Binding affinity of surface functionalized gold nanoparticles to hydroxyapatite

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Abstract: Gold nanoparticles (Au NPs) have been investigated for a number of biomedical applications, including drug and gene delivery vehicles, thermal ablation therapy, diagnostic sensors, and imaging contrast agents. Surface functionalization with molecular groups exhibiting calcium affinity can enable targeted delivery of Au NPs to calcified tissue, including damaged bone tissue. Therefore, the objective of this study was to investigate the binding affinity of functionalized Au NPs for targeted delivery to bone mineral, using hydroxyapatite (HA) crystals as a synthetic analog in vitro. Au NPs were synthesized to a mean particle size of 10–15 nm and surface functionalized with L-glutamic acid, 2-aminoethylphosphonic acid, or alendronate, which exhibit a primary amine for binding gold opposite carboxylate, phosphonate, or bisphosphonate groups, respectively, for targeting calcium.

Bisphosphonate functionalized Au NPs exhibited the most rapid binding kinetics and greatest binding affinity to HA, followed by glutamic acid and phosphonic acid. All functional groups reached complete binding after 24 h. Equilibrium binding constants in de-ionized water, determined by nonlinear regression of Langmuir isotherms, were 3.40, 0.69, and 0.25 mg/L for bisphosphonate, carboxylate, and phosphonate functionalized Au NPs, respectively. Functionalized Au NPs exhibited lower overall binding in fetal bovine serum compared to de-ionized water, but relative differences between functional groups were similar. © 2011 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 99A: 58–66, 2011.


INTRODUCTION

Gold nanoparticles (Au NPs) have been investigated for a number of biomedical applications,1,2 including drug and gene delivery vehicles,3,4 thermal ablation therapies,4,5 diagnostic sensors,6,7 and imaging contrast agents.8–11 The utility of Au NPs in these applications is derived from multiple factors including the ease of synthesizing monodispersed nanoparticles, colloidal stability, biocompatibility, surface plasmon resonance, and high X-ray attenuation. Moreover, Au NPs are readily surface functionalized with molecular groups for cellular targeting and delivery, as well as enhancing colloidal stability. Surface functionalization with molecular groups exhibiting calcium affinity could enable targeted delivery of Au NPs to mineralized tissue, including fatigue microdamage in bone tissue,12 as well as calcifications in soft tissue associated with cancer or musculoskeletal injury.

Microdamage accumulates in bone tissue during repetitive loading in the form of microcracks and diffuse damage.13,14 The accumulation of microdamage has been implicated in clinical fracture susceptibility, including fatigue fractures in active individuals, such as military recruits, fragility fractures in the elderly, and the effects of long-term antiresorptive treatments for osteoporosis.13,14 However, the role of microdamage in clinical bone fragility remains poorly understood due, in part, to limitations in available methods for detection and imaging.14

Current methods for imaging microdamage in bone tissue are limited to histological sections, which are inherently two-dimensional (2-D), destructive, invasive, and tedious.15 The first and most common method has been en bloc staining with basic fuchsin in ethanol.15–17 Basic fuchsin exhibits protein affinity, but primarily acts as a space-occupying dye.15 Therefore, microdamage must be distinguished from nonspecific staining under observation in transmitted light or epifluorescent microscopy. More recently, fluorophores, such as xylene and calcein, which employ iminodiacetate moieties for calcium-chelation, have been used as damage-specific labels for epifluorescent microscopy.15,18,19 Moreover, sequential labeling with fluorophores exhibiting differing binding affinity enabled the measurement of spatiotemporal variation in damage events, including crack propagation18,19 and modes of loading.20 Additionally, calcium-specific photoinduced electron transfer sensor molecules have been
investigated for the ability to "switch-on" fluorescence upon chelation to calcium ions in damaged tissue. However, both light and epifluorescence microscopy are inherently 2-D. Three-dimensional (3-D) spatial information can only be ascertained from serial sectioning, which is destructive and tedious. Laser scanning confocal microscopy has been used to provide limited depth of field for 3-D imaging of damage in histological sections labeled with fluorophores.

X-ray computed tomography has been proposed for non-destructive and 3-D imaging of damage using contrast agents with higher X-ray attenuation than bone tissue. The presence, spatial variation, and accumulation of microdamage in both cortical and trabecular bone specimens was detected using micro-computed tomography (micro-CT) after staining tissue with a precipitated barium sulfate (BaSO₄) contrast agent. While this technique enabled non-destructive and 3-D detection of microdamage in vitro, the precipitated BaSO₄ stain was not damage-specific and the staining solutions were not biocompatible. Iodinated molecules were investigated as a damaged-specific X-ray contrast agent, but were only detected using micro-CT when precipitated in powder form.

Functionalized Au NPs were recently investigated as a targeted X-ray contrast agent for labeling microdamage in bone tissue. Gold exhibits greater X-ray attenuation than other commonly used X-ray contrast agents containing barium and iodine. Au NPs exhibited high X-ray attenuation and biocompatibility as an intravascular X-ray contrast agent in mice after intravenous administration. Functionalized Au NPs also exhibit relatively high water solubility and low viscosity compared to iodinated molecular contrast agents. Finally, gold surfaces are readily functionalized through adsorption of thiols and amines, which can be used to attach molecules which target calcium ions on bone mineral crystals exposed on the surface of microcracks.

The objective of this study was to investigate the binding affinity of functionalized Au NPs for targeted delivery to bone mineral, using hydroxyapatite crystals as a synthetic analog in vitro. Au NPs were surface functionalized with either l-glutamic acid, 2-aminoethylphosphonic acid, or alendronate (Fig. 1). Glutamic acid is a naturally occurring amino acid with known affinity for calcium phosphates. Moreover, glutamic acid functionalized Au NPs were previously shown to target damaged bone tissue. However, phosphonate and particularly bisphosphonate groups are known to exhibit strong binding affinity to calcium phosphates. Bisphosphonates, such as alendronate, are widely used clinically to suppress bone resorption as a pharmacological treatment for osteoporosis and other bone-related diseases.

**EXPERIMENTAL METHODS**

**Gold nanoparticle synthesis and functionalization**

Au NPs were synthesized to a mean particle diameter of 10–15 nm using the citrate reduction method. Briefly, 0.1 g HAuCl₄·3H₂O (≥99.9%, Aldrich) was added to 400 mL of de-ionized (DI) water and the solution was boiled vigorously while stirring. A 1% solution of trisodium citrate dehydrate (ACS reagent, ≥99.0%, Sigma) was added to the boiling solution at a mass ratio of 5:1 HAuCl₄ to sodium citrate and left boiling for an additional 20 min. The solution volume was then adjusted to a total of 500 mL using DI water. The resulting Au NP solution had a gold concentration of ~0.5 mM and a wine red color.

Au NPs were prepared for functionalization by first removing excess citrate ions. 1.5 mL 2% polyvinyl alcohol (PVA 10–98, Mw = 61,000, Fluka) was added to 24 mL Au NP solution, followed by 0.6 g ion exchange resin (Amberlite MB-150, Sigma). The resulting solution was stirred overnight and subsequently filtered (Grade 3, Whatman) to remove spent ion exchange resin. Au NPs were surface functionalized by adding 1 mL of a 0.01M solution of either l-glutamic acid (≥99.5%, Fluka), 2-aminoethylphosphonic acid (99%, Aldrich), or alendronate sodium trihydrate (≥97%, Sigma) to the Au NP solution (Fig. 1). The solution was left to equilibrate under mild stirring overnight. Excess functionalization molecules were removed by dialysis (Spectra/Por, MWCO = 3500, Spectrum Laboratories) against DI water for a total of 3 days, changing the water solution at least twice daily.

The mean particle diameter and particle size distributions were measured before and after functionalization by transmission electron microscopy (TEM, Hitachi H-600) at 75 kV accelerating voltage. TEM specimens were prepared by dropping a solution of functionalized Au NPs onto carbon-coated grids and evaporating the solvent. The particle diameter and aspect ratio were characterized by the mean and standard deviation from measurements on a total of 100 particles per functional group. Ultraviolet–visible (UV–vis) spectra (Varian Cary 3 spectrophotometer) were collected before and after functionalization to verify colloidal stability and relative concentrations of the particles within solution.

Surface functionalization of Au NPs was qualitatively verified using diffuse reflectance infrared Fourier transform
Binding affinity of functionalized Au NPs

Two separate binding experiments were performed to investigate the binding affinity of functionalized Au NPs to bone mineral, using hydroxyapatite (HA) crystals as a synthetic analog. Calcium-deficient HA single crystal whiskers were synthesized using the chelate decomposition method, as described in detail elsewhere.\(^{46}\) As-synthesized HA crystals exhibited a mean length of \(\sim 18\ \mu m\) and mean width of \(\sim 2\ \mu m.\)\(^{46}\) The specific surface area of the crystals was 5.63 m\(^2\)/g as measured by Brunauer–Emmett–Teller (BET) N\(_2\) adsorption (Autosorb-1, Quantachrome Instruments).

The first set of experiments investigated the kinetics of binding. HA whiskers (10 ± 0.1 mg) were added to DI water, followed by a measured volume of functionalized Au NP solution, for a final volume of 15 mL containing a gold concentration of 0.1 mg/L. Solutions were placed onto a test tube rotator and allowed to incubate for 0.25, 0.5, 1, 2, 4, 8, 12, and 24 h. After the predetermined time, solutions were centrifuged at \(\sim 700g\) for 2 min to separate HA crystals and bound Au NPs from Au NPs remaining in solution. Percent binding was defined as the concentration (mg Au/L) of Au NPs remaining in the supernatant solution after binding, subtracted from the initial concentration of Au NPs in solution, and divided by the initial concentration of Au NPs.

A second set of experiments investigated the effect of concentration on binding affinity by plotting the binding isotherm for each functional group. The binding affinity of functionalized Au NPs to HA crystals was determined separately in DI water and 10% fetal bovine serum (FBS, Omega Scientific). The binding affinity of as-synthesized, citrate stabilized Au NPs was also measured as a control. Experiments were performed using the same methods outlined above for the binding kinetics, except the incubation time was held constant at 4 h and the initial gold concentration was varied. The kinetics of binding revealed that after 4 h 30–80% of Au NPs were bound to HA crystals depending on the functional group. This incubation time was also comparable to previous studies investigating bisphosphonate functionalized protein binding to HA surfaces.\(^{43,44}\)

Binding isotherms were plotted as the amount of gold bound per mass of HA crystals added, \(V\) (mg Au/g HA), versus the initial gold concentration, \([S]\) (mg Au/L), for both DI water and FBS. Plotted binding data were modeled as a Langmuir isotherm,

\[
V = \frac{V_{\text{max}}[S]}{K + [S]}
\]

where \(V\) is the amount of functionalized Au NPs bound per mass of HA crystals (mg/g), \(V_{\text{max}}\) is the maximum surface binding (mg/g), \([S]\) is the initial concentration of gold (mg/L), and \(K\) is the equilibrium binding constant (mg/L). The equilibrium binding constant, \(K\), and maximum binding of functionalized Au NPs on HA crystals, \(V_{\text{max}}\), were measured from Langmuir isotherms using nonlinear least squares regression.

The gold concentration in control and supernatant solutions was measured using inductively coupled plasma-optical emission spectroscopy (ICP-OES, Optima 7000, PerkinElmer). Solutions were acidified to 2% v/v HCl prior to analysis. Calibration curves were created by diluting certified standard gold solutions (SPEX CertiPrep). All binding tests were performed at least in triplicate, reporting the mean and first standard deviation.

**RESULTS AND DISCUSSION**

**Characterization of functionalized Au NPs**

Functionalized Au NPs were spherical and relatively mono-dispersed (Fig. 2). There was no apparent change in particle size or morphology between as-synthesized and functionalized Au NPs. The mean (±standard deviation) particle diameter was 13.4 (1.2) nm for as-synthesized Au NPs, with an aspect ratio of 1.1 (0.1). The mean (±standard deviation) particle diameter was 11.4 (1.4), 13.6 (1.4), and 12.8 (1.6) nm for glutamic acid [Fig. 2(a)], phosphonic acid [Fig. 2(b)], and bisphosphonate [Fig. 2(c)] functionalized Au NPs,
respectively, with a mean (±standard deviation) aspect ratio of 1.1 (0.1) for each group. The plasmon resonance peak remained constant at ∼530 nm for functionalized Au NPs, indicating that the colloidal suspension remained stable after functionalization (Fig. 3). The plasmon resonance band broadened slightly upon surface functionalization, due to the adsorption of amine groups.

Gold surface adsorption was expected to occur through the terminal amine group present in each functional molecule (Fig. 1). Previous studies have reported that the amine–gold surface interaction is facilitated by a weak covalent bond and an electrostatic complex between protonated amine groups and chloroaurate ions. Adsorption of terminal amines facilitated the availability of carboxylate, phosphonate, and bisphosphonate groups for binding to calcium on HA crystals, which was qualitatively verified by FTIR (Fig. 4). Characteristic peaks for primary amines (3400–3500 cm⁻¹, N–H stretch; 1500–1650 cm⁻¹, N–H scissoring) were apparent for stock solutions of functional molecules, but were not apparent for functionalized Au NPs. A broad hydroxyl peak (2500–3600 cm⁻¹, O–H stretch) was present in all spectra. As-synthesized, citrate stabilized Au NPs and phosphonic acid functionalized Au NPs also exhibited peaks corresponding to alkyl groups (2850 and 2920 cm⁻¹, C–H stretch) which were not apparent for glutamic acid and bisphosphonate functionalized Au NPs. Carbonyl peaks (~1600 and 1710–1730 cm⁻¹, C=O stretch) were most prominent for glutamic acid functionalized and as-synthesized, citrate stabilized Au NPs. Phosphonic acid and bisphosphonate functionalized Au NPs exhibited peaks corresponding to phosphonyl groups (1220–1260 cm⁻¹, P=O stretch; 2500–2700 cm⁻¹, PO–H stretch). The location of these peaks were similar to a previous study on phosphonic acid functionalized Au NPs. Taken together, the FTIR spectra suggested that Au NPs exhibited the intended terminal surface functionality, but did not quantify the amount of functional molecules adsorbed to Au NP surfaces.

FIGURE 2. Particle size distributions and representative TEM micrographs of (a) glutamic acid (GA), (b) phosphonic acid (PA), and (c) bisphosphonate (BP) functionalized Au NPs. The scale bar in each micrograph represents 100 nm. The mean (±standard deviation) particle diameter was 11.40 (1.36), 13.57 (1.43), and 12.79 (1.58) nm for GA-, PA-, and BP-Au NPs, respectively.

FIGURE 3. UV–vis spectra of as-synthesized Au NPs compared to glutamic acid (GA), phosphonic acid (PA), and bisphosphonate (BP) functionalized Au NPs, showing a consistent plasmon resonance peak at ∼530 nm, which indicated that the particle size did not change and the colloid was stable after surface functionalization.
The mean (± standard deviation) amount of functional molecules adsorbed to glutamic acid, phosphonic acid, and bisphosphonate functionalized Au NP surfaces was 5.01 (0.02), 4.96 (0.02), and 3.25 (0.14) μmol/mg Au, respectively, as measured by ESI-MS. Therefore, the surface density of glutamic acid and phosphonic acid was similar and ~50% greater than bisphosphonate. The lower surface density of bisphosphonate molecules adsorbed to Au NP surfaces was likely due to steric interactions given the larger size of bisphosphonate molecules compared to glutamic acid and phosphonic acid (Fig. 1).

Binding of functionalized Au NPs to HA crystal surfaces was confirmed by direct observation in TEM (Fig. 5). The number of Au NPs bound to HA crystal surfaces was comparatively greatest for bisphosphonate functionalized Au NPs followed by glutamic acid and phosphonic acid. Bisphosphonate functionalized Au NPs exhibited more rapid binding kinetics compared to glutamic acid or phosphonic acid functionalized Au NPs, reaching complete binding after 8 h in DI water (Fig. 6). Glutamic acid and phosphonic acid functionalized Au NPs exhibited similar binding kinetics, reaching near complete binding by 24 h in DI water. Thus, kinetic tests suggested differences in binding affinity between different functional groups, requiring further binding experiments.

Binding affinity isotherms for functionalized Au NPs exhibited large differences between functional groups.

Functionalized Au NP binding to HA crystals
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Binding affinity isotherms for functionalized Au NPs exhibited large differences between functional groups.
Fig. 7. Bisphosphonate functionalized Au NPs exhibited the greatest binding affinity to the surface of HA crystals, as evidenced by a comparatively greater amount of gold bound per HA (mg/g) and greater saturation concentration. Binding isotherms for glutamic acid [Fig. 7(a)], phosphonic acid [Fig. 7(b)], and bisphosphonate [Fig. 7(c)] functionalized Au NPs in DI water exhibited correlation coefficients ($R^2$) of 0.88, 0.69, and 0.95, respectively, using a Langmuir model (Table I). The equilibrium binding constant, $K$, and the maximum surface binding, $V_{\text{max}}$, were greatest for bisphosphonate functionalized Au NPs followed by glutamic acid and phosphonic acid (Table I). The maximum surface binding was also normalized by the specific surface area of the HA crystals to obtain a measure of the maximum binding of bisphosphonate functionalized Au NPs.

**Table I. The Equilibrium Binding Constant, $K$ (mg/L), Maximum HA Surface Binding, $V_{\text{max}}$ (mg Au/g HA), and Maximum HA Surface Binding Normalized to the Specific Surface Area of HA Crystals, $V_{\text{max}}$ (mg Au/m$^2$ HA), for Glutamic Acid (GA), Phosphonic Acid (PA), and Bisphosphonate (BP) Functionalized Au NPs Compared to As-Synthesized (Citrate Stabilized) Au NPs**

<table>
<thead>
<tr>
<th>Group</th>
<th>$K$ (mg/L)</th>
<th>$V_{\text{max}}$ (mg/g)</th>
<th>$V_{\text{max}}$ (mg/m$^2$)</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>GA-Au NPs</td>
<td>0.69</td>
<td>1.20</td>
<td>0.21</td>
<td>0.88</td>
</tr>
<tr>
<td>PA-Au NPs</td>
<td>0.25</td>
<td>0.48</td>
<td>0.09</td>
<td>0.69</td>
</tr>
<tr>
<td>BP-Au NPs</td>
<td>3.40</td>
<td>7.75</td>
<td>1.38</td>
<td>0.95</td>
</tr>
<tr>
<td>Au NPs</td>
<td>2.14</td>
<td>0.39</td>
<td>0.07</td>
<td>0.75</td>
</tr>
</tbody>
</table>

Constants were determined by nonlinear least squares regression of Langmuir isotherms.
adsorption models, including linear, Langmuir, Freundlich, or Brunauer, Emmett and Teller (BET) isotherms. Non-equilibrium binding of functionalized Au NPs in DI water was not able to be fit using any of the common adsorption models, including linear, Langmuir, Freundlich, or Brunauer, Emmett and Teller (BET) isotherms. Non-equilibrium binding of functionalized Au NPs to HA crystals in FBS may be attributed to a number of possible competitive binding effects, including (1) competition between soluble calcium ions and HA surfaces for binding functionalized Au NPs, (2) competition of soluble proteins and functionalized Au NPs for HA surfaces, (3) interactions between bound Au NPs through shared interactions with either soluble calcium or soluble proteins, (4) place-exchange reactions between functional groups and thiol or amine containing proteins in solution, or (5) some combination of these mechanisms. Note that serum proteins have been shown to rapidly coat Au NP surfaces, resulting in a change in the hydrodynamic diameter and zeta potential, which could contribute to blocking functional group binding sites.

**Implications for targeted delivery**

A damage-specific X-ray contrast agent could enable non-invasive and 3-D imaging of fatigue microdamage in bone. The results of this study suggest that, of the functional groups investigated, bisphosphonate functionalized Au NPs would provide the highest surface density (cf., Table I) of Au NPs labeling damaged bone tissue and, therefore, the greatest contrast enhancement in X-ray tomography. This will be verified in future work. Fatigue microcracks in human cortical bone specimens labeled by bisphosphonate functionalized Au NPs were able to be detected by synchrotron X-ray tomography in a preliminary study. On the other hand, release and clearance of the contrast agent in clinical use might benefit from functional groups with intermediate binding affinity, similar to l-glutamic acid in this study. For example, γ-carboxylglutamic acid is a post-translationally modified amino acid which has been identified in proteins with hydroxyapatite affinity. Further work will be required to determine the appropriate balance between the contrast enhancement and clearance of the agent. The significance of the present study was in demonstrating the ability to tailor and quantify the binding affinity for these objectives.

An additional concern for the use of bisphosphonate functionalized Au NPs as an imaging contrast agent is the pharmacological activity of bisphosphonates, which are commonly used to treat osteoporosis by inhibiting the cellular remodeling process to prevent additional loss of bone mass. The mechanism by which bisphosphonates are able to inhibit osteoclast activity is directly related to the presence of nitrogen. Nitrogen-containing bisphosphonates, such as alendronate, have been shown to selectively inhibit a specific enzymatic pathway necessary for osteoclast function, while bisphosphonates that do not contain nitrogen are incorporated into toxic ATP analogs that cause apoptosis. However, the nitrogen in alendronate is in the form of a primary amine, which was bound to the surface of Au NPs. Therefore, the pharmacological activity may have been passivated. The effects of amine binding to gold surfaces on cellular activity is unknown and will depend on potential intracellular place-exchange reactions between thiol and amine-containing molecules.
Amongst the expansive body of research for active targeting of Au NPs for biological applications, there has been prior investigation on the binding affinity of functionalized Au NPs for mineralized tissue. While the main motivation for this study was in the use of Au NPs as a targeted contrast agent for labeling damaged bone tissue, other applications are readily envisioned for the detection of calcifications in soft tissue associated with cancer or musculoskeletal injury, and as drug delivery vehicles.

CONCLUSIONS

Au NPs were synthesized to a mean particle diameter of 10–15 nm and surface functionalized with either α-glutamic acid, 2-aminoethylphosphonic acid, or alendronate, which exhibit a primary amine for gold surface adsorption opposite carboxylate, phosphonate, or bisphosphonate groups, respectively, for targeting calcium. Bisphosphonate functionalized Au NPs exhibited the most rapid binding kinetics and greatest binding affinity to HA, followed by glutamic acid and phosphonic acid. Functionalized Au NPs exhibited lower overall binding in FBS compared to de-ionized water, but relative differences between functional groups were similar. The high binding affinity of bisphosphonate functionalized Au NPs to HA could lead to improved labeling of damage bone tissue and enhanced-contrast in X-ray tomography.

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