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Amine and Acid-Functionalized Gold Nanoparticles via Thiol-Ene Click Chemistry: A Bio-Orthogonal Route for Surface Bioconjugation

Prakash G Kshirsagar[1]* and Shiv Shankar Sangaru[2]*

[1]*Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, Nebraska, USA*

[2]*Baker Huges, Saudi Arabia*

***Corresponding author:** Shiv Shankar Sangaru, Baker Huges, Saudi Arabia and Prakash G Kshirsagar, Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, Nebraska, USA

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ABSTRACT

Surface functionalization of inorganic nanoparticles without metal catalysts plays a vital role in their application, ranging from biomedical to biotechnological fields. Herein, we described a simple, two-step surface functionalization strategy for gold nanoparticles (AuNPs) using phase transfer and a metal-free 'Thiol-Ene Click' chemistry. Briefly, a tyrosine-capped photoproduced AuNPS were synthesized and transferred into a polar solvent using Octadecylamine (ODA) and further capped with Hexadecanedithiol (HDT). Then, the clickable layer and two different functional allyl derivatives of an acid (acrylic acid) and amine (Allylamine) were used to impart chemical functionality and different charge to the surface of AuNPs. Finally, physicochemical and surface properties of both pristine AuNPs and functionalized AuNPs have been examined by spectroscopic and microscopic techniques. The ease of synthesis for the thiol labeled AuNPs and its ability to undergo a very efficient "metal-free" bio-orthogonal, chemoselective thiol-ene "click" reaction would make a desirable platform for the covalent immobilization of biomolecules and biosensing probes.

Abbreviations: ODA: Octadecylamine; HDT: Hexadecanedithiol; DMF: Dimethylformamide; FTIR: Fourier Transforms Infrared

Introduction

Surface functionalized nanomaterials have emerged as one of the most critical research areas in the field of advanced functional materials [1,2]. These functional nanomaterials are designed to perform a specific role of scientific interest because of their interesting characteristics and widespread applications in drug delivery, medical diagnostics, catalysis, and many others applications [2-5]. By modifying the surface functional group or surface charge, one can modulate the dispersion and adsorption properties of nanoparticles and facilitate the immobilization of biological or non-biological moieties, including dyes, proteins, nucleic acids, fluorophores, Raman reporters, etc. [6]. For instance, Mirkin et al. used oligomers modified AuNPs (Spherical nucleic acid) for hybridization assay [7]. Murphy et al. employed functionalized AuNPs for colorimetric assay to monitor the detection of metal ions [8]. By incorporating the miRNA-specific oligonucleotide on the surface of AuNPs, Degliangeli et al. have designed gold nanoprobes to detect and quantify miRNAs [9]. On the other hand, Carmicheal and co-workers created an anti-MUC4 antibody, and Raman reporter conjugated AuNPs adduct to detect MUC4, a pancreatic cancer biomarker in serum sample [10]. Apart from these, the AuNPs with different functionality such as folate, peptide, and saccharides have been used for targeting and sensing applications.

All these applications emphasize the importance of surface functionality. However, chemistries involved in making these functional derivatives are also an area of tremendous interest that

should include a straightforward, robust, and environmentally friendly synthetic route, which minimizes or eliminates the generation of any side products. Among many approaches, copper-catalyzed click (1,3-dipolar reaction) between azide and alkyne (Cu-AAC) has been used as an ideal route for the ligation of biomolecules. Research to date suggested that the Cu-AAC reaction has emerged as a valuable tool in biomedical fields and inorganic chemistry [11]. Click chemistry has resulted in a paradigm shift by displaying the practical implications of cycloaddition reactions on nanoparticle and cell surfaces, in the cell cytosol, or within the body, which is not easy with most other chemical reactions. Additionally, the Click chemistry *in vitro* approach allocated the specific labeling of cellular target proteins and investigated drug target engagement with drug surrogates in live cells. However, cytotoxicity of the Copper (Cu) catalyst has shown significant restriction on *in vitro* and *in vivo* applications [11-13]. To reduce this cytotoxic effect of copper, researchers have tried a variety of approaches.

It includes the use of stabilizing agents (i.e., bis(tert-butyltriazoly) ligand) or use of ring-strain (strain promoted azide-alkyne cycloaddition, SPAAC), which enables the increased reactivity of alkynes in an azide-alkyne reaction without the need for a cytotoxic copper. However, some chemists were unsatisfied with the second-order rate constant of the SPAAC reaction and the limitation of using large-sized Azide-alkyne molecules that can restrict the progress of the reaction. Thus, a simple alternative to strain-promoted cycloaddition reaction for the functionalization of nanoparticle or biomaterials without the use of copper catalyst is the need of the hour [14]. In the present study, we developed a simple, two-step surface functionalization strategy for gold nanoparticles (AuNPs) using phase transfer and a metal-free 'Thiol-Ene Click' (TEC) chemistry. We then demonstrated the ease of our TEC approach by clicking the Hexadecanedithiol (HDT) functionalized AuNPs (HDT@AuNPs) surface with allyl derivatives of an acid (acrylic acid) and amine (Allylamine). As a result, our approach imparted chemical functionality and charged on the surface of AuNPs via covalent conjugation. The generality of our assay opens up a new possibility for conjugations of different biological and biochemical analytes for biosensing, diagnostics, catalytic applications. Overall, we find this approach is straightforward, site-specific, environmentally benign, and applicable to a wide range of analytes and biomolecules.

Materials and Methods

All chemicals and reagents were purchased from Sigma Alrich and used without any further purification unless otherwise mentioned.

Synthesis of Tyrosine-Capped AuNPs

Photochemical synthesis of Au NPs was carried out using a previously reported procedure with some modifications [15,16]. Briefly, a laboratory reactor system (UV consulting Peschel) fitted with a UV lamp (150 W medium pressure Hg lamp, Heraeus TQ 150) having quartz tubing for cooling with water. Under the vigorous stirring condition, the precooled KOH solution (10^{-3}) was irradiated with a UV lamp. When the lamp glowed steadily at peak intensity, tyrosine and the $HAuCl_4$ salt were added. Irradiation was continued for 20 min. The molar ratio of $HAuCl_4$, tyrosine, and KOH was kept at 1: 3: 10.

Phase Transfer of Tyrosine-Capped AuNPs

Gold nanoparticles prepared by photoreduction method were phase transferred into an organic medium (i.e., chloroform) by using Octadecylamine (ODA) as a phase transfer agent. To a 50 mL as prepared Au NPs, 50 mL of $2x10^{-4}$ M ODA in chloroform was added, followed by 200 µL of 1 M HCl, and the mixture was agitated vigorously for a minute. The red wine color was observed in the lower organic phase while the upper aqueous phase becomes colorless. We recovered phase transferred AuNPs into an organic medium.

Thiol Functionalized Au NPs

Phase transferred Au NPs were further surface-functionalized using Hexamethylene Dithiol (HDT). In typical reaction to a 100 mL of AuNPs in $CHCl_3$ (conc. Ca. ~10 nM) was sonicated for 15 min. The dispersion of Au NPs was mixed with a solution of HDT in $CHCl_3$ (5×10^{-5} M). The reaction mixture was stirred vigorously at RT for 24 h under an inert atmosphere. At the end of the reaction, purification of AuNPs was carried using 3 rounds of centrifugation (15000 rpm, 30 min) interspersed and washing with ethanol. Finally, all AuNPs were dispersed in Dimethylformamide (DMF) and sonicated further for 30 min.

Thiol Click Reaction

In typical experiments, 50 mL of thiol functionalized AuNPs (thiol@Au NPs) dispersed in DMF (Conc. ca. ~ 5 nm) was taken in a 100 mL round bottom flask and sonicated for 15 min. Following complete dispersion of gold NPs, allylamine (Conc. Ca. 5×10^{-5} M) in DMF (1mL) was added dropwise under vigorous stirring and in an inert atmosphere for 24 h. At the end of the reaction, washing these NPs was carried out using two rounds of centrifugation (13000 rpm, 30 min) and three rounds of water (13000 rpm, 30 min) and redispersed in 10 mL of Mili-Q water. Following a similar procedure, thiol functionalized Au NPs also clicked with acrylic acid (5×10^{-5} M). Finally, all the above AuNPs were further characterized with UV- Vis spectroscopy on a carry 300 UV- Visible spectrometer at a resolution of 1 nm using a 5 mm path length quartz cuvette. Transmission Electron Microscopy (TEM) measurements were recorded by a JEOL Jem 1011 microscope (Accelerating voltage = 100kV). TEM analysis samples were prepared on carbon-coated copper grids (carbon 300 mesh Cu grid) by drop-casting Au NPs

samples and adequately dried in a desiccator. Fourier transforms infrared (FTIR) spectra of Au samples were recorded on Bruker Vertex-70 FT-IR spectrometer. All FTIR samples were prepared by making a KBr pellet of Au NPs and dried well under a vacuum desiccator overnight.

Results and Discussion

Gold nanoparticles (AuNPs) are valuable backbone materials for developing sensing systems and bioimaging techniques in biology, biotechnology, and nanomedicine [17-22]. During the fabrication process, an important role is played by the functionalization of the NP surface, frequently achieved with thiol ligands. For instance, this step is used to impart water solubility to the particles [23-28], an essential feature for applying AuNPs in physiologically relevant media. Functionalized nanoparticles that specifically interact with cellular targets are of great interest in biotechnology, biomedicine, and biodiagnostic development [22,29,30]. Especially, functionalized gold nanoparticles (AuNPs) have been demonstrated as versatile tools for various biotechnological applications [17-19,31]. For instance, cell-penetrating peptides conjugated with AuNPs to improve internalization, [32-35] peptides to target the cell nucleus, [34,36-38] oligonucleotides–functionalized AuNPs used in the development of a biosensor for DNA analysis, [39] cell targeting, [40] aptamer conjugated–AuNPs in making biosensor strip for protein analysis, [41] indicating the selectivity and versatility of the biomolecule functionalized nanoparticles. Understanding the importance of surface functionalization, we have developed a new synthetic route to modify AuNPs surface with both acid and amine functional group using the Thiol-ene Michael addition method (Figure 1) and characterized them with spectroscopic and microscopic techniques.

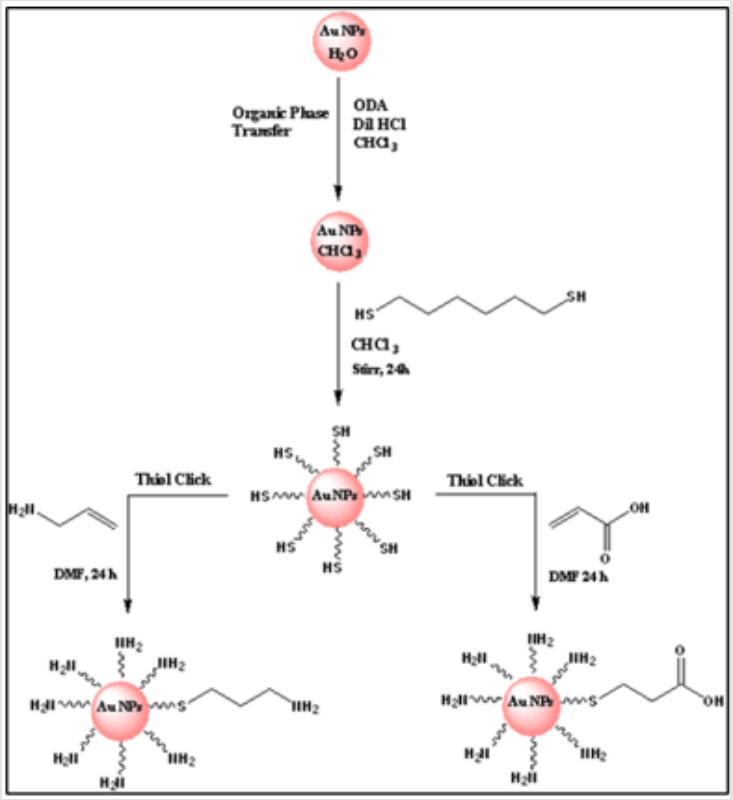

Figure 1: Representative scheme for functionalization of 5 nm AuNPs using Thiol-ene click (Michael) addition reaction.

To study the stability and surface plasmon resonance of the AuNPs during the course reaction, we collected UV-visible spectra of AuNPs. In Figure 2A, SPR spectra of photoreduction AuNPs were observed at 510 nm (curve a), indicating the characteristic plasmonic resonance band for Au NPs solution. After phase transfer of Au NPs with ODA in $CHCl_3$, a distinct redshift at wavelength 525 nm (curve b) can be observed. Further treatment of ODA@Au NPs with HDT in $CHCl_3$ shows absorbance at 542 nm (curve C). One thing to be considered here that while comparing the UV- Vis spectra of ODA and HDT-capped AuNPs with respect to as-prepared Au NPs show a distinct red shift in absorbance peaks. This shift is well known and is attributed to the changes in the electron cloud at the Au NP surface due to chemisorption of amine or thiol functionalized capping agents. Further to understand surface functionality, we performed FTIR measurement of all ODA and HDT-capped (Figure 2B) and Acid/amine-functionalized AuNPs

(Figure 2C). Evidently, the characteristic peak of -C=O stretch at 1680 cm^{-1} from acrylic acid is retained acid-functionalized AuNPs. However, the peaks observed between 3000-2850 cm^{-1} attributed to the -C-H stretch and large featured centered near 1670 cm^{-1} for allylamine-capped AuNPs are consistent with -N-H stretch of pure allylamine addition peak observed between 1500 and 1400 cm^{-1} stretched for C-C bending modes. Overall, the FTIR results revealed surface functionalization of acid and amine moieties. To further confirm these results, we determined the surface charge of AuNPs (Figure 2D). Interestingly, both the tyrosine and acrylic acid capped AuNPs showed negative zeta potential -15 mV and -27 mV due to the exposed $-COO^-$ group at the outer surface; whereas, allylamine capping AuNPs displayed a drastic change in the surface charge of 22 mV, confirming the successful collating of the allylamine.

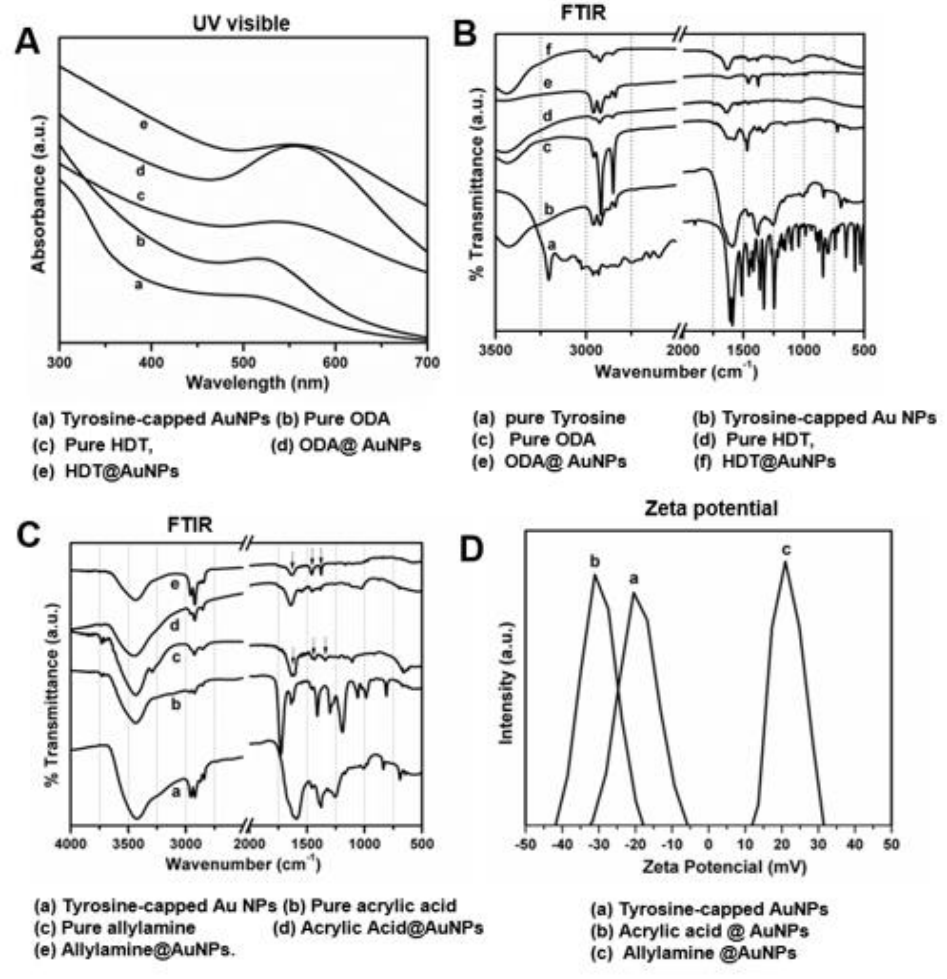

Figure 2: Representative spectroscopic analysis showing surface characterization of AuNPs
A. UV visible spectra of AuNPs and phase transferred AuNPs
B. FTIR spectra of AuNPs and phase transferred AuNPs
C. FTIR spectra of AuNPs and acid and amine functionalized AuNPs
D. Zeta potential measurement of acid and amine functionalized AuNPs.

Next, to confirm the size, morphology, and stability (i.e., aggregation status) of AuNPs before and after the coating/functionalization, the TEM imaging was carried out (Figure 3). At first glance, it can be observed that all the samples of before and after surface modification the NPs seem to have a narrow size distribution. From the Gaussian fit of the histogram, the mean particle size were calculated to be between 4.0-6.0 nm. In summary, our characterization data revealed a successful synthesis of acid- and amine-functionalized AuNPs using the thiol-ene click chemistry. Collectively, the ease of synthesis for the thiol-labeled AuNPs and its ability to undergo a very efficient "metal-free" bio-orthogonal, chemoselective thiol-ene "click" reaction would make an attractive platform for its covalent immobilization of biomolecules and biosensing probes.

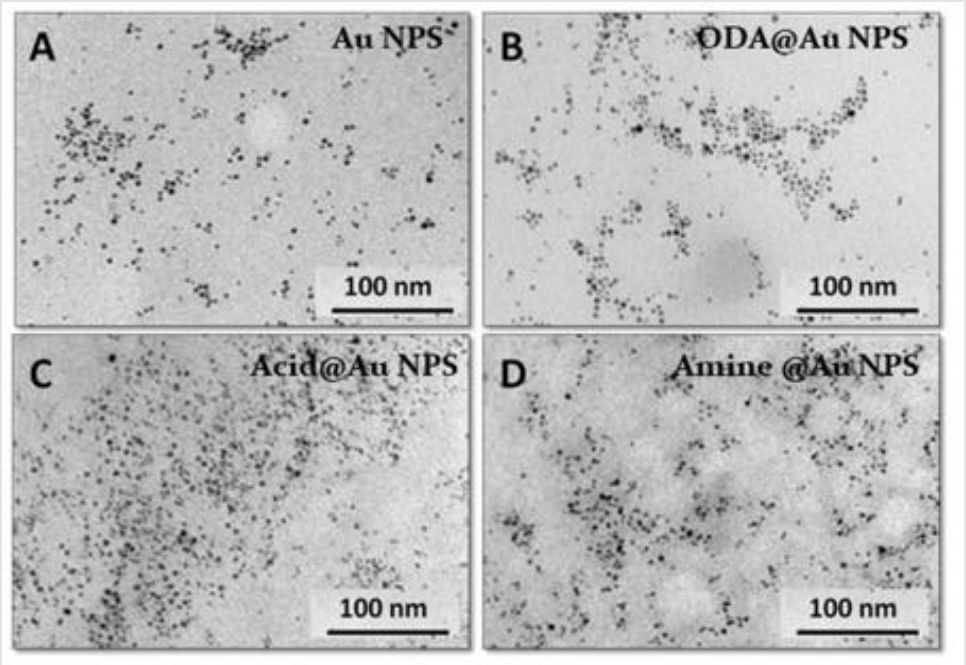

Figure 3: TEM images of
A. Tyrosine-capped AuNPs
B. ODA@AuNPs
C. Acid-functionalized AuNPs
D. Amine-functionalized AuNPs (Scale bar 100 nm).

Conclusion

In summary, we have shown the application of the Thiol-ene click chemistry approach as an efficient way to impart both acid and amine functionality on the surface of AuNPs. We have demonstrated that the thiol-coated and phase transferred AuNPs are participating in the bio-orthogonal Thiol-ene click reaction and imposing the functionality of the acid-/amine group in the presence of a polar solvent. Further, we checked the stability, surface charge, and functionality of the AuNPs using UV visible, zeta potential, and FTIR spectroscopy. The TEM imaging of functionalized AuNPs revealed a formation of stable, monodispersed AuNPs with narrow size distribution and without any significant traces of aggregation. The simplicity of the Thio-ene modified AuNPs opened up a new future perspective in designing a more rational strategy for conjugating various analytes and biomolecules to metal nanoparticles and surfaces for biosensing, biomarker diagnostics, and other bioimaging applications.

Conflicts of Interest

All other authors declare no competing financial interest.

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