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Effect of gold nanoparticle size and coating on labeling monocytes for CT tracking

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Abstract

With advances in cell therapies, interest in cell tracking techniques to monitor the migration, localization and viability of these cells continues to grow. X-ray computed tomography (CT) is a cornerstone of medical imaging but has been limited in cell tracking applications due to its low sensitivity towards contrast media. In this study, we investigate the role of size and surface functionality of gold nanoparticles for monocyte uptake to optimize the labeling of these cells for tracking in CT. We synthesized gold nanoparticles (AuNP) that range from 15 to 150 nm in diameter and examined several capping ligands, generating 44 distinct AuNP formulations. *In vitro* cytotoxicity and uptake experiments were performed with the RAW 264.7 monocyte cell line. The majority of formulations at each size were found to be biocompatible, with only certain 150 nm PEG functionalized particles reducing viability at high concentrations. High uptake of AuNP was found using small capping ligands with distal carboxylic acids (11-MUA and 16-MHA). Similar uptake values were found with intermediate sizes (50 and 75 nm) of AuNP when coated with 2000 MW poly(ethylene-glycol) carboxylic acid ligands (PCOOH). Low uptake values were observed with 15, 25, 100, and 150 nm PCOOH AuNP, revealing interplay between size and surface functionality. TEM and CT performed on cells revealed similar patterns of high gold uptake for 50 nm PCOOH and 75 nm PCOOH AuNP. These results demonstrate that highly negatively charged carboxylic acid coatings for AuNP provide the greatest internalization of AuNP in monocytes, with a complex dependency on size.

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Supporting Information

The supporting information contains synthesis details for each nanoparticle size including ligand concentrations and centrifuge parameters. Additional characterization data of the gold nanoparticle formulations are supplied. Additionally, the relationship between attenuation and uptake of gold in monocytes shown.

Keywords

gold nanoparticles; monocytes; cell tracking; computed tomography; size; uptake

Introduction

The ability to track specific cells non-invasively in biological systems is of growing importance.¹ With surging interest in adoptive cell therapies for cancer treatment and the promise of stem cell therapy, techniques to monitor the behavior of transferred cells are becoming increasingly valuable tools.^{2–4} Labeling and tracking of transferred cells can be uniquely informative, revealing insights about cell migration, differentiation and viability without invasive procedures.^{3, 5} A number of studies have explored the potential benefits of cell tracking for cellular therapies including applications for stem cell implantations, T-cell immunotherapy, and myeloid cells for treatment of myocardial infarction.^{6–10}

Only a few groups have attempted to use the advantages of x-ray computed tomography (CT) for the purpose of cell tracking.^{11–13} CT is a whole body imaging technique that has high spatial and temporal resolution, which has led it to become one of the foremost clinical imaging methodologies, with more than 70 million scans performed annually in the USA alone.^{14–15} CT is therefore the first-in-line imaging modality for a number of conditions, such as cardiovascular disease, lung cancer and trauma.^{15–16} The development of cell tracking for CT is therefore of considerable interest. For CT, attenuation from contrast agents is linearly proportional to the concentration of the agent allowing for quantification opportunities in cell tracking. For instance, Betzer *et al.*, were able to estimate the number of gold labeled cells *in vivo* in a region of the brain non-invasively using CT scans.¹⁷ Quantifying cell numbers can reveal vital information for cell tracking studies including cell migration and viability. Advances in emerging CT technology continue to open new possibilities for cell tracking; e.g., with the use of x-ray synchrotron beams, some groups were able to achieve single cell resolution for cell tracking.^{18–19} Additionally, with the emergence of spectral CT for specific differentiation of contrast agents from tissue, opportunities for cell tracking with CT continue to grow.^{14, 20}

Cell tracking requires the labeling of specific cells of interest with a reporter for imaging. The challenge for CT is its low sensitivity towards contrast agents, which demands efficient methods for labeling cells with high payloads. Gold nanoparticles (AuNP) are an attractive platform upon which to base CT contrast agents due to being biologically inert, providing strong attenuation for CT and their high density, which allows efficient payload delivery.^{21–22} Moreover, developments in AuNP chemistry have allowed for control over their size, shape and chemical functionality allowing the nanoparticle properties to be tailored for specific biomedical applications.^{23–24} Previous investigations have shown that these nanoparticle properties can affect cytotoxicity, cell uptake and serum protein interactions in biological systems.^{25–27} However, the interaction of the nanoparticles appears to be dependent on the surrounding biological environment and system of interest, so the optimal nanoparticle properties for desired interactions (*i.e.* cell avoidance or uptake) must be determined for each specific case.²⁸

In our previous work, we demonstrated the feasibility of tracking monocyte recruitment to atherosclerotic plaques using computed tomography.¹³ Monocytes play a critical role in the progression of plaques and other diseases, with monocyte recruitment being prominent in early stages of disease development.^{29–32} Imaging recruitment of these monocytes can reveal information about the disease state and allow further investigation of factors that may affect this behavior. In this study, we sought to optimize the uptake of AuNP by monocytes via examining in depth, the effects of size and chemical functionality on AuNP uptake. We synthesized and characterized a library of particles ranging from 15 nm to 150 nm in diameter, each coated with several functionally distinct ligands, totaling 44 unique formulations. We had previous success in labeling monocytes when using 15 nm AuNP coated with short chain hydrocarbon carboxylic acid ligands.¹³ Therefore, we sought to explore similarly coated large AuNP as well as poly(ethylene) glycol ligands with carboxyl acid end groups. Additionally, we explored poly(ethylene) glycol ligands with alternate end groups to explore how differences in surface properties affect interactions with monocytes. The interactions of these nanoparticles with monocytes were assessed via viability and uptake studies. Moreover, parameters of incubation time were explored to optimize cell uptake. CT contrast generation of AuNP labeled monocytes was examined with CT scans and intracellular localization studied with TEM.

Results

AuNP synthesis

Spherical AuNP of approximately 15, 25, 50, 75, 100, and 150 nm in diameter were synthesized to examine the effect of size on cellular uptake. AuNP of 15 and 25 nm were synthesized using the Turkevich method.^{33–34} Diameters of 50 nm and above were synthesized using a modified seeded growth method described by Perrault *et al.*³⁵ This method utilized 15 nm AuNP synthesized via the Turkevich method as nucleation points to further “grow” gold around these “seeds.” We used the seeded growth method for these larger diameter nanoparticles, since we found that the Turkevich method resulted in highly heterogeneous sizes and shapes when forming nanoparticles 50 nm or above. The number of seeds added to the synthesis dictated the final size of the AuNP. We empirically determined the number of seeds needed to form nanoparticles of a range of sizes with this synthesis (Supporting Fig. 1). TEM images in Figure 1 were analyzed to determine core sizes of the AuNP (Table 1). The distributions for each nanoparticle size can be seen in Supporting Figure 2. A Shapiro-Wilk test was used to test for normality. Each nanoparticle formulation was found to be normally distributed except the 15 nm particles ($p < 0.05$).

In addition to forming nanoparticles of a range of sizes, several different coatings were used for each nanoparticle size. With the use of thiol ligands, the surface coating of AuNP was exchanged to produce particles with various chemical functionalities. We examined seven different coatings for each size of AuNP (including citrate) and each formulation was characterized for UV/Vis absorption, hydrodynamic diameter (dynamic light scattering) and overall surface charge (zeta potential) (Supporting Table 1). The chemical structure for each ligand is shown in Figure 2. Straight-chain hydrocarbon ligands with distal carboxylic acid functional groups (11-MUA and 16-MHA) were used as they demonstrate high uptake in

monocytes without affecting viability as shown in our previous work with 15 nm AuNP.¹³ In addition, a variety of thiol-poly(ethylene-glycol) (PEG) based coatings were examined. PEG with a distal methoxy group (MPEG) is widely used to coat nanoparticles, typically providing high stability and resulting in low cell uptake.^{36–37} We used this coating as a control. For this study, we sought to improve AuNP uptake by monocytes, therefore we investigated PEG coatings with charged distal end groups such as carboxylic acid (PCOOH) and PEG-amine. The bulky PEG coating should help to provide AuNP stability, while we hypothesized that the charged surfaces may increase cell uptake.³⁸ We found that 100% PEG-amine coated AuNP aggregated after 24 hours (data not shown). However, we found that AuNP coated with PEG-amine mixed in a 1:4 ratio with MPEG (2N8M) or with PCOOH (2N8C) were stable, and therefore were used in this study. Characterization data for each AuNP core size coated with thiol-poly(ethylene glycol) carboxylic acid (PCOOH) are shown in Table 1 and data for each AuNP formulation used in this study can be found in Supporting Table 1. As the core diameter of the AuNP increases, the UV/Vis absorbance maxima increase in wavelength, in agreement with previously published results.³⁹ The surface potential of PCOOH coated particles for each size is highly negative as expected due to the carboxylic acid functional end group. Measurements of hydrodynamic diameters were larger than core diameters measured by TEM as expected due to the ligands attached to the nanoparticle surfaces. Large PDI values were seen for 25 nm formulations indicating some heterogeneity for these particles (Supporting Table 1). With the 11-MUA ligand, 50, 75, 100, and 150 nm particles were not stable and aggregated during ligand exchange (Supporting Figure 3) and therefore were not used in the study.

Cytotoxicity

As an initial screen for suitability for cell labeling purposes, the effect on cell viability of each AuNP formulation was examined. The cells were treated with AuNP for 24 hours at concentrations of 0.1, 0.5, and 1.0 mg Au/ml to examine the effect of each size and surface functionality over a large concentration range (Figure 3). The majority of formulations studied in the 15–100 nm diameter range were found to be biocompatible for the concentrations tested. However, significant reductions in cell viability were observed for several 150 nm AuNP that had PEG-based coatings. For 150 nm PCOOH AuNP, significant ($p < 0.05$) cytotoxicity was only observed at the highest concentration tested (1.0 mg Au/ml). In the case of 150 nm 2N8M AuNP, 0.5 and 1.0 mg Au/ml concentrations resulted in cell viability of approximately 60% but only 0.5 Au/ml was found to be statistically significant. Lastly, 150 nm 2N8C AuNP caused significant reductions in cell viability at all concentrations examined.

Formulations that did not cause adverse effects on cell viability were studied for their uptake by monocytes, as detailed in the next section. However, we found that some formulations aggregated under the conditions used for cell culture (i.e. 50 nm 2N8C, 75 nm 16-MHA, 75 nm 2N8C, 100 nm 16-MHA, 100 nm 2N8M, 100 nm 2N8C, 150 nm 16-MHA, 150 nm 2N8M and 150 nm 2N8C) likely due to a combination of particle instability and sedimentation. Since co-injection of free nanoparticles with labeled cells would result in erroneous conclusions in cell tracking experiments, these formulations were excluded from cell uptake experiments. Notably, this effect was seen in all citrate formulations regardless of

size. This effect was also more common for larger size AuNP formulations such as 100 nm and 150 nm, for which only MPEG and PCOOH were found to be stable. This is likely due to the higher van der Waals interactions for these larger AuNP overcoming the steric/charge-based repulsion of the ligands. Additionally, formulations that resulted in toxicity at 0.5 mg/ml were excluded from uptake studies.

AuNP uptake by monocytes

We first investigated the optimal incubation time to yield high AuNP uptake. Figure 4 shows the results of experiments where monocytes were incubated with 15 nm 11-MUA AuNP (0.5 mg Au/ml) for several different durations, ranging from 2 to 48 hours. From 2 to 24 hours, AuNP uptake in the cells increased with increasing time in addition to the number of cells rising. However, there was no significant difference ($p>0.05$) in gold uptake between 24 and 48-hour incubations suggesting that the maximum limit of AuNP uptake had been reached at 24 hours. Additionally, when the cells were incubated with these AuNP for 48 hours, fewer cells were present after culture than at 24 hours ($p<0.05$). From these data, we concluded that 24 hours was the best duration over which to incubate monocytes with AuNP for both maximum uptake and number of cells. We therefore used 24 hours incubation time in all subsequent experiments.

For the investigation of cell uptake, all sizes and formulations were incubated with monocytes at a concentration of 0.5 mg/ml for 24 hours. In Figure 5, a number of distinct relationships between size of nanoparticle and coating ligand on uptake can be observed. Particles coated with small molecule functionalized carboxylic acids (11-MUA and 16-MHA) show high uptake with smaller size particles between 15 nm and 50 nm. However, these ligands were increasingly unstable at larger sizes, preventing experimental measurements being acquired. MPEG AuNP exhibited low gold uptake in cells with slightly increasing values at larger sizes. We suspect this result was due to these larger AuNP settling in the cell culture media, resulting in increased cell contact and uptake. Most notably, PCOOH AuNP exhibited a complex relationship between uptake and particle size. Minimal uptake was found at 15 and 25 nm while the highest uptake values were observed at 50 and 75 nm. Interestingly, cell uptake for 100 and 150 nm PCOOH AuNP was significantly less than for 50 and 75 nm AuNP. The uptake for 50, 75, and 100 nm PCOOH AuNP was found to be significantly different than all other coatings at that size ($p<0.05$). Similar uptake values for 150 nm PCOOH and MPEG AuNP suggests that for both these nanoparticles uptake may be largely due to particle settling (non-significant, $p>0.05$). Low uptake was observed for 15 and 25 nm 2N8C and were found to aggregate at 50 nm and above.

Monocytes incubated with selected AuNP formulations were additionally examined with TEM to observe the intra-cellular localization of AuNP. Cells were treated for 24 hours with 0.5 mg/ml of 15, 75, 100, and 150 nm PCOOH AuNP. The results in Figure 6 show AuNP aggregated into intercellular vesicles located in the cytoplasm of the monocytes. With monocytes, the uptake of AuNP is likely through phagocytosis with particles concentrated into lysosomes. Further investigation would be required to confirm the route of uptake, but other researchers have shown that the major route of uptake of nanoparticles in monocytes and macrophages is phagocytosis.^{40–41} More AuNP were seen more frequently in 75 nm

PCOOH AuNP treated monocytes as compared to other sizes, in agreement with the results observed from ICP-OES measurements (Figure 5).

CT imaging

To demonstrate the contrast generating properties of the AuNP taken up in monocytes, cells were scanned with a microCT scanner at 80 kVp. Monocytes were treated with 50, 75, 100 and 150 nm PCOOH AuNP for 24 hours at 0, 0.1, 0.25, and 0.5 mg/ml. Afterwards, cells were collected in 4% PFA and allowed to settle into loose pellets before scanning (Figure 7). Figure 7A shows CT scans of cells treated at increasing concentrations for 75 and 150 nm AuNP. The contrast generated from the AuNP increases as treatment concentration increases for these formulations. Quantitative analysis of the attenuation of these cell pellets is shown in Figure 7B, and revealed that the highest contrast enhancement arose for 50 and 75 nm AuNP. This size dependent attenuation for the PCOOH AuNP mirrors the AuNP uptake results in Fig. 5, signifying that the observed attenuation is due to the internalized AuNP. The change in attenuation was measured by normalizing to values obtained from untreated monocytes. A one-way ANOVA and post-hoc tukey pairwise mean comparison was used to determine significance between sizes at each concentration group. At each treatment concentration, the quantified attenuation was found to be significant between all sizes for PCOOH ($p < 0.05$). To explore the relationship between gold uptake and CT attenuation, the attenuation of cell pellets (HU) was plotted against gold uptake (pg/cell). Supporting Figure 4 shows that there is a linear relationship ($R^2 = 0.89$) between attenuation and gold uptake.

Discussion

We studied a total of 44 AuNP formulations with a range of diameters and diverse capping ligands as potential labels for monocyte cell tracking. While the majority of the formulations studied did not affect cell viability, we observed that 150 nm AuNP with PEG-amine as part of the coating (2N8M, 2N8C) resulted in significant reductions in cell viability (Figure 3). This toxicity is likely due to the presence of the positively charged amine groups in combination with increased AuNP-cell interactions due to these large nanoparticles settling in the cell culture media. Researchers have hypothesized that cytotoxicity from cationic surface coatings could be due to increased diffusion of nanoparticles through the cell membrane, disrupting the lipid bilayer.⁴² Formation of protein coronas adsorbed onto the AuNP from the serum in the cell culture media during the incubation is also likely to play a role in their monocyte interactions.⁴³ The protein corona formed would be unique to each AuNP formulation and some speculate these adsorbed proteins are highly influential in the interactions between cells and nanoparticles.⁴⁴

The small animal microCT scanner used in the study was limited to 80 kVp as the maximum x-ray tube voltage. At 80 kVp the photons produced are below the k-edge (80.7 keV) of Au. However, at 80 kVp, the attenuation difference between Au and tissue remains substantial, allowing for strong contrast in our study. With the higher voltages typically available on clinical scanners (100–140 kVp), we would likely see greater contrast between Au and controls than seen in this study.

In the uptake experiments, high uptake of AuNP was found with short carbon chain carboxylic acid ligands, consistent with our prior results and those of others.^{13, 45–46} It may be the case that upon incubation in serum, formation of protein coronas or aggregation of these AuNP with proteins cause changes in size. The resulting nanoparticles may be highly taken up by monocytes due to their size or due to settling. Future experiments will probe the structure of these nanoparticles in serum to answer these questions. For AuNP coated with these short carboxylic acid ligands, uptake was greater for 15 nm particles than 25 nm particles, however a size trend could not be discerned due to a lack of stability of larger diameter AuNP with these coatings. 11-MUA particles were unstable during ligand exchange for particles larger than 25 nm, while 16-MHA coated were stable during synthesis but produced significant aggregation artifacts in uptake studies with particles larger than 50 nm (Figure 5). These results indicate that larger AuNP may require larger ligands for robust stability. For increased stability at larger AuNP diameters, we explored a variety of PEG-2000 ligands. PEG-2000 is well known to provide nanoparticle stability in serum as well as ‘stealth’ properties.⁴⁷ As expected, MPEG coated AuNP were taken up at low levels for all particle sizes, although slightly increased uptake was seen at the largest sizes, i.e. 100 and 150 nm (Figure 5). This uptake is likely a function of the increased settling rates for larger nanoparticles rather than due to the particle’s surface properties. Due to the lack of uptake with methoxy-PEG coating, we explored PEG coating ligands that possessed carboxyl and amine functional groups at their distal ends.

PEG functionalized carboxylic acids coated particles (PCOOH) were stable for each diameter of AuNP studied. The monocyte uptake of AuNP coated with this ligand demonstrated a complex relationship with size (Figure 5). The highest gold uptake values were seen with 50 and 75 nm PCOOH AuNP. Other researchers have suggested 50 nm as optimal uptake size for AuNP in other cell lines.⁴⁸ For instance, Chithrani *et al.*, observed the highest accumulation for 50 nm citrate coated AuNP in HeLa cells after 24 hours.⁴⁹ While the data for PCOOH AuNP from our experiments are in agreement with these prior studies, maximal uptake for 50 nm AuNP was not common to all coatings, demonstrating that uptake is dependent on both size and surface functionality. The PCOOH ligand and citrate both expose carboxylic acid groups to the surrounding milieu and may therefore form similar protein coronas around them, which may explain the similarity between our results and those of Chithrani *et al.*

Several studies have reported favorable nanoparticle uptake with amine based coatings.^{50–51} Therefore for this study, we incorporated PEG-amine ligands into MPEG (2N8M) and PCOOH (2N8C) at a 1:4 ratio (AuNP coated with 100% PEG-amine were not stable). Low uptake values with both coatings at 15 and 25 nm suggest similar properties to MPEG and PCOOH coated AuNP. However, for larger core sizes only 50 and 75 nm 2N8M AuNP were found to be without stability issues, limiting the extent to which these coatings could be explored. The gold uptake values of 50 and 75 nm 2N8M AuNP are similar to those of MPEG of the same sizes, suggesting the observed behavior is dominated by the presence of MPEG and that the availability of the amine groups was limited. A higher ratio of PEG-amine to MPEG may be necessary to fully explore the effect of size with PEG-amine coated AuNP.

In CT imaging of cell pellets, the uptake of PCOOH coated AuNP resulted in large attenuation values, <2000 HU for the formulations with the highest uptake. These values are dependent on the amount of gold present in the volume of the cell pellet. For 75 nm PCOOH, we calculated the total mass of gold in the cell pellet from the cell uptake value (174.68 pg/cell) and the number of cells in the pellet (2.5 million) to be 0.44 mg. Since the volume of the pellet was 10 μ L, we calculated the concentration of gold in the pellet to be 221.7 mM. The attenuation change relative to the concentration of gold was therefore approximately 9.65 HU/mM of Au. This attenuation per concentration of gold value is close to those reported previously in the literature when using similar CT scanners.⁵²

For the purpose of labeling monocytes, similar values of gold uptake were seen with 15 nm 11-MUA, 50 nm PCOOH, and 75 nm PCOOH AuNP. Both types of coatings have functional carboxylic acid end groups with strong negative surface charge. Previous research has shown that anionic surface charges may lead to favorable protein corona formation for uptake by preferential binding of Apolipoprotein H (ApoH) compared to positively charged particles.⁵³ The increased presence of ApoH on nanoparticles showed increased uptake in human mesenchymal stem cells compared to other highly abundant proteins composing the corona. Considering synthetic efficiency, 15 nm AuNP are easier to produce on a large scale (performing seeded growth to form 50–75 nm AuNP adds another synthetic step which we have found challenging to scale up). The scalability of a synthesis is important feature for future translation, an issue that few studies have sought to address.⁵⁴ Additionally, the low cost of the 11-MUA ligand (\$13.14/g, Sigma-Aldrich, St. Louis, MO) compared to the ligand PCOOH (\$1400/g, Creative PEGworks, Chapel Hill, NC) reinforces the justification for use of 15 nm 11-MUA coated AuNP for future monocyte labeling studies.

Conclusion

In this study, we explored the effect of size and surface coating on AuNP biocompatibility and uptake in monocytes. We synthesized AuNP ranging in diameter from 15 to 150 nm and examined both short carbon chain ligands and PEG-2000 functionalized ligands. The majority of these formulations had no adverse effect on cell viability except 150 nm AuNP coated with charged PEG ligands. We found that small AuNP coated with short carboxylic acid ligands were taken up extensively. We also observed a complex effect of diameter for PCOOH coated AuNP, with high uptake values at the intermediate sizes of 50 and 75 nm but lower uptake for smaller and larger diameters. Additionally, TEM and CT scans of monocytes incubated with these formulations showed similar patterns of high uptake for 50 and 75 nm PCOOH AuNP. Gold uptake values at 50 and 75 nm for PCOOH AuNP were similar to values seen for 15 nm 11-MUA particles. Similar studies with other cells of interest for cell tracking studies, such as T-cells and mesenchymal stem cells would be of value. These data continue to expand the landscape of cell tracking with CT.

Methods

Materials

Chemicals of analytical grade were purchased from Sigma-Aldrich (St. Louis, MI) unless specified elsewhere. The monocyte cell line used for cell studies, RAW 264.7, was

purchased from ATCC (Manassas, VA). Cells were cultured with Dubecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (10000 units/MI, 10000 µg/mL), purchased from Cellgro, Corning (Manassas, VA).

AuNP synthesis

Spherical AuNP of 15 nm and 25 nm in diameter were synthesized using a modified Frens/Turkevich method.^{33–34} For 15 nm AuNP, 600 µl of 1% gold(III) chloride (HAuCl₃) was dissolved in 60 mL of ultrapure water (Milli-Q, EMD Millipore, Billerica MA). The solution was brought to a boil before 1.8 mL of 1% sodium citrate dihydrate was added to the mixture. The solution was left to reflux for 15 min before cooling to room temperature. Similarly, 25 nm AuNP were synthesized by adding 1 mL of 1% sodium citrate dihydrate to the boiling gold solution.

AuNP ranging from 50 nm to 150 nm in diameter were synthesized using the seeded growth method described by Perrault *et al.*³⁵ Briefly, 15 nm spherical AuNP were used as seeds in this method and were produced as outlined above. For the synthesis, 1 mL of 1% HAuCl₃ was added to 100 mL of ultrapure water at room temperature. A number of gold “seeds” were added to the solution to create nanoparticles 50, 75, 100 or 150 nm in diameter. The number of seeds needed for each size was empirically determined (Supporting Figure 1). A typical solution of seeds contained 1.54×10^{12} seeds/mL. For example, 50 nm AuNP were synthesized by adding 3 mL of seed solution (as made in the above method) to HAuCl₃ solution. To initiate the AuNP growth reaction, 220 µL sodium citrate dihydrate (1% weight/volume) and 1 mL of 0.03 M hydroquinone solution were added quickly in succession to the seed/HAuCl₃ solution. The solution was allowed to stir for an hour before performing ligand exchange.

After AuNP core synthesis, ligand exchange was performed with a library of ligands to stabilize the AuNP for biomedical applications and provide differing chemical functionalities to explore their effect on cell uptake. AuNP that did not undergo ligand exchange remained coated with citrate and were also used in the study. Short carbon chain carboxylic acid ligands 11-mercaptopundecanoic acid (11-MUA) and 16-mercaptohexadecanoic acid (16-MHA) were examined. Additionally, 2000 MW poly(ethylene-glycol) with methoxy (MPEG), carboxylic acid (PCOOH), and amine functional groups were examined. AuNP synthesized with PEG-amine were found to aggregate during ligand exchange, however, amine containing coatings were achieved by using 1:4 ratio of PEG-amine to MPEG (2N8M) or PCOOH (2N8C). Each nanoparticle size was capped with a ligand amount that we estimated based on the total nanoparticle surface area for that size. A ratio of 1.35×10^6 mmol/cm² was used to determine the amount of ligand to use (Supporting Table 2). Each ligand was dissolved at 5 mg/mL concentration before addition to the gold solutions. 11-MUA and 16-MHA ligands were dissolved in ethanol and all PEG functionalized ligands were dissolved in water. For example, for 100 nm PCOOH, 85 µL (0.421 mg) of 5 mg/mL PCOOH solution was added to a typical 100 nm seeded growth solution (100 mL) for exchange. Gold solutions were allowed to stir overnight for complete ligand exchange. Refer to Supporting Table 2 for ligand volumes

added to each batch. Afterwards, the nanoparticles were washed twice by centrifugation and then purified through a 0.45 μm syringe filter (EMD Millipore, Billerica MA). The parameters for centrifugation were specific to each size and can be found in Supporting Table 3.

Particle characterization

The AuNP concentration after purification was determined through inductively coupled plasma optical emission spectroscopy (ICP-OES). Samples (typically around 10 μL) were dissolved in 1 mL aqua regia (3 parts HCl:1 part HNO_3) for 30 mins before making the final volume of the sample to 3 mL with ultrapure water. The resulting solutions were analyzed with a Spectro-Genesis ICP (Spectro Analytical Instruments GmbH, Kleve, Germany) for gold content. Transmission electron microscopy (TEM) was used to visualize the AuNP and evaluate core size and was performed on a JEOL 1010 (JEOL USA Inc., Peabody MA) at 80 kV. Over 200 AuNP in the TEM images were measured with ImageJ (National Institutes of Health) to determine the average diameter for each core size. UV/Vis spectra were recorded using an Evolution 201 UV-Visible Spectrophotometer (Thermo Scientific, Waltham, MA) scanning from wavelengths of 700 to 300 nm. Typically, 10 μL of sample was added to a cuvette and diluted to 1 mL with ultrapure water. The hydrodynamic diameters (dynamic light scattering) and zeta potentials of the AuNP were evaluated using a Nano ZS-90 Zetasizer (Malvern Instruments, Worcestershire, UK). AuNP samples were diluted to 1 mL with ultrapure water for analysis with these techniques.

In vitro studies

The effects of AuNP size and coating were studied using a monocyte cell line, RAW 264.7. The LIVE/DEAD assay (Life Technologies, Frederick, MD) was used to study cytotoxicity. Monocytes were cultured in 20 mm glass bottom dishes at 7.5×10^4 cells per dish for 24 hours at 37 C, 5% CO_2 with DMEM (10% FBS, 1% streptomycin/penicillin). After this time, the media was removed and fresh media containing AuNP at 0.1, 0.5, 1.0 mg/ml or without AuNP was added. The cells were incubated with the AuNP for 24 hours before performing analyses, unless specified otherwise. After incubation, cells were washed twice with DPBS before 400 μL of LIVE/DEAD cocktail (2 mL DPBS, 2 μL stock Ethidium-1 homodimer, 0.5 μL stock Calcein AM, 0.5 μL 3.2 mM Hoechst 33342) was gently added to the cells. The cells were incubated for 20 mins with the LIVE/DEAD cocktail. Afterwards, the cells were imaged with Nikon Eclipse Ti-U fluorescence microscope. Filters for DAPI (Ex. 359, Em. 461 nm), FITC (Ex. 495, Em. 519 nm), and Texas Red (Ex. 595, Em. 613 nm) were used to visualize cell nuclei, living cells and dead cells respectively. Four separate fields were taken for each dish for each filter channel. The images were analyzed by a custom MATLAB (MathWorks, Natick MA) program to count the number of stained cells in each field. The viability percent is expressed as number of living cells divided by total number of cells present in each field of view. Viability experiments were repeated 3 times (n=3).

Cell Uptake Studies

For uptake studies, monocytes were cultured in 12 well plates, 8.0×10^5 cells per well for 24 hours at 37 C, 5% CO_2 with DMEM (10% FBS, 1% streptomycin/penicillin). At this point,

the media was removed and replaced with fresh media containing AuNP. Various concentrations, formulations and timeframes were used, see results for details. After incubation with AuNP, the media was removed and the cells were washed twice with DPBS and then scraped for collection in 1 mL of DPBS. The cells were counted with a hemocytometer and centrifuged at 200 g for 5 mins to form a pellet. The supernatant was discarded and the cells were re-suspended in 200 μ L of 4% paraformaldehyde (PFA) until preparation for ICP-OES. Cells were dissolved in 1 mL of aqua regia (3 parts HCl:1 parts HNO₃) for 30 mins and then centrifuged at 200 g for 5 mins to pellet and discard cell debris. The supernatant was collected and diluted to 3 mL with ultrapure water and analyzed with ICP-OES.

Cells treated with AuNP for 24 hours were also collected and fixed in 2% PFA/2.5% glutaraldehyde, cut into 60 nm sections and prepared using standard techniques for examination with TEM.⁵⁵ Sections were viewed with a JEOL 1010 Electron Microscope at 80 kV. For CT scans, after treating cells with AuNP, monocytes were collected and fixed with 200 μ L 4% PFA. The cells were allowed to loosely settle into pellets in 250 μ L tubes before scanning with a small animal MicroCAT II scanner (Imtek Inc., Knoxville, TN). The parameters used for the scan were 100 μ m slice thickness (100 x 100 μ m in-plane spatial resolution), 51.2 mm x 76.8 mm field of view, 80 kVp tube voltage, and tube current of 500 μ A. The microCT contains a 1.0 mm aluminum beam filter. Image reconstruction was done using a Feldkamp cone beam correction and a Shepp-Logan filter, which produced images scaled in Hounsfield units (HU). Images were analyzed with OsiriX v3.7.1 64-bit software. Identical regions of interest (1.5 mm²) were drawn on 3 sequential slices in the cell pellets and the recorded attenuation values averaged. The HU was defined as the difference in attenuation between non treated cell pellets and samples treated with AuNP.

Statistical Methods

All error bars shown on figures are standard errors of means. In Table 1, the $\pm$ values for core diameter and zeta potential represent standard deviations of measurements. Statistical analysis was performed using Stata-14 (64-bit, College Station, Texas). For viability of 150 nm PCOOH, 2N8M, and 2N8C, a one-way ANOVA was used to determine significance among concentrations. Post-hoc, Tukey pairwise mean comparison was used to compare the interactions between each concentration. For our time course experiment, a one-way ANOVA was used to analyze significance for cell numbers and AuNP uptake. Tukey pairwise mean comparison was used to compare interactions between each time point for both cell number and uptake. For uptake experiments, a two-way ANOVA was used to analyze significance differences caused by both size and coating on uptake. Afterwards, a post-hoc Tukey pairwise mean comparison was used to analyze differences between coatings at each size. For CT attenuation experiments, a one-way ANOVA was performed to determine significant differences at each concentration examined. A post-hoc Tukey pairwise comparison was used for the coatings at each concentration examined.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. TEM images of spherical gold nanoparticle of increasing size from 15 to 150 nm. 15 and 25 nm AuNP were synthesized through the Turkevich method. 50, 75, 100, and 150 nm AuNP were synthesized through a seeded growth method.

Figure 2. Schematic depiction of the range of AuNP sizes used in this study and the chemical structures of the ligands used as coatings. PEG-amine coatings were mixed in 1:4 ratios with MPEG and PCOOH to provide particle stability. Ligands examined represent different functionalities and charges.

Figure 3.

Cell viability of monocytes incubated with AuNP of a range of diameters and coatings. Cells were incubated with AuNP for 24 hours at 0.1, 0.5, 1.0 mg Au/ml. Viability was assessed with the LIVE/DEAD assay. Values are normalized to those for untreated monocytes. Values represent the mean of 3 replicates (n=3) and error bars represent standard error of mean. Significance for 150 nm AuNP was examined with one-way ANOVA and post hoc Tukey pairwise comparisons. * indicates significance of treated groups compared to non-treated group (p<0.05).

Figure 4.

Evaluation of gold content in monocytes incubated with 15 nm 11-MUA AuNP (0.5 mg Au/ml) for up to 48 hours. In addition, the total number cells at each time point is displayed. Values represent mean of 3 replicates (n=3) and error bars are standard error of mean. A one-way ANOVA was used to determine significance for both uptake and number of cells. Post-hoc Tukey pairwise comparison was used to compare significance between time points ($p < 0.05$). Results for statistical comparisons between 24 and 48 hours shown only.

Figure 5.

Gold uptake in monocytes incubated with AuNP of a variety of sizes and coatings. Formulations exhibiting particle instability (significant aggregation and sedimentation) or cytotoxicity were excluded from the study. The cells were treated for 24 hours at 0.5 mg Au/ml and evaluated for gold content with ICP-OES. Values represent mean of typically six replicates (n=6) and error bars are standard error of mean. A two-way ANOVA was used to determine significance of AuNP uptake for size and coating. A Tukey pairwise mean comparison was used to determine significance between coatings at each size. * indicates significance of PCOOH compared to all other coatings at that size.

Figure 6.

TEM of monocytes treated with 15, 75, 100, and 150 nm PCOOH AuNP. Cells were treated at 0.5 mg/ml for 24 hours before being harvested and fixed for TEM.

Figure 7.

(A) CT scans of monocytes settled into pellets after treatment with 75 and 150 nm PCOOH AuNP for 24 hours at 0, 0.1, 0.25 and 0.5 mg/ml. Cells were collected in 4% PFA solution after treatment. Scans performed on small animal microCT scanner with 100 μ m spatial resolution at 80 kVP and 500 μ A. (B) Quantification of attenuation in Fig. 7A of monocytes incubated with PCOOH AuNP of various sizes. Values normalized to untreated cells. Error bars are standard error of the mean with (n=3 for each data point). A one-way ANOVA and post-hoc Tukey pairwise mean comparison used to compare different sizes at each concentration group. * indicates significance between all sizes at that concentration (p<0.05).

Characterization of AuNP of a range of diameters coated with PCOOH. Absorbance values measured with UV/Vis scan from 700 to 300 nm. Hydrodynamic diameter and polydispersity index (PDI) measured with dynamic light scattering. Core diameter sizes measured with TEM. Surface charge measured with zeta potential assays. “ $\pm$ ” represent standard deviations of the measurements.

Table 1

Size (nm)	Coating	Abs (nm)	HydrodynamicDiameter (nm)	PDI	Core Diameter (nm)	Surface charge (mV)
15	PCOOH	520.2	31.89	0.164	14.0 $\pm$ 1.7	-36.5 $\pm$ 3.0
25	PCOOH	532.7	43.6	0.688	21.2 $\pm$ 3.9	-39.7 $\pm$ 1.1
50	PCOOH	538.4	57.37	0.140	48.5 $\pm$ 6.0	-41.5 $\pm$ 0.3
75	PCOOH	554.1	96.24	0.178	73.6 $\pm$ 7.6	-37.4 $\pm$ 1.4
100	PCOOH	559.8	110.5	0.163	102.4 $\pm$ 10.4	-44.1 $\pm$ 0.1
150	PCOOH	550.8	169.9	0.281	153.9 $\pm$ 12.9	-45.6 $\pm$ 1.6