Quantifying the Coverage Density of Poly(ethylene glycol) Chains on the Surface of Gold Nanostructures

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Inorganic nanomaterials have attracted widespread interest as a multifunctional platform for various applications in biology and medicine.1–3 Among them, Au nanostructures have been used as both imaging and therapeutic agents for diagnosis and treatment of diseases such as cancer.4–10 To be useful in vivo, it is critical to have the nanoparticles delivered to the site of interest without being accumulated in healthy tissues and organs. The nanoparticles, therefore, must have the ability to bypass the reticuloendothelial system (RES), circulate in the bloodstream for a long period of time, and preferentially accumulate at the site of interest. Poly(ethylene glycol) (PEG) is the most widely used polymer for masking nanoparticles from clearance by RES due to its ability to resist protein adsorption, excellent biocompatibility, and commercial availability.11–14 The success of PEGylation critically depends on the coverage density of PEG chains on the surface of a nanoparticle, herein referred to as the number of PEG chains per nm² of the particle surface. Essentially, the coverage density of PEG chains represents one of the key parameters in determining the efficiency of PEGylation and thereby the protein repelling capability of resultant nanoparticles and their circulation half-life.15,16

PEGylation of Au nanostructures is typically achieved through a ligand exchange process by using thiol-terminated PEG molecules.17–19 For chemically synthesized Au nanostructures, they are often covered and stabilized by different capping ligands depending on the protocols. During PEGylation, the capping ligands are displaced by –S–PEG chains due to a stronger Au–S linkage and an energy gain associated with the intermolecular interaction.20 Different methods have been employed to assess the efficiency of PEGylation, including those that directly measure changes to the physical properties of nanoparticles such as solubility, stability, hydrodynamic diameter, and zeta potential. None of these methods, however, can provide quantitative information with regard to the number of PEG

ABSTRACT

The coverage density of poly(ethylene glycol) (PEG) is a key parameter in determining the efficiency of PEGylation, a process pivotal to in vivo delivery and targeting of nanomaterials. Here we report four complementary methods for quantifying the coverage density of PEG chains on various types of Au nanostructures by using a model system based on HS–PEG–NH₂ with different molecular weights. Specifically, the methods involve reactions with fluorescamine and ninhydrin, as well as labeling with fluorescein isothiocyanate (FITC) and Cu²⁺ ions. The first two methods use conventional amine assays to measure the number of unreacted HS–PEG–NH₂ molecules left behind in the solution after incubation with the Au nanostructures. The other two methods involve coupling between the terminal –NH₂ groups of adsorbed –S–PEG–NH₂ chains and FITC or a ligand for Cu²⁺ ion, and thus pertain to the “active” –NH₂ groups on the surface of a Au nanostructure. We found that the coverage density decreased as the length of PEG chains increased. A stronger binding affinity of the initial capping ligand to the Au surface tended to reduce the PEGylation efficiency by slowing down the ligand exchange process. For the Au nanostructures and capping ligands we have tested, the PEGylation efficiency decreased in the order of citrate-capped nanoparticles > PVP-capped nanocages ≈ CTAC-capped nanoparticles > CTAB-capped nanorods, where PVP, CTAC, and CTAB stand for poly(vinyl pyrrolidone), cetyltrimethylammonium chloride, and cetyltrimethylammonium bromide, respectively.

KEYWORDS: Au nanostructure · PEGylation · ligand exchange · capping ligand

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chains on the surface of a particle. Thermal gravity analysis (TGA) has been widely used to estimate the number of PEG chains on the surface of a nanoparticle.\textsuperscript{21} It measures the mass difference before and after removal of PEG chains due to thermal desorption and decomposition. To measure the mass change accurately, it requires the use of a relatively large quantity of the sample. The coverage density calculated from TGA data corresponds to the total number of PEG chains in the sample, including those loosely trapped among the particles. When the other end of a PEG chain is terminated in a different functional group like /C0NH2, not all the terminal groups can be activated and coupled to another ligand such as a targeting moiety. In this case, there is also a critical need to quantify both the total and “active” /C0PEG/C0NH2 chains on the surface of a Au nanoparticle. Here we accomplish this goal by using a combination of four complementary methods.

We focused on HS—PEG—NH2 molecules with different molecular weights because —NH2 is one of the most commonly used functional group for further conjugation through amide coupling with the carboxylate or carboxylic acid group.\textsuperscript{22,23} Various reagents such as fluorescamine and ninhydrin have been used to quantitatively analyze the number of primary amines in small molecules, peptides, and proteins.\textsuperscript{24–26} The fluorescamine-based assay involves the production of a fluorescent compound that can be quantified by fluorescence spectroscopy with high sensitivity while the ninhydrin-based assay yields a chromophore with strong absorption in the visible that can be measured using ultraviolet–visible (UV–vis) spectroscopy. In this article, we apply these two assays to quantify the total number of HS—PEG—NH2 molecules left behind in the reaction solution after incubation with various types of Au nanostructures. We determined the coverage density of —S—PEG—NH2 chains on the surface of a Au nanostructure and systematically studied the adsorption kinetics of HS—PEG—NH2 with different molecular weights. We also compared the PEGylation efficiencies for Au nanostructures with different morphologies and/or initially capped by different types of ligands. At the same time, we developed two new methods for quantitatively measuring the number of “active” —S—PEG—NH2 chains on the surface of a Au nanostructure using assays based on dye- and Cu2+-labeling. This study provides a set of useful guidelines for the PEGylation of Au nanostructures toward a range of biomedical applications.

**RESULTS AND DISCUSSION**

We used four different methods to measure the coverage density of —S—PEG—NH2 chains on the surface of a Au nanostructure, including fluorescence assays based on fluorescamine and fluorescein isothiocyanate (FITC)-labeling, UV–vis spectroscopy assay involving ninhydrin, and inductively coupled plasma mass spectrometry (ICP–MS) analysis based on Cu2+-labeling. Figure 1 schematically illustrates the principles of these four methods. Both the fluorescamine- and ninhydrin-based assays measure the concentration of unreacted HS—PEG—NH2 molecules left behind in the supernatant of a sample using fluorescence and UV–vis absorption, respectively. These two methods yield the total number of —S—PEG—NH2 chains on the entire surface of all Au

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**Figure 1.** Schematic illustration of the four methods for quantifying the average number of —S—PEG—NH2 chains on the surface of one single Au nanostructure: (i) fluorescamine-based assay, (ii) ninhydrin-based assay, (iii) FITC-labeling assay, and (iv) Cu2+-labeling assay.
nanostructures in the sample after the amount of HS—PEG—NH₂ remaining in the supernatant has been subtracted from the amount of HS—PEG—NH₂ added to the original solution. For the other two methods, the Au—S—PEG—NH₂ conjugates are labeled with FITC molecules or Cu²⁺ ions and precipitated out from the solution for fluorescence and ICP—MS measurements, respectively, after the Au nanostructures have been selectively dissolved with an etching solution. The outputs of these two methods are the coverage densities of “active” —S—PEG—NH₂ chains on the surface of a Au nanostructure that could be labeled with FITC or Cu²⁺.

Synthesis of Au Nanostructures and PEGylation of Their Surfaces via Ligand Exchange. Gold nanostructures were prepared with four different capping ligands: poly(vinyl pyrrolidone) (PVP), cetyltrimethylammonium chloride (CTAC), cetyltrimethylammonium bromide (CTAB), and citrate ions. Their surfaces were then modified with HS—PEG—NH₂ using a ligand exchange process. Figure 2 shows TEM images of PVP-capped Au nanocages (AuNCs) with edge lengths of 30, 50, and 60 nm, respectively; CTAC-capped AuNPs with an average diameter of 40 nm; citrate-capped AuNPs with an average diameter of 42 nm; and CTAB-capped Au nanorods (AuNRs) with average dimensions of 80 nm × 22 nm.

Figure 2. TEM images of the different types of Au nanostructures used in this work: (A, B, C) PVP-capped AuNCs with average edge lengths of 30, 50, and 60 nm, respectively; (D) CTAC-capped AuNPs with an average diameter of 40 nm; (E) citrate-capped AuNPs with an average diameter of 42 nm; and (F) CTAB-capped AuNRs with average dimensions of 80 nm × 22 nm.

During ligand exchange, HS—PEG—NH₂ could displace the original capping ligand on the surface of a Au nanostructure thanks to a relatively strong Au—S bond and the energy gained through intermolecular interactions between the PEG chains. Interestingly, the outcome of a ligand exchange process was found to have a strong dependence on the reaction temperature for PEG chains with relatively low molecular weights (e.g., PEG3000 and PEG5000). The localized surface plasmon resonance (LSPR) peak of the AuNCs only showed very minor red-shifts after incubation with HS—PEG—NH₂ in three different molecular weights at 4°C (Figure 3A), which can be ascribed to the slight changes in refractive index at the interface. The LSPR peak was essentially retained in shape and width, implying that the AuNCs remained to be well dispersed in the medium after ligand exchange and no aggregation had occurred. When conducted at 22°C, however, the LSPR peak of the AuNCs was significantly broadened after surface modification with PEG chains of 3000 and 5000 in molecular weight (Figure 3B), indicating that aggregation had occurred in the system. When a PEG of 20000 in molecular weight was used, no aggregation was observed. In addition, the PVP-covered AuNCs showed no change to its UV—vis spectrum when the sample was aged at 4 and 22°C (Supporting Information, Figure S1). We observed no change to the spectrum either when the PVP-covered AuNCs were conjugated with HS—PEG—COOH at 4°C while the

CTAB-capped Au nanorods (AuNRs) with average dimensions of 80 nm × 22 nm.

Figure 3. UV—vis spectra of 50-nm AuNCs before and after functionalization with HS—PEG—NH₂ at two different temperatures: (A) 4 and (B) 22°C. Note that the AuNCs showed significant broadening for their peaks due to aggregation when they were functionalized with HS—PEG3000—NH₂ or HS—PEG5000—NH₂ at 22°C.
peak intensity was slightly reduced when the conjugation was conducted at 22 °C. These results were consistent with the particle sizes and zeta potentials measured by dynamic light scattering (DLS) (Supporting Information, Table S1). The aggregation observed for both samples involving HS–PEG\textsubscript{3000}–NH\textsubscript{2} and HS–PEG\textsubscript{5000}–NH\textsubscript{2} at 22 °C can be attributed to the direct connection of two AuNCs by HS–PEG–NH\textsubscript{2} and/or to the presence of some thiol groups on the outermost surface of AuNCs, which are susceptible to cross-linking via the formation of a disulfide (S–S) bond between two AuNCs. At 22 °C, both the –NH\textsubscript{2} and –SH groups of a HS–PEG–NH\textsubscript{2} molecule could interact with a Au surface to generate Au–S and Au–N bonds with energies in the range of 30–40 kcal/mol\textsuperscript{27} and 5–10 kcal/mol\textsuperscript{28,29} respectively. At 4 °C, however, the Au–S bond was preferentially formed over the Au–N bond probably due to a kinetic reason, leading to the presence of fewer –SH groups on the outer surface. Such a dependence of selectivity on temperature was also observed by other groups for thiol molecules terminated in the amino group.\textsuperscript{30} As a result, we observed a more positively charged surface and less significant aggregation for the AuNCs conjugated with HS–PEG–NH\textsubscript{2} of the same molecular weight at 4 °C than those at 22 °C. This phenomenon, however, was not observed for PEG of 20000 in molecular weight probably due to its randomly coiled conformation.\textsuperscript{31,32}

**Quantification Using the Fluorescamine-Based Assay.** The fluorescamine-based assay was originally developed for quantification of primary amines in biomolecules with sensitivity on the pM scale.\textsuperscript{24} In this assay, non-fluorescent fluorescamine reacts with a primary amine to generate a fluorescent product that emits light at 480 nm when excited at 390 nm (Figures S2 and S3 in the Supporting Information). Figure 4A shows a calibration curve that correlates the fluorescence intensity at 480 nm with the concentration of HS–PEG–NH\textsubscript{2}. The assay was performed under a basic condition (pH 10), and the typical fluorescence spectra are shown in Supporting Information, Figure S4A. Similar to primary aliphatic amine, the pK\textsubscript{a} of the amine terminus in HS–PEG–NH\textsubscript{2} is in the range of 9–11.\textsuperscript{33} At an acidic pH (e.g., 6.5), the protonated amine could not react with fluorescamine effectively, resulting in a poor linearity for the calibration curve (Supporting Information, Figure S5). In a basic solution, the primary amine was deprotonated and thus became highly reactive toward fluorescamine. Linear relationships were found up to μM concentrations with the slopes decreasing in the order of HS–PEG\textsubscript{3000}–NH\textsubscript{2} > HS–PEG\textsubscript{5000}–NH\textsubscript{2} > HS–PEG\textsubscript{20000}–NH\textsubscript{2}. This trend suggests that the amine terminus of PEGs with low molecular weights reacted with fluorescamine more efficiently than those with high molecular weights. Previous studies showed that a helical conformation was preferred by PEG due to the formation of hydrogen bonds between the neighboring oxygen atoms and water molecules.\textsuperscript{34,35} As such, all the amino groups will be completely exposed at the surface. When the molecular weight of PEG is increased beyond a certain number, however, the helical structure will become less favored. The random coil conformation taken by the polymer chains tends to engulf the amino groups, reducing their accessibility and reactivity.\textsuperscript{36,37}

We then applied the assay to measure the coverage density of HS–PEG–NH\textsubscript{2} chains on the surface of Au nanostructures. Figure 4B shows fluorescence spectra taken from the original solution and the supernatant after incubation with the 50-nm AuNCs for 12 h, respectively.

![Image](image-url)
the inner surface due to a relatively small pore size for the AuNC (ca. 5 nm in diameter for the 50-nm AuNC). Accordingly, the surface area of a 50-nm AuNC was estimated to be 15000 nm². As such, the footprint of an individual −S−PEG−NH₂ chain on the surface of 50-nm AuNC could be derived as 0.61, 1.18, and 7.14 nm² for −S−PEG₃₀₀₀−NH₂, −S−PEG₅₀₀₀−NH₂, and −S−PEG₂₀₀₀₀−NH₂, respectively. Accordingly, the coverage densities of PEG chains were 1.64, 0.85, and 0.14 per nm² for −S−PEG₃₀₀₀−NH₂, −S−PEG₅₀₀₀−NH₂, and −S−PEG₂₀₀₀₀−NH₂, respectively.

Quantification Using the Ninhydrin-Based Assay. In the ninhydrin-based assay, ninhydrin reacts with the primary amine to generate a chromophore in deep blue or purple color, known as Ruhemann's purple (Figure S6). In a typical reaction, the color was developed over a short period of time depending on the reactivity of the amine group and could reach a maximum intensity in 4 min. Figure 5A shows the calibration curves for HS−PEG−NH₂ that correlate the absorbance at 565 nm with the concentration of HS−PEG−NH₂. Similar to the fluorescamine-based assay, linear relationships were found up to μM concentrations with the slopes decreasing in the order of HS−PEG₃₀₀₀−NH₂ > HS−PEG₅₀₀₀−NH₂ > HS−PEG₂₀₀₀₀−NH₂. In general, the detection sensitivity of ninhydrin-based assay was much lower than that of fluorescamine-based assay. For example, HS−PEG₃₀₀₀−NH₂ could still be detected at a concentration of 250 nM by the fluorescamine-based assay (Supporting Information, Figure S4A) while it became very difficult to measure using the ninhydrin-based assay at a concentration of 500 nM (Figure S4B). Figure 5B shows the UV–vis spectra taken from the original HS−PEG−NH₂ solution and from the supernatants after incubation with 50-nm AuNCs. The drop in absorbance was directly proportional to the number of HS−PEG−NH₂ molecules that had been attached to the surface of AuNCs. The average number of −S−PEG−NH₂ per AuNC were found to be 33170 ± 2750, 20000 ± 2400, and 3200 ± 1770 for −S−PEG₃₀₀₀−NH₂, −S−PEG₅₀₀₀−NH₂, and −S−PEG₂₀₀₀₀−NH₂, respectively. The coverage densities of PEG chains were 2.21, 1.33, and 0.21 per nm² for −S−PEG₃₀₀₀−NH₂, −S−PEG₅₀₀₀−NH₂, and −S−PEG₂₀₀₀₀−NH₂, respectively.

Both fluorescamine- and ninhydrin-based assays gave the number of unreacted HS−PEG−NH₂ molecules in the solution, which could then be converted to the number of −S−PEG−NH₂ chains on the surface of AuNCs. As shown in Table 1, the ninhydrin-based assay was found to consistently give a larger number of PEG chains per AuNC as compared to the fluorescamine-based assay. The discrepancy between these two assays could be attributed to a phenomenon known as metal-enhanced fluorescence (MEF). Although both assays measured the number of unreacted HS−PEG−NH₂ molecules in the supernatant, any small amount of AuNCs left in the supernatant might enhance the fluorescence intensity while causing no change to the absorbance because there was no overlap between the absorption peaks of AuNCs and the dye. As a result, the fluorescamine-based assay tended to produce a smaller difference for the samples before and after PEGylation, thus giving a smaller number of PEG chains on each AuNC in comparison with the ninhydrin-based assay. In general, the ninhydrin-based assay is less sensitive to the sample preparation procedure (e.g., complete sedimentation of all AuNCs or not) and should be more reliable for quantifying the number of −S−PEG−NH₂ chains. As a compromise, the ninhydrin-based assay has a much lower detection sensitivity compared to the fluorescamine-based assay. Depending on the physical properties of the sample, one needs to carefully choose a proper quantification method.

Quantification Using an Assay Based on FITC-Labeling. In this assay, FITC molecules were added and coupled to the −NH₂ groups on Au−S−PEG−NH₂ conjugates through a reaction involving isothiocyanate and amino groups. After conjugation, the fluorescence from FITC attached to the surface of Au nanostructures is typically quenched due to a strong interaction between the electrons on the metal surface and the dipole of the dye, a phenomenon known as nanosurface energy transfer (NSET). To recover the fluorescence, an
aqueous KCN solution was added to completely dissolve the Au nanostructures, releasing the $-S-\text{PEG}-\text{FITC}$ chains from the metal surface, probably in the form of disulfide. The intensity of fluorescence from the released $-S-\text{PEG}-\text{FITC}$ is directly proportional to the number of “active” $-\text{NH}_2$ groups that could react with FITC. Figure 6 shows fluorescence spectra recorded from the $-S-\text{PEG}-\text{FITC}$ molecules in three different lengths that had been released from the surface of 50-nm AuNCs. By comparing with a calibration curve (Figure S7), the average numbers of “active” $-\text{NH}_2$ groups were found to be 8860 ± 990, 4760 ± 870 and 1010 ± 620 for $-S-\text{PEG}_{3000}-\text{NH}_2$, $-S-\text{PEG}_{5000}-\text{NH}_2$, and $-S-\text{PEG}_{20000}-\text{NH}_2$, respectively. The percentages of “active” $-\text{NH}_2$ groups for FITC conjugation versus the total $-S-\text{PEG}-\text{NH}_2$ chains measured using the fluorescamine-based assay were 35.9%, 37.5%, and 48.1% for $-S-\text{PEG}_{3000}-\text{NH}_2$, $-S-\text{PEG}_{5000}-\text{NH}_2$, and $-S-\text{PEG}_{20000}-\text{NH}_2$, respectively.

**Quantification Using an Assay Based on Cu$^{2+}$-Labeling.** In this method, the $-\text{NH}_2$ groups on the surface of Au$- S-\text{PEG}-\text{NH}_2$ conjugates were initially coupled with DOTA-$\text{NH}_3$ through an amide coupling reaction via NHS-activated ester, followed by loading of Cu$^{2+}$ ions.42-44 The resultant Au$- S-\text{PEG}$-DOTA-Cu$^{2+}$ conjugates were then digested using *aqua regia* for ICP-MS measurement. The numbers of “active” $-\text{NH}_2$ groups on each AuNC were then derived from the numbers of loaded Cu$^{2+}$ ions, which were found to be 7000 ± 3200, 3800 ± 2100 and 760 ± 400 for $- S-\text{PEG}_{3000}-\text{NH}_2$, $-S-\text{PEG}_{5000}-\text{NH}_2$, and $-S-\text{PEG}_{20000}-\text{NH}_2$, respectively, per 50-nm AuNC. The percentages of “active” $-\text{NH}_2$ groups for DOTA conjugation and Cu$^{2+}$ loading relative to the number of $-S-\text{PEG}-\text{NH}_2$ chains measured by the fluorescamine-based assay was 28.3%, 29.9%, and 36.2% for $-S-\text{PEG}_{3000}-\text{NH}_2$, $-S-\text{PEG}_{5000}-\text{NH}_2$, and $-S-\text{PEG}_{20000}-\text{NH}_2$, respectively. This trend was similar to what was obtained using an assay based on FITC-labeling. However, the number of active PEG-$\text{NH}_2$ units per 50-nm AuNC measured using the Cu$^{2+}$-labeling assay was lower than what was obtained using the FITC-labeling method. The discrepancy can be attributed to the difference in conjugation procedure: The FITC-labeling assay involved one-step conjugation while the Cu$^{2+}$-labeling assay required the use of two steps. In general, the
(positive) \(\Rightarrow\) AuNC—S—PEG\(_{20000}\)−NH\(_2\) (slightly negative) > PVP-capped AuNC (negative). This trend is in agreement with the hydrodynamic diameter measurement and could provide some indirect information about the coverage density of −S−PEG−NH\(_2\) chains on AuNCs, as well as the polymer chain conformation.

**Adsorption Kinetics of HS−PEG−NH\(_2\).** We also studied the adsorption kinetics of HS−PEG−NH\(_2\) for the functionalization of different types of Au nanostructures. The adsorption of a HS-containing aliphatic molecule onto a Au surface occurs very quickly, typically in few minutes, at room temperature.\(^{20,46}\) To monitor the adsorption kinetics, the conjugation of Au nanostructures with HS−PEG−NH\(_2\) was performed at 0 °C in an ice bath. The fluoresceamine-based assay was chosen to quantify the coverage density of −S−PEG−NH\(_2\) chains at different reaction times \(t\) due to its high sensitivity. Figure 7 compares the adsorption kinetics for HS−PEG\(_{20000}\)−NH\(_2\) and Au nanostructures covered with different capping ligands. For PVP-capped AuNCs, fast kinetics was involved at the initial stage, by which 50% of the −S−PEG−NH\(_2\) chains had been adsorbed onto the surface of AuNCs at \(t \approx 10−15\) min. After 20 min, the −S−PEG−NH\(_2\) chains seemed to undergo a reorganization process through desorption and adsorption and finally the coverage density of PEG−NH\(_2\) chains reached a plateau at \(t \approx 100\) min. The CTAC-capped AuNPs showed kinetics similar to what was observed for the PVP-capped AuNCs. For citrate-capped AuNPs, at \(t \approx 5−10\) min, 50% of −S−PEG−NH\(_2\) chains were found to be adsorbed onto the surface of AuNPs, which was faster than the above two cases. An equilibrium state was reached at \(t \approx 60\) min. In the case of CTAB-capped AuNRs, however, much longer periods of time were required to reach both 50% coverage (at \(t \approx 20−30\) min) and an equilibrium state (at \(t \approx 150\) min).

**The Effects of Particle Size and Capping Ligand on PEGylation.** We investigated the effect of particle size on the efficiency of PEGylation by employing AuNCs with different edge lengths. For this purpose, 30-, 50-, and 60-nm AuNCs (Figure 2A–C) were conjugated with HS−PEG\(_{20000}\)−NH\(_2\) using the standard procedure at 4 °C. The average numbers of −S−PEG\(_{20000}\)−NH\(_2\) chains per AuNC were found to be 3300 ± 1040, 12700 ± 3340 and 29500 ± 8980, respectively, using the fluoresceamine-based assay. The coverage densities of −S−PEG\(_{20000}\)−NH\(_2\) chains on the 30-, 50-, and 60-nm AuNCs were calculated as 0.61, 0.85, and 1.36 per nm\(^2\), respectively. As the AuNCs became smaller, the areas of both corners and edges would increase relative to the side faces.\(^{47}\) Because of the presence of curvature at the corner or edge site, the PEG chains would not be able to interact strongly or pack densely, so it is not unreasonable to expect the coverage density of PEG chains to drop as the particle size was reduced.

![Figure 7. Adsorption kinetics for the PEGylation of various types of Au nanostructures with HS−PEG\(_{20000}\)−NH\(_2\) as measured by the fluoresceamine-based assay. The error bars represent standard deviation from six replicas for each measurement. The inset depicts the morphology of each type of Au nanostructure and its surface capping layer.](image-url)
TABLE 2. The Coverage Densities of \( -S-\text{PEG}-\text{NH}_2 \) Chains on Various Types of Au Nanostructures\(^a\)

<table>
<thead>
<tr>
<th>gold nanostructures</th>
<th>coverage density of ( -S-\text{PEG}-\text{NH}_2 ) chains(^b)</th>
<th>coverage density of active ( -\text{NH}_2 ) groups(^c)</th>
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</thead>
<tbody>
<tr>
<td>PVP-capped AuNCs(^d)</td>
<td>0.61</td>
<td>0.19</td>
</tr>
<tr>
<td>CTAC-capped AuNPs</td>
<td>0.41</td>
<td>0.22</td>
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<tr>
<td>citrate-capped AuNPs</td>
<td>1.63</td>
<td>1.01</td>
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<tr>
<td>CTAB-capped AuNPs</td>
<td>0.052</td>
<td>0.029</td>
</tr>
</tbody>
</table>

\(^a\) The coverage density is defined as the number of \( -\text{PEG}-\text{NH}_2 \) chains per \( \mu \text{m}^2 \) of Au surface. \(^b\) Measured by the fluorescamine-based assay. \(^c\) Measured by the FITC-labeling assay. \(^d\) The inner surface and pores of the AuNCs were not included.

“active” \( -\text{NH}_2 \) groups versus total \( -S-\text{PEG}-\text{NH}_2 \) chains on the AuNCs was \(
\approx 30\% \) as compared to a value of \(
\approx 50\% \) for all three other types of Au NPs. These results imply a low coupling efficiency between FITC and the amino groups on the AuNCs, which is possibly because some of the \( -S-\text{PEG}-\text{NH}_2 \) chains diffused through the pores and thus trapped inside the AuNCs. These \( -S-\text{PEG}-\text{NH}_2 \) molecules might be poorly accessible to the dye molecules. On the other hand, as compared to AuNPs with large curvature on the surface, the relatively flat surface of AuNCs could increase the packing density of PEG chains and thus reduce the accessibility and reactivity of terminal amino groups on the PEG chains.

**CONCLUSIONS**

In summary, we have demonstrated four complementary methods for quantitative analysis of the PEGylation efficiency of Au nanostructures, including the coverage density of \( -S-\text{PEG}-\text{NH}_2 \) chains and the “active” \( -\text{NH}_2 \) groups, by using assays based on reactions with fluorescamine or ninhydrin, as well as labeling with a dye or \( \text{Cu}^{2+} \). For a model system based on HS-PEG-\( \text{NH}_2 \) with different molecular weights and AuNCs with 50 nm in edge length, we found that: (i) the number of \( -S-\text{PEG}-\text{NH}_2 \) chains per AuNC decreased in the order of \( -S-\text{PEG}_{5000}-\text{NH}_2 > -S-\text{PEG}_{30000}-\text{NH}_2 > -S-\text{PEG}_{50000}-\text{NH}_2 \); and (ii) the percentage of “active” \( -\text{NH}_2 \) groups versus the number of \( -S-\text{PEG}-\text{NH}_2 \) chains decreased in the same order. While the fluorescamine-based assay was much more sensitive than the ninhydrin-based assay, the latter was less sensitive to the experimental details and thus most reliable among all four methods. The \( \text{Cu}^{2+} \)-labeling assay was less efficient than the assay based on FITC-labeling because of the involvement of a two-step procedure. The initial capping ligand on the Au nanostructures played a key role in determining both the efficiency and kinetics of the ligand exchange process. This study provides a set of useful guidelines for PEGylation of Au nanostructures, as well as for surface modification with other compounds or polymers, in an effort to improve the in vivo delivery of nanomaterials.

**METHODS**

**Chemicals and Materials.** Fluorescamine was purchased from Invitrogen (Carlsbad, CA). HS-PEG\(_{5000}\)-\( \text{NH}_2 \) (MW \( \approx 5000 \)) and HS-PEG\(_{30000}\)-\( \text{NH}_2 \) (MW \( \approx 30000 \)) was obtained from Rapp Polymere GmbH (Tubingen, Germany). 1,4,7,10-Tetraazaacyclododecane-1,4,7,10-tetraaetatic acid mono(N-hydroxysuccinimide ester) (DOTA-NHS, \( \geq 90\% \)) was obtained from Macrocyclics (Dallas, TX). The Kaiser test kit was obtained from Rapp Polymere (Tubingen, Germany). Gold(III) chloride trihydrate (HAuCl\(_4\), \( 3\text{H}_2\text{O}, \geq 99\% \)), gold(III) chloride (HAuCl\(_4\), \( > 99\% \)), silver nitrate (AgNO\(_3\), \( > 99\% \)), poly(vinyl pyrrolidone) (PVP, MW \( \approx 20000 \)), 1,10-phenanthroline ethylenediaminetetraacetic acid (EDTA, \( \geq 99\% \)) was all obtained from Sigma-Aldrich (St. Louis, MO). All other chemicals and materials used for conjugation with FITC or DOTA-NHS for corresponding measurements.

**Quantification of –S–PEG–NH\(_2\) Chains on Au Nanostructures Using Fluorescamine-Based Assay.** Prior to analysis, a calibration curve was obtained from a series of HS-PEG-\( \text{NH}_2 \) solutions with known concentrations. Briefly, to each 3 mL of HS-PEG-\( \text{NH}_2 \) standard phosphate buffered (PB, \( \text{PH} = 10 \)) solution, 0.25 mL of 2 \( \mu \text{M} \) fluorescamine solution in acetone was added. After 15 min, fluorescence spectra (\( \lambda_{\text{ex}} \approx 390 \text{ nm}, \lambda_{\text{em}} \approx 480 \text{ nm} \)) were recorded. The fluorescent intensities at 480 nm for each solution were plotted as a function of the concentration of HS-PEG-\( \text{NH}_2 \) to generate a calibration curve. For sample measurements, 100 \( \mu \text{L} \) of the supernatant solution was diluted into 3 mL with the PB buffer and treated using a procedure similar to what was used for the calibration curve. By comparing the fluorescence intensities with the calibration curve and multiplying the dilution factors, we obtained the concentrations of HS-PEG-\( \text{NH}_2 \) in the supernatant solutions. The number of HS-PEG-\( \text{NH}_2 \) on the Au nanostructures was obtained by subtracting the number of HS-PEG-\( \text{NH}_2 \) in the supernatant from the total number of HS-PEG-\( \text{NH}_2 \) added into the suspension of Au nanostructures. This number was then converted to the coverage density by taking into account the total number of Au nanostructures and their total surface area. Each data point represents an average of three replicas.
Quantification of S-PEG-NH₂ Chains on Au Nanostructures Using Ninhydrin-Based Assay. All reagents used for the assay were prepared according to the literature. Typically, 6% ninhydrin ethanol solution was prepared by dissolving 2.5 g of ninhydrin in 50 mL of anhydrous ethanol. The KCN pyridine solution and 80% phenol solution in ethanol from the Kaiser test kit were combined at a 1:1 volume ratio to give a KCN/phenol solution. Prior to assay, a calibration curve was obtained from a series of HS-PEG-NH₂ standard solutions with known concentration N₄ (μg/mL), and S-PEG-NH₂ standard solutions, 100 μL of 6% ninhydrin ethanol solution and 200 μL of KCN/phenol solution were added, followed by heating at 100 °C for 4 min. After being cooled down in an ice bath, 200 μL of 60 wt % ethanol in water was added. UV–vis spectra were then recorded. The calibration curve was generated by plotting the absorbance at 565 nm as a function of the HS-PEG-NH₂ concentration. Samples of 250 μL (the supernatant solution) were treated using the same procedure as that used for standard solutions. The number of overall HS-PEG-NH₂ on the Au nanoparticle was calculated using the same method as that of the fluorous-based assay. Each data point represents an average of three replicas.

Quantification of Active –NH₂ Groups on Au Nanostructures by Dye-Labeling Assay. To 500 μL of Au/S-PEG-NH₂ (with a known concentration), 2 μL of 50 mM FITC in dimethyl sulfoxide (DMSO) was added. The reaction mixture was incubated at room temperature for 15 min, followed by five times washing with water. The pellet was dispersed in 500 μL of water. To the pellet suspension, 2.5 mL of 40 mM KCN aqueous solution and 2.0 mL of water was added to dissolve the Au nanoparticles at room temperature for 30 min. After that, fluorescent spectra (λmax ~ 488 nm, λexc = 520 nm) were taken from the sample. The concentration of FITC in each sample was calculated by comparing with the calibration curve obtained for free FITC aqueous solution. The number of active –NH₂ groups per Au nanoparticle was calculated from the numbers of FITCs and Au nanoparticles. Each data point represents an average of three replicas.

REFERENCES AND NOTES

