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Interaction of colloidal gold nanoparticles with human blood: effects on particle size and analysis of plasma protein binding profiles

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Abstract

Nanoparticle size and plasma binding profile contribute to a particle's longevity in the bloodstream, which can have important consequences for therapeutic efficacy. In this study an approximate doubling in nanoparticle hydrodynamic size was observed upon in vitro incubation of 30- and 50-nm colloidal gold in human plasma. Plasma proteins that bind the surface of citrate-stabilized gold colloids have been identified. Effects of protein binding on the nanoparticle hydrodynamic size, elements of coagulation, and the complement system have been investigated. The difference in size measurements obtained from dynamic light scattering, electron microscopy, and scanning probe microscopy are also discussed.

Keywords

Nanoparticles; Gold colloids; Plasma protein binding; Hydrodynamic size; Complement

There has been recent success in the use of nanoparticles as platforms or carriers for otherwise insoluble or poorly soluble drugs, and nanoparticle reformulations of cancer chemotherapeutics often have reduced side effects and improved efficacy due to active and passive targeting of the carried drug to locations in the body where it can be most effective.¹ Colloidal gold nanoparticles in particular are a promising drug delivery platform for targeted cancer therapies, and recent years have seen nanomedicine formulations containing colloidal gold enter clinical trials as anticancer therapeutics.^{2,3} The success of gold colloids in this domain will hinge on their safety for repeated systemic administration and their ability to deliver drugs specifically and efficiently to tumors. Both safety and drug-delivery performance depend (to some extent) on the disposition and clearance of the colloids. The disposition and clearance, in turn, depend on a variety of characteristics of the nanoparticles, including their size and surface charge. As with many nanoparticles, citrate-stabilized gold

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Appendix A. Supplementary data

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colloids injected into the bloodstream are quickly coated by serum proteins; most cells and tissues never encounter the naked particles. Some of the absorbed proteins remain associated with the particle for a significant portion of its therapeutic lifetime, and the properties of the protein coat may ultimately define the biological response to the nanoparticle, including influence on cellular uptake, organ accumulation, and route of clearance.^{4,5}

Chithrani et al have shown that colloidal gold particles adsorb serum proteins and that this binding influences clearance by immune cells,⁶ but neither the size of the protein-bound colloid particles nor the individual molecular identities of the bound proteins have been examined. Other groups have developed methods for determining the exchange rates of adsorption of different plasma proteins to polymeric particles,^{7,8} from which the stoichiometries (number of protein molecules bound) and degree of surface coverage can be estimated. Here as well, the size of the protein-bound particles is not known.

One reason the size of blood protein-bound gold colloids has not been examined before is the available instrumentation. Because metals scatter light efficiently, electron microscopy is the technique of choice for physical characterization of colloidal gold nanoparticles.^{2,9,10} Transmission electron microscopy (TEM) uses powerful electron beams and can provide a great amount of detail at the atomic scale—such as information about the crystal structure and granularity of a sample. However, many biological compounds (e.g., proteins) are invisible to TEM without heavy metal staining procedures, because these compounds do not sufficiently deflect an electron beam. Atomic force microscopy (AFM) is another method that can provide a measure of nominal colloid size for nanoscale particles. However, AFM relies on tapping a particle (either in solution or dried to a surface) and so has limited resolution of flexible compounds (e.g., proteins), which may move under the force applied by the instrument tip. Because dynamic light scattering (DLS) measures hydrodynamic diameter, it provides a fundamentally different measure of particle size from TEM or AFM. DLS is very sensitive to “soft” flexible biological molecules such as polymers, proteins, and antibodies because they cause significant frictional drag,¹¹ which can dramatically influence the rate of the particle’s motion under Brownian diffusion. DLS is therefore appropriate for measurement of the hydrodynamic size of protein-bound nanoparticles. Complementary size characterization by TEM and AFM can be useful in resolving ambiguities due to the different measurement techniques. For example, the DLS-measured size may be influenced by particle agglomeration, and further analysis by TEM is required to ensure that the DLS-measured sizes represent primary particle size (i.e., discrete particle size), and not the size of an agglomerate.¹²

This study examines the size and charge of colloidal particles incubated with plasma and also provides insight into the molecular identities of the plasma proteins that bind the colloids. Here we use a combination of DLS, TEM, and AFM to examine gold colloids with 30-nm and 50-nm nominal diameters before and after incubation in pooled plasma. We use microscopy (TEM and AFM) to verify that the colloids do not agglomerate.

The effects of protein binding on particle surface charge are also studied. The zeta potential (the electrostatic potential generated by the accumulation of ions at the surface of the colloidal particles) is monitored before and after plasma incubation. Two-dimensional polyacrylamide gel electrophoresis (2D PAGE) and mass spectrometry (MS) are used to identify bound proteins. Finally, we examined coagulation and complement activation of the colloids, to determine if these biological responses are influenced by the spectrum of proteins bound to the particles.

Methods

Reagents

Colloidal gold nanoparticles (30- and 50-nm nominal size) were purchased from TedPella (Redding, California). Particle concentration according to the manufacturer was 2×10^{11} particles/mL for 30-nm gold colloids and 4.5×10^{10} particles/mL for 50-nm gold colloids. The particles were concentrated 10-fold for this study. Surface area was calculated according to the formula $A = 4\pi R^2$. Total surface area was calculated by multiplying single-particle surface area by particle number. Cobra venom factor and veronal buffer were from Quidel Corporation (San Diego, California) and Boston Bioproducts (Boston, Massachusetts), respectively. Collagen was from Helena Laboratories (Beaumont, Texas). Normal and abnormal coagulation controls, buffers, and activators for coagulation assays were from Diagnostica Stago (Parsippany, New Jersey). Anti-fibrinogen polyclonal antibodies and donkey anti-goat IgG conjugated to horseradish peroxidase (HRP) were from Abcam (Cambridge, Massachusetts) and Jackson ImmunoResearch Laboratories (West Grove, Pennsylvania), respectively. Enhanced chemi-luminescence (ECL) detection reagents were from Pierce (Rockford, Illinois).

Research donor blood

Healthy volunteer blood specimens were drawn under NCI-Frederick Protocol OH99-C-N046. Blood was collected in BD Vacutainer tubes (BD Diagnostics Franklin Lakes, New Jersey) containing sodium citrate as anticoagulant. For evaluation of platelet aggregation, coagulation time, and complement activation assays, specimens from at least three donors were pooled. Incubation of nanoparticles and sample preparation for 2D PAGE, AFM, DLS, and TEM were done using pooled sodium citrate plasma from BioChemed Pharmacologicals, Winchester, Virginia.

Sample preparation and 2D PAGE

According to the manufacturer, concentration of gold in the stock solution was 0.01%. Colloidal gold particles were concentrated to 1 mg/mL by centrifugation at 18,000g according to the manufacturer's instructions. The 30-nm and 50-nm colloidal samples that were used for this study contained 0.450 and 0.420 mg/mL of gold, respectively, as determined by inductively coupled plasma (ICP)-MS. Three milliliters of concentrated particles were mixed with 3 mL of pooled plasma and incubated for 30 minutes at 37°C. Particles were then pelleted in a microcentrifuge at 18,000g for 30 minutes, excess of plasma was removed, and 1.5 mL of phosphatebuffered saline (PBS) was added to the tube to reconstitute particles; the centrifugation step was then repeated. A total of four wash steps with 1× PBS, followed by two washes with 0.1× PBS were conducted using centrifugation settings and volumes described above. After the final wash, 200 µL of protein rehydration buffer containing 8 M urea, 2% (w/v) CHAPS buffer, 2% (v/v) immobilized pH gradient (IPG) pH 3–10 buffer, 40 mM dithiothreitol, and 0.01% (w/v) bromophenol blue were added to the particles pellet, vortexed, and incubated at room temperature (20°–22°C) for 10 minutes. Particles were removed from rehydration buffer by centrifugation at 18,000g for 15 minutes; protein-containing rehydration solution was collected, transferred into a fresh tube, and stored at –80°C until analysis.

An additional plasma sample was used in each experiment when the particles were omitted so as to assess the nonspecific protein binding by polypropylene tubes. Aliquots of each sample were also dialyzed against PBS to determine the total amount of adhering polypeptides.

The entire volume (200 μ L) of IPG gel rehydration containing protein samples isolated from gold particles was loaded onto 11-cm-long Immobiline DryStrip pH 3–10 L gels (GE Healthcare, Piscataway, New Jersey), allowing proteins to enter the gel during an overnight rehydration and equilibration process. After equilibration the proteins were separated by isoelectric focusing using an Ettan IPGphor II electrophoresis unit (GE Healthcare). The strips were run for a total of 25 kVh. After the isoelectric focusing the strips were equilibrated for 15 minute with sodium dodecyl sulfate (SDS) equilibration buffer containing 50 mM Tris-HCl pH 8.8, 6 M urea, 30% (w/v) glycerol, 2% (w/v) SDS, and a trace of bromophenol blue. The equilibrated gel strips were used in second dimension electrophoresis.

2D PAGE

The 2D PAGE was performed using a Multiphor II (GE Healthcare) flatbed system with precast ExcelGel SDS, 12–14% gradient gel. Sea Blue protein standard (Invitrogen, Carlsbad, California) was used as molecular weight marker. The gels were stained using an MS-compatible silver staining kit SilverQuest (Invitrogen). The gel images were analyzed using PDQuest software (BioRad, Hercules, California) (http://ncl.cancer.gov/NCL_Method_JTA-4.pdf).

Analysis of particle size and shape (TEM, AFM, DLS)

Particle concentration and incubation with plasma were performed as described above. After two washes with 1 $\times$ PBS or saline (0.9% sodium chloride), particles were reconstituted in 1 mL of PBS or saline and either analyzed fresh or stored at 4°C. The data shown here are for particles washed in PBS, because this buffer was used for all other biological experiments with protein-coated particles. The samples were also prepared in water by repeating the final three washings with deionized water followed by centrifugation. Alternately, ultrafiltration in a 10K molecular-weight-cutoff Microcon device (Millipore, Billerica, Massachusetts) is used to exchange PBS buffer with deionized water.

Dynamic Light Scattering (DLS)

Batch mode hydrodynamic size (diameter) measurements were performed on a Malvern Zetasizer Nano ZS (Malvern Instruments, Southborough, Massachusetts) equipped with a back-scattering detector (173 degrees). Samples were prepared as described above and filtered through a pre-rinsed 0.2- μ m filter followed by equilibration (typically 5 minutes) to 25°C before a minimum of three measurements per sample were made.

Zeta potential

A Malvern Zetasizer Nano ZS instrument was used to measure zeta potential at 25°C for all samples. Samples prepared for the DLS measurements were loaded into a prerinsed folded capillary cell for the zeta potential measurements. An applied voltage of 150 and 100 V was used for the 30-nm and 50-nm gold colloids, respectively. An applied voltage of 80 V was used for the plasma-incubated samples. A minimum of three measurements were made per sample.

AFM imaging

The AFM measurements were performed on a PicoPlus SPM II (Molecular Imaging, Tempe, Arizona) atomic force microscope with magnetic alternating current (MAC) mode in liquid. Type II MACLever silicon tip (spring constant 2.8 N/m; Molecular Imaging) was used at a driving frequency of 30 kHz in liquid for all experiments. The samples were prepared by adding 250 μ L gold colloidal solutions either in PBS or water on freshly cleaved mica. Incubation in plasma and conditions are described above. The surface was covered

with a fluid cell and allowed to stand for 1 minute before imaging. For imaging in the dry state, colloidal preparations in deionized water were allowed to air-dry before imaging.

TEM measurement

TEM images were obtained using a Hitachi H7600 instrument (Hitachi, Tokyo, Japan) operated at 80 kV. A 400-mesh formvar carbon-coated copper grid was glow-discharged in a vacuum evaporator (Denton, Moorestown, New Jersey) for 30 seconds to make the grid hydrophilic and thus attractive to particles. The sample was prepared by dropping 2 μ L gold colloid particle solution onto the grids and wicking off the excess sample with filter paper after 30 seconds. Negative sample staining was obtained by adding 2 μ L of 1% pH 7.0 phosphotungstic acid (PTA) to the sample solutions on the TEM grids. Excess PTA was blotted, then allowed to air-dry. Sample grids containing salt were washed with twice-distilled water before PTA stain application. Statistics describing the size distribution of the observed colloids was analyzed using Lispixl (NIST freeware, www.nist.gov/Lispixl/index.htm). Incubation in plasma is described above.

MS analysis

Excised gel spots were processed according to published procedures.¹ Proteins were in-gel digested with trypsin and the peptides extracted. Each sample was purified using C-18 Zip Tips (Millipore, Billerica, Massachusetts) before analysis by nano-electrospray ionization (ESI) reversed-phase liquid chromatography using an Agilent 1100 nano-flow LC system (Agilent Technologies, Santa Clara, California) coupled online to an ion trap (IT) mass spectrometer (LCQ Deca XP; Thermo Finnigan, Waltham, Massachusetts)². Reversed-phase separations were performed using 75 μ m inner diameter $\times$ 360 μ m outer diameter $\times$ 10-cm-long columns (Polymicro Technologies, Phoenix, Arizona) that were slurry-packed on site with 5- μ m Jupiter C-18 stationary phase (Phenomenex, Torrance, California). After sample injection a 20-minute wash with 95% buffer A (0.1% v/v formic acid in water) was applied, and peptides were eluted using a linear gradient of 5% solvent B (0.1% v/v formic acid in acetonitrile) to 45% solvent B over 40 minutes with a constant flow rate of 0.5 μ L/min. The IT-MS instrument was operated in a data-dependent mode wherein each full MS scan was followed by three tandem MS scans in which the three most abundant peptide molecular ions were dynamically selected for collision-induced dissociation (CID) using a normalized collision energy of 36%. The temperature of heated capillary and electrospray voltage (applied on column base) was 180°C and 1.2 kV, respectively. The CID spectra were searched against the human proteome database (UniProt release 26 October 2005) using SEQUEST (Thermo Finnigan).

Complement activation, plasma coagulation, and western blotting

Platelet-poor plasma was prepared by centrifugation of freshly drawn whole blood for 10 minutes at 2500g and either used fresh or stored at -20°C before use. Complement activation experiments were performed as described (http://ncl.cancer.gov/NCL_Method_ITA-5.pdf). Briefly, equal volumes (10 μ L each) of nanoparticles, freshly prepared plasma, and veronal buffer were mixed together and incubated at 37°C for 60 minutes. The reaction was stopped by addition of 4 $\times$ NuPAGE sample buffer (Invitrogen, Carlsbad, California), and after heating for 5 minutes at 90°C, 30 μ L of each sample were resolved on 10% Trisglycine gel. For quantitative analysis, plasma samples untreated or treated with gold nanoparticles were studied using Human Anaphylatoxin kit (BD Biosciences San Jose, California) according to the manufacturer's instructions. For western blot analysis of fibrinogen, proteins separated by 2D PAGE were transferred onto a Westran S nylon (Whatman Inc., Florham Park, New Jersey) membrane and incubated with anti-fibrinogen antibodies followed by detection with anti-goat IgG-HRP and ECL reagent. For plasma coagulation experiments, platelet-poor plasma was treated with gold nanoparticles

for 30 minutes at 37°C, after which coagulation time was measured on STArt4 coagulometer (Diagnostica Stago) using prothrombin time (PT), activated partial thromboplastin time (APTT), and thrombin reagents (Diagnostica Stago) according to the manufacturer's instructions. Detailed protocol is available at http://ncl.cancer.gov/NCL_Method_ITA-12.pdf. World Health Organization–certified normal plasma (normal control) and abnormal plasma (abnormal control) were used for internal control of the instrument and assay reagents. Both normal and abnormal plasma controls were from Diagnostica Stago.

Platelet aggregation

To study particles' effects on platelet aggregation, whole blood was centrifuged 8 minutes at 200g to obtain platelet-rich plasma. Platelet-rich plasma was treated with nanoparticles, PBS (negative control), or collagen (positive control) for 15 minutes at 37°C. Collagen was from Helena Laboratories. After that single-platelet count was conducted using a Z2 counter and size analyzer (Beck-man Coulter Inc., Fullerton, California). A decrease in the single-platelet count occurring due to the platelet aggregation was used to calculate percentage aggregation. A detailed protocol is available at http://ncl.cancer.gov/NCL_Method_ITA-2.pdf

Results

Plasma protein binding increases particle hydrodynamic size

The sizes of two sets of citrate-stabilized gold nanoparticles with nominal diameters of 30 nm and 50 nm were examined by DLS, TEM, and AFM before and after 30-minute incubation with human blood plasma. Particles were washed at least three times in PBS following incubation, before further analysis. DLS measurements before incubation revealed a scattered-light intensity-weighted average particle hydrodynamic diameter (Z-avg) of 33.3 nm and 55.0 nm for the two sets. The distributions of particle diameters are approximately Gaussian. The hydrodynamic diameters of both sets of particles increased approximately 50 nm upon incubation (Figure 1, *A* and *B*). After incubation with plasma the intensity-weighted average particle diameters increased to 76.1 nm and 100.0 nm, respectively (Table 1). Furthermore, trypsin digestion of the particle-bound plasma proteins returns the colloids to approximately their pre-incubation sizes (Figure 1, *C*). When uncoated gold colloids are diluted in PBS or saline, these particles aggregate, evidenced by a change in the solution color (turns black) and appearance of visible black aggregates in the test tube (see Supplemental Figure 1, in Supplementary Material, which can be found in the online version of this article). The ions in PBS probably lessen the coulombic repulsion previously stabilizing the sol. For this reason, the DLS size of the uncoated colloids was measured in water. After plasma incubation the proteincoated particles were washed with excess PBS and reconstituted for DLS analysis in PBS, with no changes in color or visible particle aggregation. In this case the protein coatings stabilize the sol by steric hindrance. Incubation with trypsin results in complete removal of protein from the particle surface and the formation of visible black aggregates and change in the color of the solution. DLS measure of the particle size is not possible under these conditions, so Figure 1, *C* shows gold particles with partially digested proteins. Analysis of gold nanoparticle in PBS before and after incubation in plasma by ultraviolet-visible (UV-Vis) provided further evidence that the protein coating prevents the gold nanoparticles from aggregating in PBS (see Supplemental Figure 2, in Supplementary Material, which can be found in the online version of this article).

Because DLS measures a particle's hydrodynamic diameter, this measurement can also be influenced by particle agglomeration.¹² To determine if the observed increase in particle

hydrodynamic diameter upon plasma incubation could be attributed to a change in agglomeration state, the same samples measured by DLS were also studied by TEM and AFM, where particle agglomeration can be assessed from the micrograph images. Representative electron micrographs before and after plasma incubation are shown in Figure 2, along with the size distributions obtained from counting at least 45 discrete particles. The TEM measurements show no significant difference in mean electron density diameter between the before-incubation and after-incubation samples. There is no visible difference in aggregation tendency between the before-incubation and after-incubation TEM micrographs. Representative AFM micrographs are also shown in Figure 3 along with the particle height distributions obtained by counting at least 100 particles from these images. The AFM measurements show no significant difference in average particle height between the before-incubation and after-incubation samples (there is a 1.8-nm increase in mean AFM-measured particle diameter of the 30-nm particles upon incubation and a 0.5-nm decrease for the 50-nm particles). Both the TEM and AFM data suggest, then, that plasma incubation did not change the agglomeration state of the particles.

Plasma protein binding decreases particle surface charge

The surface charge (zeta potential) of the gold particles before and after plasma incubation was also measured. Before incubation both sets of particles were negatively charged. Upon plasma incubation the mean particle surface charge of the 30-nm gold particles increased (became less negative) from -38.2 mV to -16.4 mV, and the same measurement for the 50-nm gold particles increased from -33.4 mV to -17.6 mV (Table 1).

Amounts and identities of particle-bound proteins

The plasma proteins adsorbed to the gold particles were separated and examined by 2D PAGE. Proteins or protein fragments present in at least three (out of four) gels were excised and identified by MS (Table 2). There were 45 protein spots excised from the gel containing the elution from the 50-nm gold colloid. These spots were composed of 21 individual proteins. A total of 114 spots were excised from the gel of the elution of the 30-nm colloid, composed of 48 individual proteins. Only 14 proteins were common to both the 30-nm and 50-nm particles, with 8 of these demonstrating apparent higher binding affinities for the 30-nm particles (higher abundance on the 30-nm particles). There were 7 proteins found to adhere selectively to the surface of 50-nm particles, whereas 34 proteins appeared to bind specifically to the 30-nm particles. Fibrinogen was the most abundant protein on both the 30-nm and 50-nm particles.

Aliquots of each sample were also dialyzed against PBS to determine the total amount of adhering polypeptides: 23 μ g of total protein adsorbed to the 30-nm particles, and 8 μ g to the 50-nm colloids. This initial 3:1 ratio (23 μ g versus 8 μ g) is reduced to 2:1 (14.4 μ g versus 8 μ g) when the samples are adjusted for surface area ($[23 \mu\text{g} \times 3534 \times 10^{12} \text{ nm}^2]/5652 \times 10^{12} \text{ nm}^2 = \sim 14.4$ μ g of total protein bound to $3534 \times 10^{12} \text{ nm}^2$ surface of 30-nm particles).

Complement activation

One of the proteins observed in the elution from both the 30-nm and 50-nm colloids is the C3 component of complement. To examine potential effects on the complement activation cascade, we assessed the degree of complement activation in an in vitro assay in human plasma aliquots pretreated with gold particles. Neither species of colloid induced detectable activation of the complement system in a C3-specific qualitative assay (Figure 4, A). Similarly, complement activation was not observed using a sensitive cytometry-based quantitative assay specific for C3a, C4a, and C5a components of complement (Figure 5). Thus, no complement activation through both classical (C4a) and alternative (C3a, C5a) pathways was detected for 30- and 50-nm colloidal gold nanoparticles.

Coagulation and platelet aggregation

Because fibrinogen, a critical component of the blood clotting cascade, was the most abundant protein in the elution from the colloidal surfaces, we further examined the interaction of fibrinogen with the particles and their influence on blood coagulation. Plasma protein samples isolated from the surface of colloidal gold particles were separated by 2D PAGE, transferred to a nylon membrane, and probed with fibrinogen-specific antibodies. Plasma sample that had not been incubated with gold particles was used as a control. The control plasma and the plasma containing the gold particles contained equal amounts of total protein, yet a larger range of fibrinogen isoforms were observed in the colloid-containing samples (Figure 4). Analysis of total fibrinogen spot density between 30- and 50-nm particles did not reveal a significant difference. Despite the observed difference, no delay in coagulation time was observed in plasma samples containing either the 30-nm or 50-nm particles in all of these assays (Figure 6, A–C). Gold colloids did not induce any detectable platelet aggregation (Figure 6, D).

Discussion

In this study we observed and characterized an increase in the hydrodynamic size of citrate-stabilized gold colloids when the particles were incubated with plasma. The adsorbed plasma proteins decreased the absolute charge on the particles, yet the solutions remained stable as evidenced by the DLS-measured hydrodynamic size distributions and UV-Vis absorbance after the incubation. The colloidal stability is presumably due to coulombic repulsion between the charged particles or steric hindrance of the protein coatings, and is consistent with examples in the literature of protein-stabilized colloidal solutions.⁶ This increase in particle diameter and decrease in absolute zeta potential observed upon plasma incubation are consistent with adsorption of positively or neutrally charged proteins to the surface of the colloidal particles. In the bloodstream such proteins could bind the colloid by coating the negatively charged nanoparticles and thus facilitate their interaction with the immune cells either through receptor-mediated endocytosis or through reduction of the coulombic repulsion between the particles and negatively charged cell membranes. The increased size of the protein-bound particles will probably affect the particles' rate of uptake from the bloodstream and route of clearance from the body, because particle size has been demonstrated to influence these outcomes.^{6,13}

The proteins that bind nanoscale particles (polymeric nanoparticles, iron-oxide particles, liposomes, and carbon nanotubes) have been examined before, and our novel results for gold colloids are consistent with these studies, which found albumin, apolipoprotein, immunoglobulins, complement, and fibrinogen to be the most abundantly bound species.^{14–21} Some of these studies suggest that particle surface charge or hydrophobicity is the primary determinant of protein binding.^{16,19} Here we have shown that for colloids, particle size also plays a role, in that the 30-nm gold colloids bound almost twofold more protein mass than the 50-nm colloids. This difference reflects the greater total surface area of the 30-nm colloids. The surface area ($A = 4\pi R^2$) of a single 30-nm particle is smaller than that of a single 50-nm particle. However, because this study used 30- and 50-nm with equivalent gold concentrations, the 30-nm sample contains more particles per unit of volume than the 50-nm sample; thus, the total surface area of the 30-nm particles is larger than the total surface area of the 50-nm particles. We have also analyzed 30- and 50-nm particles in other amounts: equivalent number of particles (i.e., the total surface area of 50 nm is larger than that of 30 nm) and equivalent total surface area (i.e., total surface area of 30-nm particle is equal to that of 50-nm particles). If surface area completely determined the amount of bound protein, we would see more protein on the 50-nm particles in the first instance and roughly equivalent protein on the 30-nm and 50-nm particles in the second. However, we did not observe a statistically significant difference in the amount of bound protein for these

samples (unpublished observation). We attribute this to an inaccuracy in the number of particles per volume (this is not empirical data but is supplied by the colloid manufacturer TedPella Inc., based on the amount of gold chloride used in the reaction). Another source of error comes from the assumption of spherical colloid particles to calculate the surface area ($A = 4\pi R^2$). Our TEM data show that there are actually a variety of shapes, including spherical, triangular, rhomboid, and hexagonal shapes, although the nonspherical shapes generally make up less than 1% of the sample.

The 30-nm colloids also bind a greater range of proteins; significantly more proteins were detected in samples isolated from the 2D PAGE of the 30-nm colloidal gold particles than from the 50-nm particles. These data suggest that interaction may be multilayered in that a cationic protein binds anionic gold surface at one site and brings another anionic protein on the other site.

We also observed a difference in size measurement of the particles following incubation, depending on the instrumentation used. DLS was able to detect an increase in size due to opsonization, whereas TEM and AFM were not able to detect this difference (i.e., under the sample conditions described here). Other sample preparation methods, such as cryogenic methods in the case of electron microscopy, would probably overcome this limitation.

Electrostatic effects are important in protein binding to nanoscale gold colloid. The measured zeta potentials of the colloids were highly negative before plasma incubation and approached neutrality with bound protein, and the majority of bound protein species identified by MS are neutral or positively charged at physiological pH. The proteins that are negatively charged can be explained by a sequential model of protein binding, in which cationic proteins initially bind the colloid (essentially coating the negative charges) followed by binding of anionic proteins to the cationic protein coat.

Nanoparticle binding of plasma proteins may lead to the protein activation and result in inflammatory responses. For example, carbon nanotubes have been reported elsewhere to bind complement proteins, and this interaction was associated with complement activation via both classical and alternative pathways. In contrast to carbon nanotubes, adsorption of complement proteins on the surface of gold colloids observed in our studies was not associated with complement activation, under in vitro test conditions. Detection of fibrinogen on the surface of gold colloids reported in this study is in agreement with studies investigating other types of nanoparticles.^{14–21} There may be several reasons why fibrinogen was seen in greater abundance than albumin (another very abundant plasma protein) on 30- and 50-nm colloidal gold particles in our in vitro study. Fibrinogen is composed of α , β , and γ chains, and it has an elongated configuration in comparison to albumin, a single-chain globular protein.²² Although both fibrinogen and albumin are neutral at physiological pH (zeta potential between -10 and $+10$ mV), the isoelectric point (pI) of albumin (4.7) is less than that of fibrinogen (pI = 5.8).²² These data suggest that slight variation in protein charge may contribute to the difference in protein binding to the surface of colloidal gold in that the more anionic albumin will less efficiently bind the anionic gold surface than fibrinogen. A significant difference between total fibrinogen spot density in control plasma and plasma isolated from gold nanoparticles was observed. This difference may exist because an equal amount of total protein was loaded on the gel. Untreated plasma contains more than 3700 proteins,²³ whereas fewer than 100 of them are present in samples isolated from nanoparticle surfaces; thus, gold particles may concentrate fibrinogen and make it more available on the western blot.

Our study revealed 69 different proteins bound to the surface of gold nanoparticles. The functional consequences of such binding are unknown. Experiments conducted within this

study did not reveal any detectable platelet aggregation, change in plasma coagulation time, or complement activation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Analysis of nanoparticle size by DLS. The averaged (minimum of three measurements per sample) intensity size distribution plots for the (A) 30-nm and (B) 50-nm gold colloids before (red trace) and after plasma incubation (green trace) are displayed here. An increase in size by approximately 50 nm was observed for both particles after plasma incubation.

Figure 2.

Analysis of nanoparticle size by TEM. Representative TEM micrograph of 30-nm gold colloids before (A) and after (B) plasma incubation, and that of 50-nm gold colloids before (C) and after (D) plasma incubation. The colloid particles are equally dispersed in the before-incubation and after-incubation TEM images. No significant change in particle size or agglomeration tendency was observed from these images.

Figure 3.

Analysis of nanoparticle size by AFM. Representative AFM micrograph of 30-nm gold colloids before (A) and after (B) plasma incubation, and that of 50-nm gold colloids before (C) and after (D) plasma incubation.. The colloid particles are equally dispersed in the before-incubation and after-incubation AFM images. No significant change in particle height or aggregation tendency was observed from these images. All AFM measurements were performed by tapping-in-liquid mode to minimize lateral interaction between the AFM tip and the gold colloid and plasma proteins.

Figure 4.

Western blot analysis of particle effects on complement and fibrinogen. **(A)** Qualitative assay of complement activation. Western blot with C3-specific antibodies was used to monitor cleavage of C3 protein (*) into low-molecular-weight split products (**). **(B–D)** Western blot analysis using fibrinogen-specific antibodies of plasma proteins from **(B)** native plasma; **(C)** plasma sample isolated from the surface of 50-nm gold particles (Au-NP); **(D)** plasma sample isolated from the surface of 30-nm gold particles.

Figure 5.

Analysis of complement activation by quantitative flow cytometry-based assay. Plasma samples derived from at least three healthy donors were pooled and either left untreated or treated with cobra venom factor (CVF; positive control) or nanoparticles. Quantification of C3a, C4a, and C5a components of complement was done using Cytometry Based Array Human Anaphylatoxin kit (BD Biosciences). Each sample was analyzed in duplicate. Only samples with percentage coefficient of variation between individual replicates less than 25 were included in the analysis. Each bar shows the mean of four separate experiments conducted using four different pooled plasma specimens.

Figure 6.

Analysis of nanoparticle effects on blood coagulation. Plasma specimens derived from at least three donors were either untreated or treated with gold colloids (30 nm and 50 nm). Plasma coagulation times were measured on a STAart4 coagulometer using reagents specific for prothrombin time (PT) (A) activated partial thromboplastin time (APTT) (B) and thrombin time (C) from Diagnostica Stago according to the manufacturer's instructions. World Health Organization-certified plasma specimens (normal and abnormal) were used to control the instrument. Normal coagulation time in the PT test is 13.4 seconds, that of APTT is 34 seconds and that of thrombin time is 21 seconds. Normal plasma standard, untreated plasma specimens, and plasma samples treated with gold colloids showed coagulation times within these normal limits. Abnormal plasma control in all three tests showed a delay in coagulation time above the limits. Each bar represents a mean result from three independent experiments. Each sample was analyzed in duplicate, and analysis was repeated three times within an experiment. Four experiments were conducted. In each experiment samples with percentage CV between replicates above 5% were excluded from analysis. Platelet aggregation (D) was studied in platelet-rich plasma specimens untreated or treated with positive control (collagen), or nanoparticles (30 nm and 50 nm). For each experiment each sample was analyzed in duplicate; results with percentage CV between replicates above 20% were excluded from analysis. Experiment was repeated three times using three separate plasma sets. Each bar represents the mean result from three independent experiments.

Table 1

Summary of size and surface charge measurements on 30-nm and 50-nm gold colloids

Sample	DLS Z-Average hydrodynamic diameter (nm)	TEM Average diameter (nm)	AFM Average height (nm)	Zeta potential (mV)
30 nm before incubation	33.3	27.2	29.4	−38.2
30 nm after incubation	76.1	28.4	31.2	−16.4
50 nm before incubation	55.0	47.1	50.1	−33.4
50 nm after incubation	100.0	49.5	49.6	−17.6

Gold colloids before and after 30-minute incubation in pooled normal human plasma anticoagulated with sodium citrate at 37°C. Particle size was measured by DLS, TEM, and AFM. The average particle diameter is shown in nanometers as determined from the DLS intensity-weighted distributions, and from the TEM and AFM micrographs. Zeta potential was measured on the same samples used for DLS size measurement.

Table 2

Proteins isolated from the surface of colloidal gold particles and identified by MS

50 nm Au-NP		30 nm Au-NP	
Protein ID	Description (No. of spots in 2D PAGE gel)	Protein ID	Description (No. of spots in 2D PAGE gel)
P02671	Fibrinogen α/α E precursor (11)	P02671	Fibrinogen α/α E precursor (18)
P02675	Fibrinogen β -chain precursor (9)	P02675	Fibrinogen β -chain precursor (11)
P02679	Fibrinogen γ -chain precursor (3)	P02679	Fibrinogen γ -chain precursor (6)
Q14624	Inter- α -trypsin inhibitor heavy-chain H4 precursor (2)	Q14624	Inter- α -trypsin inhibitor heavy-chain H4 precursor (4)
P06396	Gelsolin precursor (1)	P05154	Plasma serine protease inhibitor precursor (5)
P01042	Kininogen precursor (1)	P06396	Gelsolin precursor (1)
P01009	α -antitrypsin precursor (1*)	P01042	Kininogen precursor (1)
Q5WW9	Protocadherin 17 (3)	P01011	α 1-antichymotrypsin precursor (1*)
P82279	Crumbs protein homologue precursor (1)	Q5VW9	Protocadherin 17 (13)
P67936	Tropomyosin 4 chain (1)	P82279	Crumbs protein homologue precursor (1)
P62736	α -actin-2 (1)	P60709	β -actin (1)
P01024	Complement C3 precursor (3*)	P01024	Complement C3 precursor (7*)
P04004	Vitronectin precursor (3)	P01028	Complement C4 precursor (2)
P01620	Ig κ -chain V-III region SIE (1*)	P02746	Complement Clq subcomponent B-chain precursor (1)
Q9NYT6	Zinc-finger protein 226 (1)	P02747	Complement Clq subcomponent C-chain precursor (1)
Q8NEM0	Microcephalin (1)	P10909	Clusterin precursor (complement-associated protein Sp40,40/complement cytolysis inhibitor) (1)
Q86YG0	Albumin (1)	P04004	Vitronectin precursor (8)
Q5EFE6	Anti-RhD monoclonal T125 κ -light chain precursor (4)	P01620	Ig κ -chain V-III region SIE (1*)
Q5RGS4	Plekstrin Homology domain containing, family A (phosphoinositide-binding specific) member 1 (1)	Q9NYT6	Zinc-finger protein 226 (1)
Q8NBZ3	Hypothetical protein FLJ90646 (2)	Q86YG0	Albumin (3)
Q8N1K5	Hypothetical protein FLJ40584 (1)	Q5EFE6	Anti-RhD monoclonal T125 κ light-chain precursor (2)
		P05090	Apolipoprotein D precursor (2)
		P02647	Apolipoprotein A1 precursor (1)
		P02649	Apolipoprotein E precursor (1)
		Q9H293	Interleukin-25 (2)
		Q5T714	Ash-1-like protein (3)
		P15169	Carboxypeptidase N catalytic-chain precursor (1)
		P15924	Desmoplakin (1)
		Q5DT20	Hornerin (1)
		Q02413	Desmoglein-1precursor (1)
		P14923	Junction plakoglobin (desmoplakin3) (1)
		P05089	Arginase1 (1)
		Q5D862	Ifapsoriasis (2)

50 nm Au-NP		30 nm Au-NP	
Protein ID	Description (No. of spots in 2D PAGE gel)	Protein ID	Description (No. of spots in 2D PAGE gel)
		Q9BXP5	Arsenite-resistance protein 2(1)
		P03952	Plasma kallikrein precursor (1)
		P29466	Caspase-1 precursor (2)
		Q9UF47	DnaJ homolog subfamily C member 5B (1)
		Q9GZR7	ATP-dependent RNA helicase DDX24 (1)
		Q6UWB4	Tryptophan/serine protease (1)
		Q8TE73	Ciliary dynein heavy chain 5 (1)
		P31040	Succinate dehydrogenase flavoprotein subunit, mitochondrial precursor (1)
		Q10466	Titin (1)
		Q8WYA0	Carnitine deficiency-associated protein expressed in ventricle 1 (1)
		Q9H2F5	Enhancer of polycomb homolog 1 (2)
		Q6NTH6	Chromosome 14 open reading frame 50 (1)
		Q6PJ95	Hypothetical protein (1)
		Q6MZQ6	Hypothetical protein DKFZp686G11190 (3)
		Q8N9N3	Hypothetical protein FLJ36848 (1)

A total of 69 proteins were identified: 48 from the 30-nm particles and 21 from the 50-nm particles. Proteins are listed in order of abundance. The text coloration is as follows: A protein listed in red adsorbed only to the 50-nm colloid, and a protein listed in blue was observed only on the 30-nm colloid. A protein listed in black adsorbed to both the 30-nm and 50-nm particles in approximately equivalent abundance, and a protein listed in green adsorbed to both the 30-nm and 50-nm colloids but with different abundance on the two sets of particles. Numbers in parentheses=number of spots.