanol (–OH) on the gold surfaces could also decide the coupling efficiency. Our work found that Au MPCs begin to aggregate when the terminal –OH functionality of the protecting ligands reaches 40% or higher, presumably because of increasing –OH intermolecular hydrogen bonding interactions between different Au MPC molecules.

Subsequent efforts to construct longer peptide chains were carried out on the Au MPC that were protected with 1-dodecanethiol (C-12, $n = 11$) and 11-mercapto-1-undecanol with a ratio of 4:1 for optimal coupling efficiency (Scheme 2). The removal of the *N*-Boc protecting group was carried out by using 50% TFA in methylene chloride at room temperature. ^{19}F NMR analysis of the corresponding Mosher's amide **4** generated from intermediate **2** found no racemization during the PyBop coupling and TFA deprotection steps (Fig. 4). Linkage of **2** with *N*-Boc-Ala under the aforementioned PyBop conditions led to dipeptide **5** in a yield of 95%. Further elongation of the peptide chain on Au supports was executed similarly. A pentapeptide Phe-Ala-Ala-Ala-Ala was constructed on the gold surfaces with an average yield of 95% for each coupling step. The chiral integrity of each amino acid α-chiral center was found to be maintained during the peptide elongation processes via ^{19}F NMR analysis of the corresponding Mosher's amides.

The presence of the pentapeptide Phe-Ala-Ala-Ala-Ala on the Au surfaces could be indirectly supported by ^{1}H NMR analysis of the pentapeptide–gold conjugates (Fig. 5A), but unambiguously confirming the structure of this pentapeptide could only be achieved after the peptide was removed from the gold surfaces (Scheme 3). I_2 oxidation etching of gold followed by saponification of the ester bond linking the C-terminus of the peptide and 11-mercapto-1-unde-

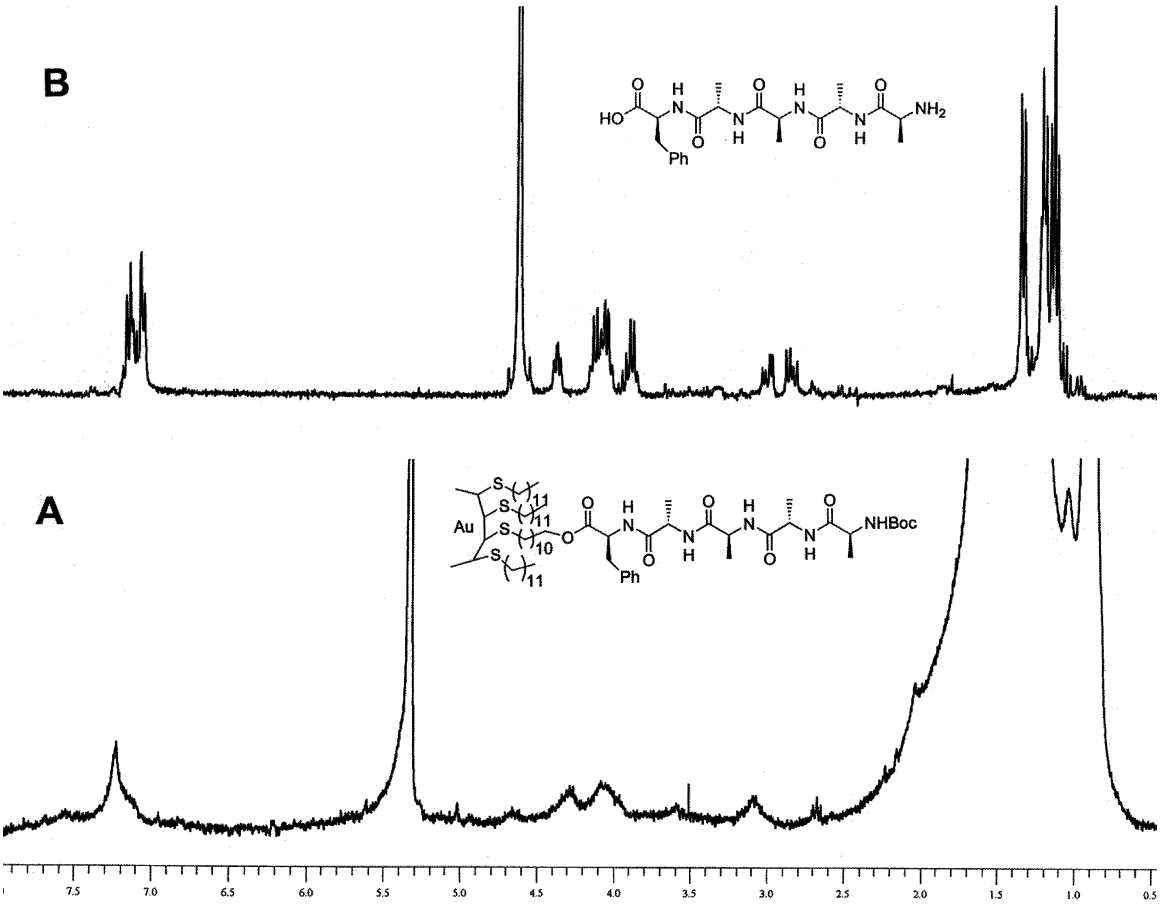
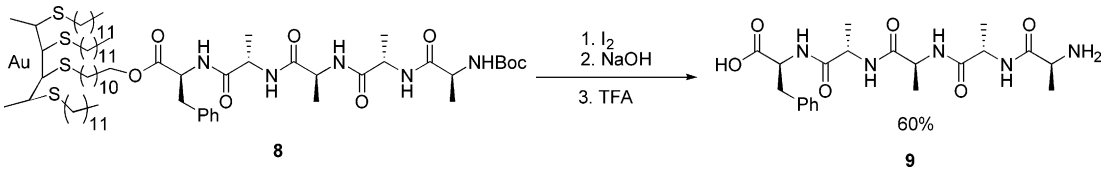


Fig. 5. ^{1}H NMR spectra of the pentapeptide Phe-Ala-Ala-Ala-Ala on the Au MPC in $CDCl_3$ (A); and the peptide after cleavage in D_2O (B).



Scheme 3. Removal of the pentapeptide from the Au nanoparticle surfaces for the structure characterization and purity analysis.

canol with NaOH, and removal of *N*-Boc using TFA gave rise to the final peptide as white powders with an overall yield of about 60%. ^{1}H NMR (Fig. 5B) and mass spectroscopy were used to confirm the structure of the peptide, and the purity of the final product was found to be greater than 95% by HPLC analysis (Fig. 6).

In conclusion, an average coupling yield of 95% could be achieved if the stable Au MPCs are used for elongating peptide chains. The length of alkanethiolate protectors and the percentage of the –OH functionality were found to affect the stability of Au MPCs. During peptide elongation, the chiral integrity of α-carbon center of each

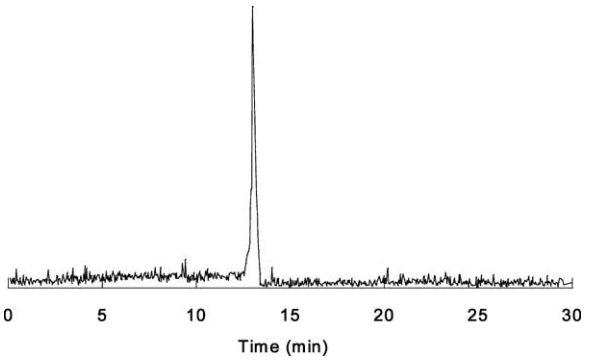


Fig. 6. HPLC analysis of the pentapeptide cleaved from the Au nanoparticle surfaces.

coupled amino acid were maintained. No significant incomplete peptide molecules and other impurities were found in a pentapeptide Phe-Ala-Ala-Ala-Ala synthesized on Au surfaces. Although continuous work is still needed in this area, our work here suggests that the peptide elongation approach can be used as an alternative for coating peptide molecules onto Au MPCs, and this strategy could be potentially expanded for covering other nanomaterials such as nanotubes with biologically active ligands [9].

Acknowledgements

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[15] The similar strategy has been adopted for peptide elongation using *N*-Fmoc amino acids, but very low coupling yields ($<10\%$) were achieved. The details of the low coupling efficiency are still under investigation.