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Oligothiol Graft-Copolymer Coatings Stabilize Gold Nanoparticles Against Harsh Experimental Conditions

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Abstract

We report that poly(L-lysine)-*graft*-poly(ethylene glycol) (PLL-*g*-PEG) copolymers that bear multiple thiol groups on the polymer backbone are exceptional ligands for gold nanoparticles (AuNPs). In general, these graft copolymer ligands stabilize AuNPs against environments that would ordinarily lead to particle aggregation. To characterize the effect of copolymer structure on AuNP stability, we synthesized thiolated PLL-*g*-PEGs (PLL-*g*-[PEG:SH]) with different backbone lengths, PEG grafting densities, and number of thiols per polymer chain. AuNPs were then combined with these polymer ligands, and the stabilities of the resulting AuNP@PLL-*g*-[PEG:SH] particles against high temperature, oxidants, and competing thiol ligands were characterized using dynamic light scattering, visible absorption spectroscopy, and fluorescence spectrophotometry. Our observations indicate that thiolated PLL-*g*-PEG ligands combine thermodynamic stabilization via multiple Au-S bonds and steric stabilization by PEG grafts, and the best graft copolymer ligands balance these two effects. We hope that this new ligand system enables AuNPs to be applied to biotechnological applications that require harsh experimental conditions.

INTRODUCTION

The stability of a nanoparticle suspension is often critically dependent on the structure of ligands that are bound to the nanoparticle surface.¹⁻² Surface ligands stabilize nanoparticle suspensions in a variety of ways—they block physical and chemical access to the nanoparticle surface, compatibilize the nanoparticle with solvent, sterically and/or electrostatically inhibit particle-particle interactions, slow the loss of surface atoms to solution or other particles (via Ostwald ripening), and provide functional groups for conjugating biological or other molecules to the particle without interfering with the particle surface. Because of the many roles that surface ligands play, and the different types of materials they are bound to, there is no single ligand structure that stabilizes all nanoparticles in every application. However, the most successful nanoparticle ligands do have some structural features in common. Good surface ligands typically have functional groups that bind the nanoparticle surface strongly, self-interacting (often solvophobic) segments that discourage ligand dissociation, and charged and/or polymer segments that provide electrostatic and steric stabilization.

Designing surface ligands for Au nanoparticles (AuNPs) is easier than for other nanoparticles because of the strong interaction between the Au surface and thiol functional groups.³ As a result, many thiol-functionalized macromolecules have been used to stabilize

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ASSOCIATED CONTENT **Supporting Information.** Details of chemical synthesis, ¹H NMR spectra, and GPC chromatograms of graft copolymers; visible absorption spectra and fluorescence spectra of AuNPs. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Notes The authors declare no competing financial interest.

Au nanoparticle suspensions,⁴ including DNA⁵⁻⁶ and RNA,⁷ peptides⁸ and proteins,⁹ oligosaccharides,¹⁰ and synthetic homopolymers (connected to Au via either terminal¹¹⁻¹² or repeat-unit¹³⁻¹⁵ thiols) and copolymers, including random,¹⁶ alternating,¹⁷ graft,¹⁸ and block¹⁹⁻²⁰ copolymers. Among the polymers, poly(ethylene glycol) (PEG) chains and segments have been used extensively in biotechnological and biomedical applications involving AuNPs²¹⁻²² because of PEG's biocompatibility, its solubility in a broad range of solvent conditions, and the degree of steric stabilization it confers to bound particles. As a result, AuNPs used in room-temperature aqueous buffers are very commonly passivated with thiolated PEG ligands.

However, we and others have found that monothiol ligands, including monothiolated PEGs, are not always stably bound to AuNP surfaces at high temperatures,²³⁻²⁵ in the presence of competing thiols²⁶⁻²⁹ or oxidizing agents,^{23, 30} and in solvents where the ligand is extremely well solvated—conditions that are often encountered in biotechnological protocols. Nuzzo et al. measured the enthalpy of desorption of thiolates (as disulfides) from Au surfaces into the gas phase to be only $\Delta H_{\text{des}} = -28$ kcal/mol,³¹ and others have measured even smaller ΔH_{des} (or related T_{des}) values for solvophilic thiols desorbing into solution.³²⁻³³ The desorption of hydrophilic polymer or biomolecular thiols into water or aqueous buffer from nanoparticle surfaces have small enough ΔH_{des} values that, even though stable Au-S bonds are formed at room temperature, the ligand-surface bonds are thermodynamically disfavored at higher temperature. In biological or biotechnological environments—such as in PCR or other enzymatic reactions, cell preparations, or *in vivo*—ligands are also readily displaced from Au surfaces by competing thiols³⁴⁻³⁵ such as glutathione, mercaptoethanol and dithiothreitol, or by oxidation,³⁶⁻³⁷ and this exchange is accelerated at high temperatures.

One way to enhance the stability of solvophilic, thiol-functionalized ligands on Au surfaces³⁸ and nanoparticles³⁹⁻⁴² has been to increase the number of thiols per ligand. Researchers have investigated the relative stability of AuNPs passivated with ligands bearing two to four thiols per ligand, and have found that they are generally more stable to harsh experimental conditions than AuNPs protected with monothiol ligands.^{40, 43} However, to our knowledge there has been no systematic demonstration of the relationship between the stability of an AuNP in aqueous suspension against heat, corrosion and competing ligands, and the number of thiols in a hydrophilic ligand greater than four.

In this report, we describe the synthesis of different thiolated poly(L-lysine)-*graft*-poly(ethylene glycol) (PLL-*g*-PEG) copolymers and the use of these graft copolymers to passivate the surfaces of AuNPs (Figure 1). We also describe how different numbers of thiols in a ligand affect the thermal stability of AuNPs under harsh conditions—including high temperatures, competing thiols, and chemical etchants. Dynamic light scattering (DLS) analysis and visible absorption spectroscopy were used to monitor the kinetic stability of AuNP suspensions under these conditions. In addition, fluorescence spectrophotometry was used to characterize the desorption of the different graft copolymer ligands at the molecular level. KCN etching experiments were performed to investigate how surface coverage by graft copolymers was related to the resistance of the AuNP surface against chemical attack. Our results provide important guidelines for the design of thiolated ligands for AuNPs, and especially for those used in biotechnological applications,⁴ such as thermocycled PCR, that require harsh experimental conditions.

EXPERIMENTAL SECTION

Poly(L-lysine) trifluoroacetate (PLL₁₁, M_n 2700, PDI 1.1; PLL₃₄, M_n 8200, PDI 1.05; PLL₅₀, M_n 12100, PDI 1.04) was purchased from Alamanda Polymers, Inc. (Huntsville, AL). Methoxy-poly(ethylene glycol)-succinimidyl carboxymethyl (mPEG-SCM, MW

2000), methoxy-poly(ethylene glycol)-thiol (mPEG-SH, MW 2000), and fluorescein poly(ethylene glycol)-succinimidyl carboxymethyl (FAM-PEG-SCM, MW 3400) were purchased from Laysan Bio, Inc. (Arab, AL). *N*-succinimidyl 3-(2-pyridyldithio)-propionate (SPDP) was purchased from ProteoChem, Inc. (Denver, CO). All other reagents were purchased from Aldrich (St. Louis, MO) unless otherwise noted. Ultrapure water was generated from a Milli-Q water purification system (Millipore Inc.; Billerica, MA, $R > 10 \text{ M}\Omega\cdot\text{cm}$). PD-10 desalting columns were purchased from GE Healthcare (Pittsburgh, PA). ^1H NMR spectra were recorded on a Varian Unity (500 MHz) using solvent peaks as internal standards. Visible absorption spectra were obtained on a Hewlett-Packard 8453 UV-Vis spectrophotometer. Gel permeation chromatography (GPC) was conducted on an Agilent 1100 HPLC system (Santa Clara, CA) equipped with two Waters HT4 Styragel columns ($7.8 \times 300 \text{ mm}$, $10 \mu\text{m}$ bead size, Milford, MA) in series, and a refractive index detector (G1362A, Santa Clara, CA). GPC experiments were performed using DMF (0.1 M LiBr) as the eluent with a flow rate of 0.4 mL/min at 50°C . GPC data was calibrated using linear PEG standards (Agilent Tech., Santa Clara, CA). When GPC traces showed multiple peaks, the traces were deconvoluted by assuming multiple polymer populations, and each peak was separately analyzed by fitting to an independent Gaussian lineshape.⁴⁴ Transmission electron microscopy (TEM) images were obtained on a JEOL 1210 electron microscope equipped with a Gatan video camera and a Gatan Multiscan CCD camera (1024×1024 pixels). One drop of nanoparticle solution was placed on copper grid (formvar/carbon, 300 mesh, Electron Microscopy Science) and air-dried. All images were obtained at an operating voltage of 120 kV.

Synthesis of PLL₁₁-*g*-PEG_{*x*}

PLL₁₁ (100 mg) and *N,N*-diisopropylethylamine (100 μL) were dissolved in DMSO (2.3 mL) in a 4 mL polypropylene (PP) vial, and the solution was stirred until the polymer was completely dissolved. Aliquots of this solution were transferred into separate 4 mL PP vials. With stirring, different concentrations of mPEG-succinimidyl carboxymethyl (mPEG-SCM, MW 2000) in DMSO (150 μL) were then added dropwise to each aliquot (Table S1). After reaction overnight, each solution was dialyzed against water using a 2000 Da molecular weight cut-off (MWCO) membrane for 1 d, and then lyophilized to obtain product as a white powder. The resulting polymers were characterized by ^1H NMR (D_2O) and GPC. Both methods indicated that the polymer product contained a small amount ($< 10 \text{ wt}\%$) of mPEG-COOH, presumably due to hydrolysis of mPEG-SCM, that could not be removed by purification; this impurity was retained in subsequent experimental steps. We refer to the product graft copolymers as PLL₁₁-*g*-PEG_{*x*}, where *x* represents the fraction of PLL lysines converted to PEG, as measured by NMR.

Synthesis of PLL₁₁-*g*-[PEG_{*x*}:PDP]

PLL₁₁-*g*-PEG_{*x*} was dissolved in 1X phosphate-buffered saline (PBS, 60 μL) in a 1.5 mL Eppendorf tube. Excess SPDP (at least 1.5 equiv) in DMSO (70 μL) was added into the solution (Table S1). For the synthesis of higher graft ratios of pyridyldithiopropionate (PDP), an additional 100 μL of DMSO was added to dissolve the SPDP. After 10 h, the reaction mixture was dialyzed against water using a 2000 Da MWCO membrane for 1 d and then lyophilized. This yielded polymer products as white powders. Incorporation of PDP groups was confirmed by ^1H NMR (D_2O).

Synthesis of PLL₁₁-*g*-[PEG_{*x*}:SH_{*y*}]

PLL₁₁-*g*-[PEG_{*x*}:PDP] (6.0 mg) was dissolved in 1X PBS (900 μL) in an Eppendorf tube. Then, excess dithiothreitol (DTT; 0.389 M, 100 μL 1X PBS) was added to the solution. After 2 h, the resulting polymer was isolated from small-molecule reactants with a PD-10 size exclusion column, using water as the eluent, according to the manufacturer's

instructions. Water was removed by lyophilization to yield product as a white powder, which was characterized by ^1H NMR (D_2O). We refer to these polymers as $\text{PLL}_{11}\text{-}g\text{-}[\text{PEG}_x\text{:SH}_y]$, where x and y represent the fractions of PLL lysines converted to PEG and thiol groups, respectively, as measured by NMR.

Synthesis of $\text{PLL}_{34}\text{-}g\text{-PEG}_x$

$\text{PLL}_{34}\text{-}g\text{-PEG}_x$ polymers with $x < 0.6$ were synthesized as described above for $\text{PLL}_{11}\text{-}g\text{-PEG}_x$ (Table S2), using PLL_{34} as the starting material. For polymers with $x > 0.6$, pre-formed $\text{PLL}_{34}\text{-}g\text{-PEG}_x$ with $x < 0.6$ was used as a starting material in place of PLL_{34} . To the solution of $\text{PLL}_{34}\text{-}g\text{-PEG}_x$ in DMSO (150 μL) was added DIPEA (0.8 μL) and mPEG-SCM in DMSO (200 μL). After reaction overnight, the solution was dialyzed against water using a 3500 Da molecular weight cut-off (MWCO) membrane for 1 d. Finally, the dialyzed solution was lyophilized to obtain product as a white powder. The product $\text{PLL}_{34}\text{-}g\text{-PEG}_x$ was characterized by ^1H NMR (D_2O) and GPC.

Synthesis of $\text{PLL}_{34}\text{-}g\text{-}[\text{PEG}_x\text{:SH}_y]$

$\text{PLL}_{34}\text{-}g\text{-}[\text{PEG}_x\text{:SH}_y]$ polymers were synthesized as described above for $\text{PLL}_{11}\text{-}g\text{-}[\text{PEG}_x\text{:SH}_y]$ (Tables S2 and S3).

Synthesis of $\text{PLL}_{50}\text{-}g\text{-}[\text{PEG}_x\text{:SH}_y]$

The graft copolymers were synthesized as described above for $\text{PLL}_{11}\text{-}g\text{-}[\text{PEG}_x\text{:SH}_y]$, except that no attempt was made to isolate or purify intermediate polymers $\text{PLL}_{50}\text{-}g\text{-PEG}_x$ or $\text{PLL}_{50}\text{-}g\text{-}[\text{PEG}_x\text{:PDP}]$. PLL_{50} (30 mg) and *N,N*-diisopropylethylamine (30 μL) were dissolved in DMSO (0.6 mL) in a vial, and the solution was stirred until the polymer was completely dissolved. Aliquots of this solution were transferred into separate vials. While stirring, mPEG-succinimidyl carboxymethyl (mPEG-SCM, MW 2000) in DMSO (150 μL) was then added dropwise to each aliquot. After 6 h, excess SPDP in DMSO (70 μL) was added to the aliquots. After the reaction overnight, the crude solution was diluted to 500 μL with DMSO. A portion of this solution (200 μL) was combined with excess TCEP-HCl (10 mg, 35 μmol) for 3 h. The resulting polymers were purified by centrifugal filtration (3000 Da MWCO) using D_2O as solvent. This D_2O solution was characterized by ^1H NMR, and then used directly in subsequent experiments.

Synthesis of fluorescein (FAM)-modified graft copolymers

$\text{PLL}_{11}\text{-}g\text{-}[\text{PEG}_x\text{:SH}_y\text{:FAM}]$ graft copolymers were synthesized by the method described above for $\text{PLL}_{11}\text{-}g\text{-}[\text{PEG}_x\text{:SH}_y]$, with some modification. Before incorporating PEG grafts or thiol groups, PLL_{11} (100 mg, 37 μmol) was initially combined with 5(6)-carboxyfluorescein *N*-hydroxysuccinimide ester (1.8 mg, 3.8 μmol , 0.1 equiv) and *N,N*-diisopropylethylamine (100 μL) in DMSO (1.1 mL) to modify a fraction of the polymer with fluorescein groups. Aliquots of this solution were then used directly as starting materials in reactions with mPEG-SCM as described above. The resulting solutions were loaded onto a PD-10 size exclusion column, using water as the eluent. Two separate fluorescent fractions were collected, corresponding to fluorescein-labeled $\text{PLL}\text{-}g\text{-PEG}$ (eluting first) and free fluorescein. The first fraction was combined with excess SPDP and reduced using DTT as described above for $\text{PLL}_{11}\text{-}g\text{-}[\text{PEG}_x\text{:SH}_y]$ to yield samples of graft copolymer containing a minority of fluorescein groups. Incorporation of fluorescein into the polymer was confirmed by ^1H NMR.

Synthesis of FAM-modified PEG-SH

FAM-PEG-SH was synthesized by the reaction between cystamine and amine-reactive FAM-PEG-NHS. Cystamine dihydrochloride (2.2 mg, 9.8 μmol) was dissolved in a mixture

of 1-methyl-2-pyrrolidinone (200 μ L) and *N,N*-diisopropylethylamine (20 μ L). FAM-PEG-SCM (100 mg, 29.4 μ mol) was added, and this mixture was allowed to react overnight. The solution was loaded onto a PD-10 size exclusion column, using water as the eluent, and the initial colored fraction ($\sim$ 7 mL) was collected. This fraction was extracted with dichloromethane (3×20 mL). The organic extracts were combined, dried over anhydrous sodium sulfate, and filtered, and solvent was removed *in vacuo*. The remaining solids were recrystallized from cold ethanol. The recrystallized material was redissolved in 1X PBS (1 mL), and combined with DTT (0.1 mmol) overnight to reduce disulfide bonds. The reaction mixture was dialyzed against water using a 1000 Da MWCO membrane for 1 d and then lyophilized. This yielded FAM-PEG-NHS as a yellow-orange powder (11.6 mg, 17 %). Successful incorporation of the thiol group was confirmed by ^1H NMR (D_2O).

Grafting ratio determination by ^1H NMR

The PEG grafting ratio, x —defined as the fraction of PLL sidechains that bear a PEG group—and the thiol grafting ratio, y —the fraction of PLL sidechains that are thiol-modified—were calculated from peak integrals (I) in the NMR spectrum, according to the equations below:

$$x = \frac{I(\text{mPEG-OC}_3\text{H}_7)}{N_{\text{PLL}} \times I(\text{PLL-NHC}_5\text{H}_{10}\text{C}_3\text{H}_7)}$$

$$y = \frac{I(-\text{NHCOCH}_2\text{C}_2\text{H}_4\text{SH})}{N_{\text{PLL}} \times I(\text{PLL-C}_5\text{H}_{10}\text{C}_3\text{H}_7)}$$

Preparation of citrate-capped AuNPs

AuNPs were prepared by sodium citrate reduction of HAuCl_4 .⁴⁵ All glassware was cleaned with aqua regia and all H_2O used was ultrapure and filtered through a 0.02- μm pore nylon membrane. A roundbottom flask was charged with 470 mL H_2O and 10 mL of 11.9 mM HAuCl_4 . This solution was stirred vigorously and refluxed. After 30 min, 20 mL of 40 mM sodium citrate was quickly added. As the solution was refluxing, the color of the solution changed from colorless to deep red within 10 min. After refluxing for 1 h, the solution was allowed to cool, and the AuNPs were analyzed by absorption spectroscopy, TEM and DLS; $\lambda_{\text{max}} = 519$ nm, $d(\text{TEM}) = 17.0 \pm 1.1$ nm, and $d_z(\text{DLS}) = 20.3 \pm 0.5$ nm. The amount of Au used in the synthesis and $d(\text{TEM})$ were used to calculate the particle concentration of AuNPs.⁴⁶

Surface modification of AuNPs with PLL-g-[PEG:SH]

The solution of AuNPs was reacted with graft copolymers to modify the surface. To 20 mL of AuNP solution (1 nM) was added excess graft copolymer (at least 1000 polymer molecules/particle) in a 20 mL vial. After vigorous mixing at room temperature, 10 mL of the solution was transferred to a 15 mL screw-top vial, and the vial was heated to 90 $^\circ\text{C}$ in a sand bath. Both the room-temperature and 90 $^\circ\text{C}$ solutions were incubated for three days. Solutions were then cooled, transferred to a 15 mL centrifuge tube, and centrifuged at $8000 \times g$ for 30 min. The supernatant was discarded to remove unreacted polymer, and the AuNP centrifugate was re-dispersed in 15 mL H_2O . Centrifugation, supernatant removal, and re-dispersion were repeated 3 times.

Dynamic light scattering (DLS) analysis

The intensity (z)-averaged hydrodynamic diameter (d_z) of AuNPs in solution was measured with a Nano ZS instrument (Malvern, Worcestershire, England). All AuNP samples were centrifuged briefly ($3000 \times g$, 60 s) to remove dust. The concentration of PBS (1X, 10 mM) and DTT (10 mM) were adjusted by addition of 10X PBS and 100 mM DTT solution,

respectively. The diameter d_z was obtained by cumulant fitting, using an initial decay time of up to 100 μ s to obtain the best fit.

Fluorescence experiments

For fluorescence experiments on polymers bound to AuNPs, the absorbance of the solution was adjusted to 0.1 to minimize inner-filter effects. All samples contained 1X PBS (pH 7.3). The temperature of each capped fluorescence cuvette was controlled in an oil bath. Fluorescence data were collected by a Quantamaster fluorimeter (PTI, London, Ontario; λ_{ex} = 470 nm) using optical filters (FF01-492/SP-25 and BLP01-488R-25, Semrock) to eliminate scattered light. Before and after each measurement, the cuvette was shaken and inverted to homogenize the solution. After 6 h of measurements, AuNPs were etched by adding 50 μ L of 10 M KCN to each sample, incubating at 90 °C for 1 h, and then at room temperature overnight, in order to release all fluorophore still bound to the particles. The fraction of polymer dissociated from the particles over time was calculated by dividing fluorescence intensity at 523 nm by the final fluorescence intensity at 523 nm after KCN etching.

RESULTS AND DISCUSSION

The primary goal of this research was to evaluate thiolated PLL-PEG graft copolymers as stabilizing ligands for Au nanoparticles, and to determine the contributions of different copolymer characteristics—backbone lengths, PEG grafting densities, and number of thiol groups per polymer chain—on the effectiveness of the copolymer ligands. Non-thiolated PLL-PEG graft copolymers have been previously shown to passivate both monolithic⁴⁷⁻⁴⁸ and colloidal⁴⁹⁻⁵⁰ surfaces against adhesion of proteins and cells; a goal of this work was to translate the advantages of PLL-*g*-PEG copolymers to the passivation of AuNPs. Detailed experimental⁴⁷ and theoretical⁵¹⁻⁵² studies on PLL-*g*-PEG-coated surfaces are consistent with past work on the general biocompatibility of PEG coatings, which resist nonspecific adsorption via steric and excluded-volume effects.⁵³⁻⁵⁴ PLL-*g*-PEG has also been used to stabilize suspensions of inorganic nanoparticles,⁵⁵ where the same steric and entropic effects are responsible.¹ In all of these studies, PLL-*g*-PEG was anchored by electrostatic or covalent interactions between lysine side-chains on the polymer and the material surface. We found that combining citrate-capped Au nanoparticles with unmodified PLL-*g*-PEG yielded colloid that was stable at room temperature, similar to previous studies performed with other PEGylated polyamines.⁵⁶ But we found that the PLL-*g*-PEG did not significantly improve the nanoparticles' stability against high temperatures or etchants (data not shown), presumably because the interaction between the polymer lysine groups and the Au surface was not strong enough to ensure a dense layer under these conditions.

Synthesis of oligothiolated graft copolymers

In order to strengthen the interaction between PLL-*g*-PEG and Au colloid surfaces, we incorporated thiol groups onto the PLL backbone to convert the polymer into a multidentate ligand for Au. This was accomplished by sequential addition of NHS-ester-terminated PEG (mPEG-SCM) and NHS-ester-containing thiol linker (SPDP) to monodisperse PLL backbone starting materials, similar to the method reported by Kataoka and coworkers.⁵⁷ The goal of the synthesis was to convert every lysine in the original PLL to either a PEG or thiol group, but we anticipated that some lysine groups would remain unconverted by this approach. As a result, we expected $x + y = 1$ for products PLL-*g*-[PEG_{*x*}:SH_{*y*}], accounting for incomplete conversion of lysine groups in the PLL starting material.

Prior to introducing thiol groups, intermediate PLL-*g*-PEGs were characterized by NMR (Figure S1) and GPC (Figure S2) to verify the grafting ratio (*x*) of PEG. These intermediate

copolymers were then combined with excess SPDP to exhaustively convert remaining lysine groups to pyridyldithiopropionate (PDP) groups. Although the incorporation of PDP groups into PLL-*g*-[PEG_{*x*}:PDP] could be verified by NMR, it was impossible to calculate the degree of PDP incorporation because of band broadening in the NMR spectra (Figure S3A). However, once the PDP groups were reduced to reveal thiols, grafting ratios in the resulting PLL-*g*-[PEG_{*x*}:SH_{*y*}] copolymers were characterized by integration of the sharp peaks in their NMR spectra (Figure 2, S3B, and S4; Tables S1-S4). These results showed that we were only able to synthesize thiolated graft copolymers for $0.25 < x < 0.65$. For $x < 0.25$, no polymer was isolated from the synthesis, presumably because addition of PDP groups made the polymer too hydrophobic or subject to oxidative (disulfide) crosslinking; and for $x > 0.65$, the procedure returned PLL-*g*-PEG_{*x*} intermediates with no thiol groups attached, possibly due to steric crowding by the attached PEGs.⁵⁸

Surface modification of AuNP with PLL-*g*-[PEG_{*x*}:SH_{*y*}]

Citrate-capped Au nanoparticles were combined with PLL-*g*-[PEG_{*x*}:SH_{*y*}] polymers in H₂O at room temperature for 3 d to yield polymer-capped AuNPs that withstood cycles of centrifugal concentration and heating. No color change or precipitation was observed during surface modification, indicating that the oligothiol polymers did not aggregate the AuNPs by crosslinking. After excess copolymer was removed by centrifugation and redispersion in aqueous solution, the polymer-stabilized AuNPs were characterized by DLS analysis and visible absorption spectroscopy (Table 2). The absorption spectra of all polymer-stabilized AuNP suspensions were red-shifted relative to that of the original, citrate-protected particles (λ_{max} , AuNP-citrate = 519 nm; $\Delta\lambda_{\text{max}} = +2\text{--}5$ nm). This shift in the surface plasmon resonance absorption of the AuNPs is consistent with a change in their surface dielectric, induced by surface functionalization.⁵⁹ DLS was used to measure the z-averaged hydrodynamic particle diameter (d_z) of the nanoparticles before and after polymer stabilization.⁶⁰ In general, polymer functionalization consistently increased d_z of the AuNPs by 10–14 nm over the diameter of the original particles. The grafted PEG chains on all of the PLL-PEG copolymers used in this study had a starting degree of polymerization of 41, and assuming the structural model in Figure 1, we estimate that these chains would extend from the particle-bound PLL backbone into solution between 3 nm (random coil) and 14 nm (fully extended). The measured increases in d_z are consistent with this model,⁶¹ though it is difficult to draw firm structural conclusions from hydrodynamic measurements. Not all of the polymers tested were successful; one, PLL₁₁-*g*-[PEG_{0.28}:SH_{0.64}], generated AuNP suspensions with anomalously large d_z and polydispersity index (PDI) values. This polymer had the highest thiol functionalization density in the PLL₁₁ series, and the high d_z value may be due to particle crosslinking by multiple functional groups on the polymer. All of the other polymers tested generated monodisperse particle dispersions (PDI < 0.10 for PLL₁₁ and PLL₃₄ graft copolymers and 0.25 for PLL₅₀ graft copolymers). These dispersions were stable not only in water, but also in solutions with high ionic strength (up to 1 M MgCl₂) over hours at room temperature, where other stabilized AuNP suspensions can aggregate (Figure S5).

Previous studies have shown that ligand passivation of AuNPs can sometimes be improved by “annealing” the product particles at elevated temperatures.^{62–63} To test the effect of temperature on the synthesis of PLL-PEG-protected AuNPs, we also combined AuNPs and PLL-*g*-[PEG_{*x*}:SH_{*y*}] copolymers at 90 °C for 3 d. After purification, the annealed particles exhibited slightly larger d_z and λ_{max} values than those prepared at room temperature, consistent with a denser degree of functionalization and a more extended corona of graft copolymer on the particle surface. One hypothesis of this study was that these characteristics would confer higher stability to the annealed, PLL-PEG-protected AuNPs.

Comparative stability of AuNP@PLL-*g*-[PEG:SH] suspensions

A central goal of this work was to develop AuNPs that are stable to the presence of the free thiols and dithiols used in biotechnology, like DTT, which typically displace surface ligands from the AuNP surface and cause irreversible particle aggregation.⁴³ To test their stability, we gradually heated AuNP@PLL-*g*-[PEG:SH] suspensions in aqueous buffer to 90 °C in the presence of 10 mM DTT, and monitored the particles by DLS and visible absorption spectroscopy. In general, time-resolved DLS experiments (Figure 3) showed that most of the particle suspensions were stable (did not experience an increase in d_z corresponding to aggregation) over hours. An increase in d_z was observed for AuNPs modified with polymers containing either low or high ratios of PEG grafts to thiol groups, and annealed particles were more stable than those prepared at room temperature. In addition, graft copolymers with shorter PLL backbones appeared to be marginally better at stabilizing AuNPs than longer ones. Importantly, all of the AuNP@PLL-*g*-[PEG:SH] particle suspensions were far more stable to heat and DTT than AuNPs functionalized with monothiolated mPEG-SH molecules, which were observed to aggregate even before the solution reached high temperature (data not shown).

These DLS studies were supported by visible absorbance spectroscopy experiments using the same solution conditions and temperature ramp to 90 °C (Figure 4, S6-S9). Using a standard 1-cm cuvette, we observed that all of the particle suspensions exhibited a slow decrease in optical absorbance (~30% over 90 min) as particles adhered to the cuvette walls (and out of the optical path of the instrument); this is frequently observed for polymer- and biomolecule-stabilized AuNPs.⁶⁴ But the $\lambda_{\max}$ of most of these particle samples was not changed by heating in the presence of DTT. We did observe large increases in $\lambda_{\max}$ for AuNPs modified with PLL-*g*-[PEG_x:SH_y] copolymers with the lowest thiol-to-PEG ratios. These large increases in $\lambda_{\max}$ are characteristic of aggregating AuNPs.⁶⁵

From this data, we argue that PLL-*g*-[PEG:SH] graft copolymers successfully stabilize AuNP suspensions by a balance of steric stabilization by PEG chains and thermodynamic stabilization by the oligothiol polymer ligands. In principle, polymers with too many thiol groups might crosslink the target AuNPs, and offer insufficient steric shielding by PEG against agglomeration; polymers with too few thiols might not bond strongly enough to the particle surfaces, or even at all, if the thiolated PLL backbone were occluded by too many grafted PEG chains. Our DLS and absorption spectroscopy experiments do not provide information on the molecular details of the polymer-particle interaction, but they are consistent with this hypothesis of a balance between steric and thermodynamic stabilization.

Stability of AuNP-copolymer interactions

In order to more specifically characterize the interaction between graft copolymers and AuNP surfaces at the molecular level, we performed time-resolved fluorescence experiments on the dissociation of fluorescein-labeled PLL-*g*-[PEG:SH] from AuNPs. AuNPs are outstanding fluorescence quenchers,⁶⁶ and so fluorescent graft copolymer molecules bound closely to AuNP surfaces show no observable fluorescence intensity.²³ However, as the polymers desorb from the surface of the AuNPs into solution, their fluorescence is recovered. Thus, monitoring fluorescence intensity provides a proportional measure of the amount of polymer bound to and released from the AuNP surface.²⁹ In these experiments, AuNP@PLL-*g*-[PEG:SH] suspensions were heated to 90 °C, either in the presence or absence of DTT, and the fluorescence intensity of the suspension was monitored over time (Figure 5). We also performed the same experiments on AuNPs modified with monothiolated FAM-PEG-SH for comparison. Then, at the end of the experiment, the total amount of fluorophore present in each sample was determined by etching away the supporting AuNPs with KCN, and measuring the final fluorescence. In this way, the

fractional fluorescence intensities shown in Figure 5 should represent the fraction of polymer dissociated from the particle surface over time.

Even in the absence of competing DTT, a substantial fraction (30%) of bound, monothiol FAM-PEG-SH was released from AuNPs at 90 °C within 30 min, and 100% of the monothiol dissociated in the presence of 1 mM DTT over the same period. By contrast, oligothiolated PLL-*g*-PEG graft copolymers showed dramatically less desorption under all conditions, demonstrating stronger binding than PEG-SH. Among the different PLL₁₁-*g*-[PEG_{*x*}:SH_{*y*}] polymers studied, ligand desorption was inversely related to the number of thiols per polymer. For example, only 20% of polymers with an average of 6 thiols per PLL chain ($y = 0.55$) dissociated from the AuNP surface at 90 °C over 6 h, while 60% of those with an average of 4 thiols per chain ($y = 0.35$) dissociated over the same time (Figure 5B). Increasing the concentration of DTT led to more rapid ligand dissociation, but even at high temperature and DTT concentrations, some oligothiolated PLL-*g*-PEG remained on the AuNP surface (Figure 5C). These results are consistent with a model for ligand binding in which multivalency leads to stronger surface interactions and resistance against ligand loss.^{14, 67}

Stability of AuNP@PLL-*g*-[PEG:SH] against chemical etching

The instability of ligand-protected AuNPs can often be traced to chemical reactions at the nanoparticle surface. As a result, AuNP suspensions can be stabilized by restricting physical access to nanoparticle surfaces. One general way to test this access to chemical attack is to observe cyanide-induced Au etching in water.⁶⁸ Even though cyanide is not used in typical biotechnology protocols, it is a good proxy for other small molecules that react with Au surfaces, and cyanide etching of AuNPs can be easily monitored by absorption spectroscopy of the disappearing Au surface plasmon band.⁶⁹ We found that the rate of cyanide etching of AuNP@PLL_{*n*}-*g*-[PEG_{*x*}:SH_{*y*}] particles depended upon the relative fraction of thiol groups in the copolymer (Figure 6). For example, AuNP@PLL₁₁-*g*-[PEG_{*x*}:SH_{*y*}] particle suspensions prepared at room temperature lost 80% of their absorbance at λ_{max} within 2 h for $y < 0.5$, but just 20% of their absorbance for $y > 0.6$ (Figure 6A). Particles made with longer PLL backbones were generally less stable to etching for similar grafting ratios (Figure 6B). All AuNP@PLL-*g*-[PEG:SH] particles were made more resistant to KCN etching by first annealing them at 90 °C (Figure 6C). All of these results point to a relationship between the density of thiol functionalization of the AuNP surface—achieved either by controlling the number of thiols per PLL-*g*-[PEG:SH] chain, or by thermodynamically annealing the modified particles—and protection against chemical attack.

Steric and thermodynamic stabilization in AuNP@PLL-*g*-[PEG:SH] particles

We chose thiolated PLL-*g*-PEG graft copolymers as stabilizers for AuNPs because of the potential for combining steric stabilization of the colloidal suspension by PEG chains with strong ligand attachment by multiple thiol groups. This same combination has previously been studied for PLL-*g*-PEG adsorption on flat surfaces. Textor and co-workers have analyzed the effect of grafting ratios on PLL-*g*-PEG_{*x*} adsorption on oxide surfaces, and found that the density of the protective PEG layer and resistance against non-specific protein adsorption was maximized at intermediate values of x .^{47, 51} In these authors' analysis, this represented a balance between affinity of lysine amines for the oxide surface on the one hand, and steric shielding by PEG on the other. Although stabilization of AuNP suspensions by PLL-*g*-[PEG:SH] copolymers is different from passivation of flat oxide surfaces, we argue that many of the same factors that make PLL-*g*-PEG copolymers effective coatings for oxides also apply to the success of the AuNP@PLL-*g*-[PEG:SH] materials reported here. Researchers have also shown that, if the ends of the grafted PEG chains in PLL-*g*-PEG are appropriately functionalized, it is possible to use PLL-*g*-(PEG-X) coatings as scaffolds for

attaching other molecules—including proteins and peptides, affinity tags, and small organic molecules—to inorganic surfaces.⁷⁰⁻⁷¹ Alternately, unconverted lysine amines in the particle-bound PLL-*g*-[PEG:SH] ligands could be used to attach other molecules to the AuNP surface, if shielding by the PEG chains allowed. Although we have not demonstrated this for AuNPs in this report, one could imagine AuNP@PLL-*g*-[PEG-X:SH] as a vehicle for combining the recognition properties of biomolecules and the unique physical properties of AuNPs into an extremely stable nanobioconjugate.

CONCLUSION

We have demonstrated that thiolated PLL-*g*-PEG graft copolymers are exceptional stabilizers for AuNPs, and have explored the relationship between graft copolymer structure and colloidal stability. In general, this study illustrates a balance between thermodynamic and steric factors in graft copolymer stabilization of AuNPs. On the one hand, having multivalent interactions between multiple thiol groups on the graft copolymer and the AuNP surface helps prevent ligand dissociation under extreme conditions, and isolates the surface from chemical attack. On the other hand, grafted PEG chains provide steric stabilization of the colloidal suspension, and keep AuNPs from aggregating. As a result, we predict that future optimization of PLL-*g*-[PEG_{*x*}:SH_{*y*}] ligands for biotechnological applications will involve balancing *x* and *y*. Moreover, we anticipate that the PLL-*g*-[PEG:SH]-stabilized AuNPs could be useful as nanoparticle supports in applications such as PCR, which require harsh experimental conditions including high salt concentrations, elevated temperature, and thiols that typically compete for surface ligands.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Schematic representation of the stabilization of AuNPs by oligothiolyated graft copolymers.

Figure 2. (A) ^1H NMR spectrum of PLL₁₁-*g*-[PEG_{0.58}:SH_{0.21}] dissolved in D₂O. (B) Closeups of ^1H NMR spectra for PLL₁₁-*g*-[PEG_{*x*}:SH_{*y*}], illustrating integration of PEG-CH₂-CONH- (A), lysinyl -CH₂NH₂- (e, e', and e''), and thiopropionate -CH₂CH₂SH (I and II) protons. Grafting ratios *x* and *y* are calculated from these integrals. The spectra also show a peak corresponding to a small amount of PEG-CH₂-COOH impurity (A').

Figure 3.

Intensity-averaged hydrodynamic diameter (d_z) of aqueous AuNP@PLL_n-g-[PEG_x:SH_y] particle suspensions containing 10 mM DTT and 10 mM PBS (pH 7), as a function of time and temperature, measured by DLS. Suspensions were prepared at room temperature (A, C, E) or annealed at 90 °C (B, D, F) with graft copolymers made from PLL₁₁ (A, B), PLL₃₄ (C, D) or PLL₅₀ (E, F) backbones. In each experiment, the solution temperature was increased stepwise from 25 °C to 90 °C as shown in the legend at the top.

Figure 4.

(A) Absorbance at 524 nm of aqueous AuNP@PLL₁₁-g-[PEG_x:SH_y] particle suspensions containing 10 mM DTT and 10 mM PBS (pH 7), as a function of time and temperature. In each experiment, the solution temperature was increased stepwise from 25 °C to 90 °C as shown in the legend at the top. (B-D) Full absorption spectra of AuNP@PLL₁₁-g-[PEG_x:SH_y] suspensions from (A).

Figure 5. Relative fluorescence intensity ($\lambda_{\text{ex}} = 470 \text{ nm}$, $\lambda_{\text{em}} = 523 \text{ nm}$) of AuNP@PLL₁₁-g-[PEG_x:SH_y:FAM] particle suspensions, incubated at 90 °C, as a function of temperature. Solutions contained (A) no DTT, (B) 1 mM DTT, and (C) 10 mM DTT. For each experiment, fluorescence intensity is normalized to the total fluorescence measured after etching away the underlying AuNP with added KCN.

Figure 6.

Absorbance at 520 nm of AuNP@PLL₁₁-g-[PEG_x:SH_y] suspensions exposed to 10 mM KCN, as a function of time. (A) AuNP@PLL₁₁-g-[PEG_x:SH_y] prepared at room temperature; (B) AuNP@PLL₃₄-g-[PEG_x:SH_y] prepared at room temperature; and (C) AuNP@PLL₃₄-g-[PEG_x:SH_y] annealed at 90 °C.

Table 1Characteristics of PLL_{*n*}-*g*-PEG_{*x*} and PLL_{*n*}-*g*-[PEG_{*x*}:SH_{*y*}]

PLL _{<i>n</i>}	PLL _{<i>n</i>} - <i>g</i> -PEG _{<i>x</i>}				PLL _{<i>n</i>} - <i>g</i> -[PEG _{<i>x</i>} :SH _{<i>y</i>}]	
	<i>x</i> NMR	<i>M</i> _{<i>n</i>} ,NMR (g/mol)	<i>M</i> _{<i>n</i>} ,GPC (g/mol)	PDI _{GPC}	<i>x</i>	<i>y</i>
PLL ₁₁	0.18	5200	4900	1.13	0.28	0.64
	0.22	6100	6100	1.28	0.27	0.61
	0.28	7300	6500	1.24	0.32	0.60
	0.32	8100	7200	1.22	0.37	0.51
	0.37	9200	8200	1.19	0.44	0.36
	0.45	11000	8600	1.21	0.50	0.29
	0.60	14000	10000	1.19	0.58	0.21
PLL ₃₄	0.17	15000	20000	1.13	0.29	0.61
	0.25	20000	20000	1.11	0.31	0.67
	0.35	27000	21000	1.09	0.41	0.58
	0.63	45000	26000	1.12	0.56	0.28
	0.77	54000	28000	1.10	0.63	0.20
PLL ₅₀					0.25	0.67
					0.33	0.57
					0.52	0.32
					0.63	0.19

^a Synthetic PLL-*g*-PEG intermediates were not isolated for PLL₅₀-based copolymers.

Table 2Characteristics of AuNP@PLL_n-g-[PEG_x:SH_y].

PLL _n	x	y	thiols per chain	M _{n,NMR} (g/mol)	prepared at RT		prepared at 90 °C	
					d _z (nm)	λ _{max} (nm)	d _z (nm)	λ _{max} (nm)
PLL ₁₁	0.28	0.64	7.04	7900	46.81	524	35.89	527
	0.27	0.61	6.71	7700	32.96	524	33.00	526
	0.32	0.60	6.60	8700	32.51	523	33.48	526
	0.37	0.51	5.61	9700	34.28	524	35.19	524
	0.44	0.36	3.96	11000	33.20	523	36.73	524
	0.50	0.29	3.19	12000	33.10	524	36.66	524
	0.58	0.21	2.31	14000	30.98	523	33.00	523
PLL ₃₄	0.29	0.61	20.77	25000	31.69	523	31.98	524
	0.31	0.67	22.84	26000	31.63	522	32.71	523
	0.41	0.58	19.88	33000	31.34	522	33.61	522
	0.56	0.28	9.43	41000	31.29	521	33.48	522
	0.63	0.20	6.96	45000	30.90	521	31.55	523
PLL ₅₀	0.25	0.67	33.50	33000	32.96	523	36.78	522
	0.33	0.57	28.50	40000	32.47	522	36.53	523
	0.52	0.32	16.00	57000	34.67	522	38.45	523
	0.63	0.19	9.50	67000	34.26	522	36.85	522