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Selective Targeting of Antibody Conjugated Multifunctional Nanoclusters (Nanoroses) to Epidermal Growth Factor Receptors in Cancer Cells

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Abstract

The ability of smaller than 100 nm antibody (Ab) nanoparticle conjugates to target and modulate the biology of specific cell types may enable major advancements in cellular imaging and therapy in cancer. A key challenge is to load a high degree of targeting, imaging, and therapeutic functionality into small, yet stable particles. A versatile method called thin autocatalytic growth on substrate (TAGs) has been developed in our previous study to form ultra-thin and asymmetric gold coatings on iron oxide nanocluster cores producing exceptional near infrared (NIR) absorbance. AlexaFluor 488 labeled Abs were used to correlate the number of Abs conjugated to iron oxide/gold nanoclusters (nanoroses) with the hydrodynamic size. A transition from sub-monolayer to multilayer aggregates of Abs on the nanorose surface was observed for 54 Abs and an overall particle diameter of ~60 to 65 nm. The hydrodynamic diameter indicated coverage of a monolayer of 54 Abs, in agreement with the prediction of a geometric model, by assuming a circular footprint of 16.9 nm diameter per Ab molecule. The targeting efficacy of nanoclusters conjugated with monoclonal Abs specific for epidermal growth factor receptor (EGFR) was evaluated in A431 cancer cells using dark field microscopy and atomic absorbance spectrometry (AAS) analysis. Intense NIR scattering was achieved from both high uptake of nanoclusters in cells and high intrinsic NIR absorbance of individual nanoclusters. Dual mode imaging with dark field reflectance microscopy and fluorescence microscopy indicates the Abs remained attached to the Au surfaces upon the uptake by the cancer cells. The ability to load intense multifunctionality, specifically strong NIR absorbance, conjugation of an Ab monolayer in addition to a strong r2 MRI contrast that was previously demonstrated in a total particle size of only 63 nm, is an important step forward in development of theranostic agents for combined molecular specific imaging and therapy.

Introduction

Development of multifunctional nanoparticles for targeted drug delivery under imaging monitoring and to determine the therapeutic response rapidly will provide a new paradigm for cancer treatment in the clinic. Whereas, nanoparticles were initially used either for

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molecular imaging or therapy,^{1–3} recent efforts are underway to do both simultaneously.^{4–7} For example, magnetic resonance imaging (MRI) and ultrasound imaging have been utilized to monitor temperature changes during photothermal therapy.^{7–9} However, nanoparticles possessing multifunctional properties for achieving both molecular imaging and real time feedback on therapeutic efficacy for cancer treatment are not yet utilized in the clinic. Thus, there is great interest in synthesis of therapeutic nanoparticles with high targeting efficiency and strong near infrared (NIR) absorbance and/or magnetic properties for imaging and therapy.¹⁰

In passive delivery of polyethylene glycol (PEG) coated gold nanoparticles, the particles permeate leaky vasculatures and accumulate in tumor interstitial space.^{11,12} An antibody (Ab) or small antibody fragment may be conjugated to the particle surface to target biomarker receptors and greatly enhance accumulation at the tumor site.^{10,13} There are multiple conjugation protocols available for attachment of peptides or proteins on the surface of nanoparticles. However, they can be broadly divided into two major strategies. The first method results in a random orientation of antibodies on nanoparticle surface. In implementation of this approach, functional groups, for example, COOH, on the polymer coatings of the nanoparticle surfaces are conjugated to primary amine groups, which may be on either the Fc (nonbinding region) or Fab region (binding region).¹⁴ For example, EDC/Sulfo-NHS chemistry has been widely adopted for nanospheres,^{13,14} nanoshells⁶ and nanorods.¹⁵ In a recent study of gold nanorods with this method, the total gold accumulation in xenograft tumor models was only marginally improved in comparison with nontargeted controls.¹⁵ In the second or directional method, the Fc region of an Ab is first coupled with a low molecular weight heterobifunctional linker such as dithiol-PEG-hydrazide, whereby the dithiol group is conjugated directly to the Au surface. The coupling is achieved with a selective reaction between the hydrazide end group and an aldehyde, formed by mild oxidation of a carbohydrate side chain on the Fc region.^{16–21} Selective binding to the Fc region of an Ab is beneficial for maximizing the biological activity of the Fab binding regions.^{19,20,22–24} This conjugation strategy allows for the multiplexing of various glycosylated Abs on a single nanoparticle that can be used for delivery and monitoring of therapeutic agents in vivo.^{19,20,25,26} Thus, gold surface can serve as a high versatile substrate for binding of multiple types of functional biomolecules with relatively simple conjugation chemistry.

Nanoparticle size, composition and surface modifications play important roles in cancer nanomedicine. In some cases, it would be desirable to load an extremely high degree of targeting, NIR absorbance, magnetization and therapeutic functionality into small, yet stable particles. Particles in the range of 35 to 65 nm have a longer blood residence time and accumulate more slowly in the reticuloendothelial system (RES) (liver and spleen) than larger particles.^{27–29} Gold coated iron oxide particles are of interest for both magnetic and optical functionality, and may exhibit relatively low toxicity.^{7,10,30} To drive nucleation of Au seeds on unfavorable low energy iron oxide surfaces, high supersaturation values are often utilized, with Au³⁺/Fe mass ratios in the order of 10.^{30–32} Excessive autocatalytic growth often produces shells on the order of 10 nm.^{33–37} The surface plasmon resonance (SPR) for the thick shells is typically in the visible, rather than the NIR region. For a given overall particle diameter smaller than 65 nm, the small total available volume must be allocated strategically to achieve the desired functionality of the various components. For typical Au coated particles, the thick Au shells take up substantial volume that would otherwise be available for the Abs, and other functional components such as iron oxide. To address these severe space requirements, 35 nm multifunctional iron oxide nanoparticles were designed with very thin Au shells (< 5 nm), yet strong NIR absorbance (cross section of $\sim 10^{-14}$ m² at 755 nm).⁷ For molecular cancer imaging/therapy, it is beneficial to conjugate target moieties such as Abs onto this type of small multifunctional nanoparticle,

while maintaining colloidal stability. Very recently, it has been found that the particle curvature and shape impact cellular targeting as seen for nanocages,³⁸ ellipsoids, rods, cylinders and disks, in addition to spheres.^{39–41} The design of multifunctional nanoparticles with various surface curvatures and control of the density and orientation of Abs on the surface has the potential to have a large impact on cellular uptake (interactions with membrane receptors and internalization within the cell) and therapy in nanomedicine.

Herein, we have varied an average number of conjugated monoclonal anti-EGFR Abs to multifunctional nanoparticles (nanorose) composed of thin knobby Au coatings on magnetic iron oxide cores from one to seventy four. As the number of Abs increased, the hydrodynamic diameter measured by dynamic light scattering (DLS), increased from 35 to 78 nm (Scheme 1). High surface density of Abs is favored by the high density of conjugation sites supplied by gold surface and the low cross sectional area of the heterobifunctional dithiol-PEG-hydrazide (M_w 708.97) linker that was used in conjugation procedure. The thin Au shells and the low M_w linker take up relatively little volume resulting in small overall particle size, with an unusually large fraction of the volume allocated to the iron oxide and Abs. Furthermore, because the Au shells are so thin, the amount of steric stabilizer (mPEG-SH, M_w 5,000) needed to counteract van der Waals (VDW) forces may be expected to be unusually low relative to more common particles with thicker Au shells.³⁴ The number of clone 225 Abs conjugated to the nanorose was tuned from sub-monolayer to multilayer coverage as characterized by labeling the Abs with AlexaFluor 488 fluorophore. For simplicity, the surface was approximated as an equivalent sphere, and thus the model does not account for Abs located in the cavities of the irregularly shaped surfaces. For ~54 Abs, the hydrodynamic diameter indicated coverage of a monolayer for Abs, in agreement with the prediction of a geometric model, by assuming a circular area for the antibody's footprint.

High uptake of conjugated nanorose by A431 skin cancer cells was observed with dark field and fluorescence microscopy and was quantified by flame atomic absorption spectroscopy (AAS). The intensive orange color under dark field illumination indicates high scattering by the thin Au shells on nanorose in agreement with previous hyperspectral images in macrophage cells for nanorose without Ab conjugation.⁷ Fluorescence images show strong green emission from AlexaFluor 488 tagged clone 225 Abs inside cancer cells. Co-localization of fluorescence and dark field reflectance signals indicates that the Abs stayed attached to the Au surfaces. The uptake of nanorose conjugated with an Ab monolayer reached 7,000 particles/cell. The high uptake is discussed in terms of the small hydrodynamic diameter, high degree of Ab conjugation, and the influence of the highly curved surfaces on the Ab orientations. These particles with NIR imaging, magnetic and therapeutic functionalities in a small overall particle size would be of interest in advancing molecular specific imaging combined with therapy.

Experimental Section

Materials

Monoclonal anti-EGF receptor antibody clone 225 (Sigma: E2156) and monoclonal anti-rabbit immunoglobulin clone RG-16 (Sigma: I0138) were obtained from Sigma-Aldrich Co. The Ab stock solutions were first purified by using a 100,000 molecular weight cutoff (MWCO) centrifugal filter (Amicon Ultra-4, Millipore Co.). Briefly, 200 μ L (1.5 mg/mL) clone 225 stock solution was transferred into one filter device with 3 mL HEPES (pH 7.4, 40 mM) addition. The filter device was centrifuged for 14 mins at 3,220 $\times g$. 300 μ L HEPES was used to form a solution of the Ab at a final concentration of 1 mg/mL. Dithiol-alkane aromatic PEG₆-NHNH₂ (C₃₃H₆₀N₂O₁₀S₂, M_w 708.97, SensoPath Technologies Inc.,

Bozeman, MT, USA), mPEG-SH (M_w 5,000) from Nanocs Inc. (New York, NY, USA), PEG (M_w 14,000) and NaIO_4 (213.89 M_w) from Sigma-Aldrich Co. were used.

Antibody Conjugation to Nanorose

The 35 nm diameter thin gold coated iron oxide “nanorose” particles were assembled by using dextran as a stabilizer as reported in our previous paper.⁷ Nanorose particles (2.4 mg Au) were then redispersed after centrifugation at $4,629 \times g$ for 6 mins by using a 50 μL of 0.05 mg/ml (10^{-5} M) mPEG-SH water solution and 2 ml deionized (DI) water to reach a concentration of ~ 1 mg Au/ml. The heterogeneous linker (dithiol-PEG-hydrazide) was used to covalently attach monoclonal Abs to gold surfaces.²⁰ Briefly, the linker consists of a short polyethylene glycol chain terminated at one end by a hydrazide group and at the other end by dithiol groups. 300 μg clone 225 Abs at a concentration of 1 mg/mL were first mixed with 300 μL of 10 mM NaIO_4 for 45 minutes at room temperature under mild agitation and protection from light. Here, the hydroxyl moieties on the Abs' Fc region were oxidized to aldehyde groups. Then, one aliquot of dithiol-PEG-hydrazide linkers (5 μL , 50mM in ethanol) was added to the oxidized Abs for 30 minutes. The hydrazide portion of the heterogeneous linker readily reacted with aldehydes on Abs' Fc region to form a stable linkage. The unreacted linker and salts were removed with a 100,000 MWCO centrifugal filter (Millipore Co.) at $3,220 \times g$ for 14 mins. The Abs (clone 225-PEG-dithiol) remained on the filter and were resuspended in 300 μL HEPES (pH 7.4, 40 mM). After purification, the modified Abs were mixed with nanoroses and dispersed in 30 mL of HEPES for 8 hours at 4°C on a shaker. Stable covalent bonds were formed between the gold surfaces and the linker's dithiol groups during mixing. Subsequently, mPEG-SH (M_w 5,000) molecules were added to passivate the gold surface on the nanoparticles unoccupied by Abs. Nanorose conjugates were centrifuged at $4,629 \times g$ for 6 mins and re-suspended in 2 mL HEPES (pH 7.4, 40mM). The concentrated nanorose-clone 225 conjugates were stored at 4°C for further characterization and cell targeting. To measure non-specific binding of nanoparticles to cancer cells, anti-rabbit IgG (clone RG-16) Abs that do not specifically bind to cancer cells were also conjugated to nanoroses by following the same procedure as for the clone 225.

Cell Culture and Nanoparticle Targeting

EGFR (+) human epithelial carcinoma cells (A431 keratinocyte) were cultured in petri dishes using a 50/50 (v/v) mixture of phenol/serum free DMEM medium at 37°C in a 5% CO_2 incubator till 80% confluence. Cells were harvested from plate by using trypsin and incubated for 5 mins. They were harvested and resuspended in 1 mL culture medium for cell counting. 10^6 cells/ml suspensions were typically harvested for nanoparticle labeling. Then, a designated number of cells were mixed with a known number of nanoparticles dispersed in 2 mL culture medium and allowed to interact for 60 mins on a shaker at room temperature. After labeling, the cells were centrifuged at $200 \times g$ for 3 mins to remove unbound particles in supernatant and re-suspended in $1\times\text{PBS}$. After a second round centrifugation at the same conditions to minimize impurities, the particle-labeled cells were dissolved in 0.5 ml 1 mM HNO_3 for AAS elemental analysis. The labeled cells were also re-dispersed in $1\times\text{PBS}$ for optical microscope imaging. EGFR (–) MDA-MB-435 breast cancer cells were cultured and labeled under the same conditions as the control cell line.

Au Elemental Analysis in the Labeled Cells

After redispersing the nanoparticle labeled cells, the Au concentrations in the cell were obtained by flame atomic absorption spectroscopy (GBC Scientific Equipment Pty Ltd, 908AA, Melbourne, Australia). The apparatus was equipped with an air-acetylene flame furnace. The absorption of Au was recorded at 242.8 nm. The instrument was calibrated using Au^{3+} standard solutions before every set of measurements. All measurements were carried in diluted samples so that the concentration of Au was between 1 and 5 $\mu\text{g}/\text{ml}$.

Hydrodynamic Diameter Measurement of Abs Conjugated Nanoparticles

DLS measurements were performed in triplicate on a custom made Brookhaven Instruments dynamic light scattering apparatus at a scattering angle of 90° and temperature of 25°C.⁴² Abs conjugated nanoparticle dispersion concentrations were adjusted with 1×PBS to give a scattering signal counting rate between 300–400 kcps. Prior to DLS measurements, the samples were bath sonicated for 2 minutes. The autocorrelation functions were analyzed with a non-negative least-squares (NNLS) method to determine distributions by volume.⁴²

Fluorescent Labeling and UV-Vis Spectrometry

Alexa Fluor 488 monoclonal antibody labeling kit (Invitrogen Co.) was used to label modified Abs (clone 225-PEG-dithiol) by following the provided procedure. The fluorophore has a tetrafluorophenyl ester moiety that reacts efficiently with primary amines on the Abs to form stable dye-protein conjugates. The excitation and emission measurements were performed at the absorption peak (494 nm) of the Alexa Fluor 488 dye by using a BioTek Synergy HT multi-mode microplate reader (BioTek Instruments, Inc.). To precisely determine the absorbance or emission of the Abs on the nanorose, freshly prepared background solutions for baseline subtraction were analyzed simultaneously with samples in a 96-well plate. The concentrations of the fluorescently labeled clone 225 Abs were measured from the absorbance at wavelengths of 280 nm (A_{280}) and 494 nm (A_{494}) in a 1 cm optical path length cuvette according to the formula suggested by the guide in the fluorescent labeling kit:

$$\text{clone 225 concentration (mg/ml)} = \frac{(A_{280} - A_{494} \times 0.11) \times 160000}{203000} \quad (1)$$

The molecular weight of the clone 225 Ab is 160,000 g/mole, and the absorbance cross section is 203,000 cm⁻¹·M⁻¹. A linear correlation between the emission intensity at 519 nm of the Alexa Fluor 488 labeled clone 225-PEG-dithiol versus concentration with a correlation coefficient $r = 0.9986$ was determined as shown in Figure 1.

A predetermined amount (Table 1) of Alexa Fluor 488 labeled clone 225-PEG-dithiol from a concentrated stock solution (0.565 mg/ml) was mixed with 6.2×10^{11} nanorose particles dispersed in 10 ml HEPES for the conjugation reaction. The number of nanorose particles was determined from elemental analysis by AAS.⁷ After mixing of the fluorescently labeled clone 225-PEG-dithiol to nanoroses as described in the Ab conjugation procedure, centrifugation at 4,629 ×g for 6 mins was used to separate the conjugated nanoroses in the pellet from unbound Abs in the supernatant. The fluorescence emission intensity of the supernatant for an excitation and emission wavelength of 494 nm and 519 nm, respectively, was measured to determine the concentration of unbound Abs by comparing with the correlation curve. The average amount of attached Abs to nanorose was determined by subtracting the unbound Abs from total.

Dark Field Reflectance Imaging and Fluorescent Imaging

Dark field reflectance images were captured with a Leica DM 6000 upright microscope using Xenon illumination along with a 20× dark-field objective (0.5 NA) and a Retiga EXi camera with 12-bit ultra-sensitive CCD camera detector, for imaging in a RGB mode (Q-imaging, Inc.). In dark-field microscopy, a sample is illuminated by a cone of light outside of the imaging angular acceptance aperture. The large scattering angle allows detection of highly scattering objects (such as nanoroses) with very little background signal. Fluorescence images were captured under the same microscope using fluorescence mode by

collecting 520 nm emission signals (emission peak at 519 nm of Alexa Fluor 488) with an excitation wavelength at 490 nm (absorption peak at 494 nm of Alexa Fluor 488).

Results

Number of Clone 225 Abs Conjugated to Nanorose and Hydrodynamic Diameter

As shown in Table 1, the number of covalently attached clone 225 molecules per nanorose particle increased monotonically with the amount of Abs used in the conjugation. When 303 Ab-PEG-dithiol molecules were added per one nanorose particle, 54 were attached as determined by the calibration curve (Figure 1). This number is on the order of a monolayer as will be quantified in detail in the discussion section.

The hydrodynamic diameter of the nanorose particles with different numbers of conjugated Abs were measured in 1×PBS dispersion, before and after filtration with a 0.22 μm filter, by DLS (Figure 2). As shown in Table 1, the hydrodynamic diameters increased monotonically from 33 nm to 80 nm as the attached number of clone 225 molecules increased from 1 to 74. The vast majority of the particle hydrodynamic diameter distribution by volume was in the primary peak. A small secondary peak ($\sim 10\%$ by volume) was observed with a larger size, indicating a small population of particle aggregates (Figure 2A and Table 1). After filtration with a 0.22 μm pore size syringe filter, the second peak from particle aggregates was fully removed as shown by DLS (Figure 2B and Table 1). The hydrodynamic sizes of the non-aggregated particles (smaller peak) did not change significantly in the filtration process. This stability of the particles suggests that the Ab molecules and mPEG-SH were effective in providing steric stabilization. We have also evaluated longer term stability of particles with 54 Ab molecules per nanorose (1 mg Au/ml) in HEPES (pH 7.4, 40 mM) stored at 4°C for 3 months. The majority of particle size distribution (80 % by volume) shifted to 65 nm indicating mild aggregation and quite reasonable colloidal stability..

The absorbance spectra of nanoroses (TEM image in Figure 3B) were obtained before and after Ab conjugation (Figure 3A). The very small change in spectra indicated high colloidal stability and stability in the Au shells for the particles. In addition, the Abs contributed little to the absorbance as expected, given the extremely strong cross section for the SPR of Au. Strong NIR absorbance of nanoroses, relative to the spectra for Au spheres, is caused by asymmetry in the thin Au shells⁷ as described below. We also determined the absorbance spectra for more well-known³⁰ spherical control particles with a 50 nm diameter and thick gold shells (TEM image in Figure 3B) on the same iron oxide core as for the nanorose in Figure 3A. At 755 nm, the extinction coefficients for the nanorose particles with thin asymmetric shells ($0.0537\text{--}0.0563\text{ cm}^2/\mu\text{g Au}$) were over five times those of the control particles with thick smooth gold shells ($0.0085\text{--}0.0106\text{ cm}^2/\mu\text{g Au}$) as shown in Table 2. For similar particles, $\sim 20\text{ nm}$ thick smooth gold shells on $\sim 10\text{ nm}$ iron oxide cores are often too thick to shift the SPR peak of pure gold at 530 nm to the NIR region that is preferred for in vivo imaging and therapy.^{16,17,30} The 6 order of magnitude larger extinction cross section of the Ab conjugated nanorose particles (10^{-14} m^2)⁷ compared with Ab conjugated fluorophore, like indocyanine green dye or Cy5.5,^{26,43,44} is highly desirable for NIR optical imaging and therapy in cancer.

Selective Cellular Uptake with Dosage Response

Dark field microscopy images were used to compare the targeting selectivity of A431 cancer cells for the EGFR specific Ab conjugated nanoroses versus the non-specific anti-rabbit IgG (RG-16) conjugated nanoroses (Control). In each case, the experiments were performed with the sample containing 54 Ab molecules per nanorose, in which the coverage was on the order of a monolayer. The A431 cells harvested from one petri dish were divided equally

and incubated with different dosage of nanorose particles. All images were acquired under the same microscope and camera settings. Since empty cells scatter light much more weakly in the visible and NIR regions (400–900 nm) (Figure 4A) relative to metal nanoparticles, the reflected light from the nanoparticles was much brighter than for the cells under dark field illumination. As shown in Figures 4A–C the non-specific anti-rabbit IgG conjugated nanoroses accumulate very little in the A431 cells, even with the highest dosage (4.0×10^5 RG-16 nanoroses/cell). On the contrary, a bright orange signal appears in the A431 cells treated with the EGFR specific Ab conjugated nanoparticles, even with a dosage of 1.0×10^4 clone 225 nanoroses/cell (Figure 4D). Similar orange colors have been observed in macrophage cells incubated with dextran-coated nanorose without Abs.⁷ The scattering signal increased monotonically with dosage.

The nanorose particle cell uptake was quantified by AAS elemental analysis by averaging over 10^5 to 10^6 A431 cells treated with different particle dosages. Figure 5 shows the cell uptake increased from 10 to 609 particles per cell with increased dosage from 1.0×10^4 to 4.0×10^5 of anti-rabbit IgG conjugated nanoroses. The cell uptake for anti-EGFR Ab conjugated nanoroses increased from 543 to 7175 particles per cell under the same dosage range. A factor of 54× higher cell uptake was achieved for molecular targeted nanoroses versus control under lowest dosage and a factor of 12× higher versus control under highest dosage. The cell uptake reached a maximum value of ~7,000 particles per cell at the dosage of 4.0×10^5 nanoroses/cell for clone 225 conjugated nanoroses.

Low Cell Uptake in EGFR(–) Control Cancer Cells

To demonstrate molecular specificity of anti-EGFR Ab conjugated nanorose particles to EGFR (+) A431 cancer cells, negative control EGFR (–) MDA-MB-435 breast cancer cells were cultured and labeled under the same conditions. For both dextran coated nanoroses (2.0×10^4 nanoroses/cell) without Abs and anti-EGFR Ab conjugated nanoroses (2.0×10^4 or 4.0×10^5 nanoroses/cell), dark field microscopy images show very little orange reflective intensity in these EGFR (–) MDA-MB-435 cells (Figure 6) compared with the as EGFR (+) A431 cells (Figure 4DEF). Thus, nonspecific interactions of the anti-EGFR nanorose particles with EGFR negative cells were very limited. Therefore, the specific interactions of these nanoparticles with the receptors on the EGFR (+) A431 cancer cells were required for the large uptake.

NIR and Fluorescent Dual Mode Imaging of Clone 225 Conjugated Nanorose Targeted to A431 Cancer Cells

The Alexa Fluor 488 labeled Abs were also used for simultaneous fluorescence imaging (right column in Figure 7) and dark field reflectance imaging (Figure 7, left column) of interactions between EGFR(+) cell and anti-EGFR nanoroses. Image D0 shows very weak auto-reflectance signal without nanoparticles under dark field mode as has been shown in Figure 4A. Upon viewing under fluorescence mode simultaneously (F0, Figure 7) with an excitation light source at 490 nm, a signal was too weak to be measurable at the detection wavelength (520 nm), indicating very low autofluorescence of the cells. A constant dosage of 5.0×10^4 nanoroses/cell was utilized in all of the other images in Figure 7. Image D1 shows a strong reflectance signal in NIR region (as seen in Figure 4E). The fluorescence image F1 shows no signal indicating no fluorescence emission without Alexa Fluor 488 labels. Image D2 and F2 were acquired with Alexa Fluor 488 labeled anti-EGFR Ab nanorose conjugates. In D2 the contrast and orange signal intensity are similar to those in D1, suggesting the reflectance intensity under dark field mode is not affected by the addition of the fluorophore. A green fluorescent signal emitted from Alexa Fluor 488 was observed in image F2. The emission was co-localized with the reflectance image in D2. Upon careful examination, a wide range of intensities is present in D2, indicating high contrast for various

degrees of aggregation of nanorose particles. Less contrast in intensity is evident in the fluorescence images. These observed differences between scattering and fluorescence signals are due to coherence light scattering from gold nanoparticles; this effect in cell imaging has been characterized in details by Aaron et al.¹⁸ Dual mode images were also obtained for nanoparticles with only 18 Abs per nanorose at the same dosage (5.0×10^4 nanoroses/cell) in D3 and F3. The images were similar to those in D2 and F2, indicating the targeting efficacy did not drop off significantly with a reduction in the number of Abs per particle. Again, higher contrast in intensity was observed in dark field imaging (D3) than by fluorescence (F3).

Discussion

Packing of Optical and Magnetic Functionality, along with Antibody in a Small Overall Particle Size

The design of a nanoparticle with a high degree of functionality and Abs conjugated to the surface requires highly efficient use of space. This challenge can become severe as the particle diameter becomes smaller than 100 nm. The mass ratio of the various components in conjugated nanoparticles was estimated from AAS and the number of conjugated Ab, as shown in Table 3. Typical mass ratios were 3.10 % for an Ab monolayer, 27.69 % for Fe cores and 69.21 % for the Au coating. For nanorose with thin Au coatings, the final Au/Fe mass ratio of 2.5 was much smaller than values of 10 for previously reported particles with spherical thick Au shells (~20 nm shell thickness).³⁰ Au was still the predominant component in the particle by volume, illustrating the importance of designing very thin Au shells (< 5 nm) to reserve a large amount of space for additional functionality and Abs.

For nanorose particles, the unusually thin Au shells were synthesized by tuning the separation of the nucleation of the Au seeds on the iron oxide surface from the autocatalytic growth on the seeds.⁴⁵ To drive nucleation of Au seeds on unfavorable low energy iron oxide surfaces, high supersaturation values have often been generated by utilization of Au^{3+}/Fe mass ratios on the order of 10.^{30–32} Consequently, as the seeds were nucleated, competitive autocatalytic growth of Au on Au often produced Au shells with thickness larger than 10 nm. Recently⁴⁵ and in the current study, extremely low Au^{3+} supersaturation values were applied to prevent excessive autocatalytic growth. The Au^{3+} addition profiles were varied during the reaction via a number of successive iterations to control the fraction of iron oxide substrate particles that were seeded.

In optical imaging and phototherapy applications, the Au shells on magnetic cores are often too thick to shift the SPR peak to the preferred NIR region (Figure 3A).³⁰ For nanorose particles, the thin Au shells with high degrees of asymmetry shifted the SPR to the NIR, with an extinction coefficient at 755 nm five times higher than for 50 nm spherical thick Au shells on iron oxide (Figure 3 and Table 2), despite the overall small diameter of 35 nm. Related types of asymmetry have been demonstrated for Au nanorod,⁵ nanocage,⁴⁶ nanorice,⁴⁷ nanoegg (asymmetric egg white shell),⁴⁸ and smooth Au shell with faceted or tetracubic core.³³ These asymmetries alter the interactions between plasmon modes and shift the SPR peak of gold nanospheres from 530 nm for symmetric systems to the NIR.^{33,47–49} In addition to nanorose, Au nanocages are also extremely small and exhibit strong NIR absorbance as a result of the morphology.³⁸

Gold has a high Hamaker constant, and thus polymer steric stabilizers are often required to prevent van der Waals attraction between Au coated nanoparticles. For example, a 10 nm polymer (HS-PEG-COOH, M_w 5kDa) shell was necessary to prevent colloid aggregation for 60 nm Au nanoparticles in buffered saline, prior to conjugation of targeting agents.¹³ For indirect conjugation of Abs to functional groups on polymeric coatings on Au surfaces, a

sufficient amount of polymer is needed to provide the required number of conjugation sites. For the low M_W bifunctional linker on Abs in the present study, the length of linker is negligible, and the density of conjugation sites (Au atoms) is extremely high. For nanorose, only thin polymer coatings are needed for steric stabilization given weaker VDW attraction for the thin Au shells, with a polymer loading of only 12 wt%, as shown recently.⁷ The weak VDW forces for nanorose particles with thin Au shells and high porosity reduce the attraction forces between particles, and allow for very thin layers of steric stabilizers. Thus, a total thickness of only ~14 nm (Table 1) for a monolayer of Abs with low M_W polymer stabilizers (mPEG-SH, M_W 5kDa) was achieved on the 35 nm thin Au coated iron oxide nanoparticles. The unusually small volume of the thin Au shells in nanorose is highly beneficial for providing space for other types of functionality, in this case, magnetic functionality and an Ab monolayer.

Packing of Antibodies on the Highly Curved Nanorose Surface

The number of Abs for formation of a monolayer was calculated from two simple geometric models to evaluate the measured surface coverage. The Fc region binds to the Au surface, while the Fab chains extend outward and occupy more surface area, as shown in Figure 8. Thus we choose to define an effective radius R from the center of the nanoparticle to the Fab region. In our case, a nanoparticle diameter of 35 nm and an Ab length of 11.7 nm give $R = 29.2$ nm.⁵⁰ In the first model, the area of the Fab region was assumed to be circular with a diameter of 16.9 nm (Figure 8). For an individual Ab, half of the end-to-end length 16.9 nm for the two Fab chains gives a radius of $r = 8.45$ nm.⁵⁰ Therefore, one Ab will occupy a circular area of 224 nm² on the surface of the particle at R . For a 2D monolayer of circles on a flat surface, where ϵ is the hexagonal close packed density (0.9069), the number of Abs

$$N_{\text{Abs}} = \frac{\text{Surface Area of Effective Sphere}}{\text{Area Occupied by Individual Antibody}} = \frac{4\pi R^2}{\pi r^2 / \epsilon} \quad (2)$$

Thus, a total of 43 Abs per particle is calculated for a monolayer of close packed Abs, assuming the native structure is maintained in the non-aggregated state.

In the second geometric model, the area of the Fab region is assumed to be a rectangle with length $L = 16.9$ nm and width $W = 4$ nm.⁵⁰ In this case, the surface area per Ab ($W \cdot L$) is 67.6 nm², such that $N_{\text{Abs}} = 4\pi R^2 / (W \cdot L) = 144$. As is expected, the number of Abs is larger for rectangles that pack more efficiently on the surface. The measured hydrodynamic diameter of 63 nm for the case of 54 Ab molecules per particle (Table 1) was close to the value of 58.4 nm predicted from R for a monolayer. The effective experimental area was 71.2 nm²/Ab. According to the first circular cross-section geometric model, the particle was covered by approximately one monolayer with 43 Abs. The deviation between the calculated 43 and measured 54 Abs was attributed to the uncertainty related to the nonspherical particle shape of nanorose, and also to the polydispersity in the particle sizes (Figure 2). The change of total hydrodynamic diameter with N_{Abs} may be attributed to a hybridization between the longer Ab chain length of 11.7 nm⁵⁰ and the shorter mPEG-SH (M_W 5,000) end-to-end chain length of 3.3 nm⁵¹ (Scheme 1). Thus for N_{Abs} values of 8 and 18, the hydrodynamic diameter fell between 35 nm and 63 nm for a monolayer. For the case with 74 Abs, N_{Abs} is above the monolayer value. The additional Abs beyond a monolayer is more likely to cause denaturation and aggregation from intermolecular interactions.^{52,53} The aggregation may even trigger an immune response and decrease the targeting efficacy.⁵⁰

In a recent study using clone 225, the same conjugation protocol and characterization methods were used for 18 nm spherical Au substrate particles.^{19,20} In this case, 9 Abs were

conjugated to each Au sphere. This corresponding area/Ab was 113.0 nm^2 , somewhat larger than the value of 71.2 nm^2 for nanorose (Table 4). Compared with the Au spheres with a radius of 9 nm, the radius of the protuberances ($\sim 5 \text{ nm}$, Figure 3B) on the surface of nanorose led to a higher actual surface area, than the surface area of a sphere with an equivalent radius of 17.5 nm. The surface roughness raised the Ab packing capacity by ~ 1.6 times. Furthermore, the varying orientations of the Abs as a result of surface roughness may further allow for higher Ab densities.

Another conjugation method is to adsorb the Ab to Au, but without the formation of covalent bonds, as has been demonstrated with anti-HER2 Ab - Herceptin (M_w 145 kDa). For 40 nm Au spheres, up to 200 protein molecules can be absorbed, which would correspond to 33 nm^2 per protein.²⁸ This high Ab density suggests multilayer absorption. Without covalent bonding of the Ab, it may be more difficult to control the Ab orientation than for conjugation with thiol-based bifunctional linkers.

Effects of Particle Size, Curvature and Shape on Selective Targeting

Studies have shown EGFR is over-expressed in human cancer cells and play a role in promoting tumor cell proliferation, invasion and metastasis.⁵⁴ Therefore, EGFR has been identified as molecular target for cancers.^{26,55} Clone 225 (Cetuximab, Erbitux)^{25,56} is a human-mouse chimeric anti-EGFR Ab that binds to the extracellular domain of EGFR with high affinity, and prevents activation of the receptor-mediated cellular survival signal. Nanoparticles conjugated with therapeutic antibodies may be used to monitor the targeted delivery of the cargo and supply real time therapeutic effects during the treatment of tumor.

For nanorose conjugated with a monolayer of clone 225, a sigmoidal curve of cell uptake versus dosage was observed in Figure 5. A high value of $\sim 7,000$ nanorose particles per cell was achieved at a dosage of 4.0×10^5 nanoroses/cell. For a cell approximated as a $10 \mu\text{m}$ diameter sphere, this cell uptake corresponds to a 0.030 % packing fraction by volume. For clone 225 coated 50 nm Au nanospheres conjugated by the same protocol, an uptake of 1,160 particle per cell (A431) was obtained at a much lower dosage level of 1.0×10^4 nanospheres/cell.⁵⁷ The resulting 0.015 % packing fraction was only half of the value for the nanorose. The high cancer cell uptake by Ab conjugated nanorose may be influenced by the small overall hydrodynamic diameter, the high Ab density on the surface, and by the orientation of the Abs with respect to each other, which is influenced by high local surface curvature.

When hydrodynamic diameters of Ab coated Au nanospheres are reduced to less than 70 nm, the biological pathways in targeted cells can undergo profound changes.^{28,58–61} The nanoparticles serve not merely as substrates for the Abs but strongly influence the effect of the Abs on the biological signaling processes. The fact that the curvature of the Au nanospheres influence binding capacities by nearly 3 orders of magnitude²⁸ suggests that interactions between multiple Abs on the surface and cell receptors play a key role. However, studies of the effect of curvature are relatively new, and it is not yet known how these effects will vary for different particle shapes, Abs, polymer stabilizers and cell types. For example, shape effects were much smaller for targeting of breast cancer cells (SK-BR-3) in a recent study of nanocages and nanospheres with anti-HER2 Abs on the surface.³⁸ Anti-HER2 Abs non-directional conjugation to 33 nm nanocages through relatively low M_w succinimidyl propionyl PEG disulfide (M_w 5kDa) gave an uptake of about 500 nanoparticles per cell. In this study, amount of Abs per nanoparticle N_{Abs} was not reported. However, the small size of the particles and the shape of the nanocage are beneficial for controlling the degree of conjugation and Ab orientation for high uptake. A related example is the highly effective cell targeting of the tyrosine kinase receptors on Jurkat T cells for DNA aptamer conjugated to viral capsid nanoclusters.⁶²

The knobby shape of the Au shells on the nanorose particles offers a rich domain of local surface curvatures that would be difficult to achieve for simpler shapes. The curvature of the protuberances (~ 5 nm, Figure 3B) on the surface of nanorose particle is similar to that of the protein spheres that make up viral capsids.⁶² The multiple number of gold domains of varying curvature for nanoclusters with convex surfaces (Figure 3B) can offer widespread opportunity for varying spacing and orientation of Abs with respect to the receptors on cell surfaces that can potentially lead to increased therapeutic efficiency. In the current study, only a single nanocluster morphology is considered. However, a homologous series in the sizes and shapes of the Au shells has been accomplished by the mechanism of thin autocatalytic growth on nanoparticles substrates (TAGs).⁴⁵ The thickness of the shells and the local surface curvatures may be adjusted independently.

Conclusion

Multifunctional nanoparticles composed of thin Au coatings on magnetic iron oxide cores, were conjugated with a monolayer of anti-EGFR monoclonal Abs (clone 225). The number of Abs, as determined from the fluorophore tag, was tuned from 1 to 74 by varying the feed Ab/particle ratio. The respective hydrodynamic diameters, from 35 to 78 nm, were extremely small given the high degree of functionality. The highly asymmetric thin Au shells, synthesized by seeded growth on iron oxide nanoclusters at ultralow supersaturations of Au precursor, produce strong NIR absorbance. The final Au/Fe mass ratio of 2.5 is smaller than previously reported values on the order of 10.³⁰ The unusually small volume of the Au is highly beneficial for providing space for other types of functionality, such as targeting and therapeutic Abs, and if desired, magnetic functionality. High surface coverage of Abs is favored by the high density of conjugation sites supplied by Au atoms on the surface and the low cross sectional area of the heterobifunctional dithiol-PEG-hydrazide linker. In contrast with this directional conjugation method, in non-directional methods the M_w of polymeric linkers is often higher and with a lower density of binding sites, resulting in lower Ab coverage. For 54 Abs, the hydrodynamic diameter indicated coverage of a monolayer for Abs, in agreement with the prediction of a geometric model, by assuming a circular area for the Fab region. Steric stabilization was provided with a combination of Ab as well as mPEG-SH in physiological media, to overcome the VDW attraction.

For the 63 nm particles with 54 Abs in a monolayer, targeting of EGFR overexpressive cancer cells was highly selective, relative to EGFR negative cancer cells. In dark field microscopy, high uptake of the conjugated nanoparticles produced an intense orange signal. The cellular uptake of nanoparticles/cell of ~7,000 was unusually large, in part because of the high concentration for incubation.

Intense NIR scattering was achieved from both high uptake of nanoparticles in cells and high NIR absorbance of individual nanoparticles, as observed in a previous study of the non-conjugated nanoparticles in macrophage cells by hyperspectral microscopy.⁷ Colocalization of the NIR scattering and fluorescence indicates the Abs remained attached to the Au surfaces upon the uptake by the cancer cells. The extremely high curvature of the Au shells with features below 5 nm influence the spacing and orientations of the Abs on the surface, resulting in the high cell uptake. Ultimately, tuning of surface curvature has the potential to have a marked effect on biological pathways within cells.^{28,39–41} The ability to load intense multifunctionality, specifically strong NIR absorbance, conjugation of an Ab monolayer and strong r_2 MRI contrast (from previous study⁷) in a total particle size only 63 nm, is an important step forward in development of theranostic agents for combined molecular specific imaging and therapy.

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Figure 1. Calibration curve for emission intensity (arbitrary units) at 519 nm of AlexaFluor 488 labeled clone 225 Ab versus concentration. The excitation at the absorption peak maximum of 494 nm of the AlexaFluor 488 dye was used. A correlation coefficient $r = 0.9986$ was determined.

Figure 2. Hydrodynamic diameter of clone 225 conjugated nanoroses. A. before filtration B. after passing a 0.22 μL filter. Insert gives numbers of Abs per particle.

Figure 3.

Extinction spectra (A) and TEM images (B) of thick gold shell coated iron oxide nanoparticle (Au@Iron Oxide) and nanorose. All spectra were normalized for 16 $\mu\text{g Au/ml}$ dispersion as quantified by AAS with 1 cm optical path length. TEM images were acquired before conjugation.

Figure 4.

Dark field microscopy images of A431 cells treated with different dosages of clone 225 conjugated nanoroses (right column) relative to control experiments using anti-rabbit IgG (clone RG-16) conjugated nanoroses (left column) under the same experimental conditions. A, B and C represent typical images with dosages of 0, 5.0×10^4 and 4.0×10^5 RG-16 nanoroses/cell. D, E and F represent typical images with dosage of 1.0×10^4 , 5.0×10^4 , 4.0×10^5 nanoroses/cell. Scale bar is 25 μm .

Figure 5.

Cell uptake of clone 225 and RG-16 conjugated nanoroses. 10^5 to 10^6 A431 cells were incubated with 2.5×10^3 , 1.0×10^4 , 2.0×10^4 , 5.0×10^4 , 1.0×10^5 and 4.0×10^5 nanoroses/cell for 1 hr. The relative standard deviation was in the range of 8–12 % for all measurements.

Figure 6.

Dark field microscopy images of EGFR negative control MDA-MB-435 cells treated with clone 225 conjugated nanoroses for 1 hr. A, 2.0×10^4 unconjugated nanoroses/cell (control); B, 2.0×10^4 particles/cell; C, 4.0×10^5 particles/cell. Scale bar is 25 μm .

Figure 7. Dark field (D) and fluorescent (F) microscopic images of A431 cells incubated with clone 225 conjugated nanoroses for 1 hour. 0, cells without nanoparticles as control; 1, 54 Abs per nanorose without fluorescent label; 2, Same as (1) with AlexaFluor 488 label; 3, same as (2) with 18 Abs per nanorose. A particle dosage of 5.0×10^4 nanoroses/cell applied to 1–3 experiments. Scale bar is 25 μm .

A **B** **Figure 8.**

A, The surface area of the particle is determined based on the nanoparticle and Ab assembly, having $R = 29.2$ nm. B, The area occupied per Ab is calculated using a circular, non-aggregated model as well as a rectangular, close packed aggregated model. The radius of the circular model, r , is the length of a Fab chain, 8.45 nm. The length of the rectangular model, L , is the length of two Fab chains, 16.9 nm. The width of the rectangular model, W , is the width of a Fab chain, 4 nm.

Scheme 1.

Geometric Properties of Antibody Layers on Model Spherical Particle Surfaces (approximately to scale)

Table 1

Clone 225 Conjugation to 6.2×10^{11} Nanorose Particles and Hydrodynamic Diameter by Dynamic Light Scattering

feed Ab mass (μ g)	feed Ab molecules/nanorose	conjugated Ab mass (μ g)	conjugated Ab molecules/nanorose	diameter, volume %	diameter after filtration
2.00	12	0.180	1	33 $\pm$ 3 nm, 92%	35 $\pm$ 4 nm
9.98	60	1.257	8	45 $\pm$ 3 nm, 89%	44 $\pm$ 6 nm
20.02	121	3.003	18	51 $\pm$ 4 nm, 87%	50 $\pm$ 5 nm
50.18	303	8.967	54	61 $\pm$ 4 nm, 92%	63 $\pm$ 5 nm
99.80	604	12.199	74	80 $\pm$ 5 nm, 91%	78 $\pm$ 7nm

Table 2

Measured Extinction Coefficient at 755 nm of Thick Gold Coated Iron Oxide Nanoparticles and Nanorose Particles

	thick Au w/o Ab	thick Au with Ab	nanorose w/o Ab	nanorose with Ab
extinction coefficient ($\text{cm}^2/\mu\text{g Au}$)	0.0085	0.0106	0.0563	0.0537

Table 3

Component of Abs Conjugated Nanorose Particles

conjugated Ab molecules/nanorose	Au w/w %	Fe w/w %	Abs w/w %
1	71.38	28.55	0.07
8	71.11	28.44	0.45
18	70.66	28.27	1.07
54	69.21	27.69	3.10
74	68.27	27.38	4.35

Table 4

Occupied Surface Area Per Ab on Nanorose and Au Sphere

	nanorose	Au sphere
diameter (nm)	35	18
conjugated Abs/particle	54	9
occupied surface area/Ab (nm ²)	71.2	113.0