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Quantitatively understanding cellular uptake of gold nanoparticles via radioactivity analysis

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Abstract

The development of multifunctional gold nanoparticles (AuNPs) underwent an explosion in the last two decades. However, many questions regarding detailed surface chemistry and how they are affecting the behaviors of AuNPs in vivo and in vitro still need to be addressed before AuNPs can be widely adapted into clinical settings. In this work, radioactivity analysis was employed for quantitative evaluation of I-125 radiolabeled AuNPs uptakes by cancer cells. Facilitated with this new method, we have conducted initial bioevaluation of surfactant-free AuNPs produced by femtosecond laser ablation. Cellular uptake of AuNPs as a function of the RGD density on the AuNP surface, as well as a function of time, has been quantified. The radioactivity analysis may shed light on the dynamic interactions of AuNPs with cancer cells, and help achieve optimized designs of AuNPs for future clinical applications.

Graphical abstract

The novel laser fabricated gold nanoparticles with clean surface allow precisely tuning of the number of functional ligands. The cellular uptake of cancer cells as a function of the RGD peptide density on the surface has been examined by radioactivity analysis method using I-125. AuNPs with 800 RGD on

1. Introduction

Multifunctional gold nanoparticles (AuNPs) have been attracting increasing interest for both fundamental science and biomedical applications^[1]. Plasmonic gold nanostructures, due to their small sizes and associated unique properties, have been widely used in drug delivery^[2-5], cellular imaging^[6-9], and biomedical diagnostics and therapeutics^[10-14]. For most practical biomedical applications of gold nanoparticles, chemical stability in a biological medium, biocompatibility, and targeting efficacy are the key requirements. Surface modifications are essential for meeting these requirements since the interactions of AuNPs with complex biological environments and biomolecules both on the surface of and inside the cells highly depend on the chemical nature of their solvent accessible surfaces^[15, 16].

In the past year, different technologies have been employed to evaluate the dynamic interactions between a variety of nanoparticles and cells or tissues, including fluorescence imaging (FI)^[17, 18], optical microscope (OM)^[19, 20], transmission electron microscope (TEM)^[21, 22], and inductively coupled plasma (ICP)^[23-25]. FI offers high detection sensitivity. However, due to the quenching by gold, this technology cannot be used to evaluate the behavior of AuNPs. OM, although can visualize AuNPs in cells when there are large amount, has only limited sensitivity. Moreover, the measurement from OM cannot be quantified. TEM provides extremely high resolution and sensitivity, and can visualize a small number of AuNPs in cells. However, TEM cannot enable quantitative evaluation of AuNPs uptakes by a large number of cells. This is also a problem for ICP. Moreover, neither ICP nor TEM can be adapted to the study on live cells. Recently, there has been increasing use of radioisotopes to study nanoparticles *in vivo*, utilizing the highly sensitive nuclear imaging technologies. Other than the extremely high sensitivity, nuclear imaging and radioactivity analysis are capable of quantitative evaluation not only *in vitro* but also *in vivo*. With nuclear imaging devices such as PET and SPECT, whole body imaging of animals or humans can be conducted, and longitudinal systemic bioevaluation of nanoparticles becomes possible. In our previous work, we have developed a highly efficient method to directly radiolabel AuNPs with I-125 radioisotope^[26-28]. Using a highly sensitive SPECT system, the *in vivo* performance of surfactant-stabilized gold nanorods, with and without pegylation, has been examined^[26-28]. Other than nuclear imaging, the radiolabeled AuNPs can also be mapped by optical imaging modalities such as photoacoustic imaging and, therefore, can facilitate multiple imaging technologies each with unique advantages.

Over the past decade, various strategies, for example, hydrophobic entrapment, electrostatic adsorption, covalent cross coupling, and ligand exchange, have been established for the conjugation of functional ligands onto surface of AuNPs. However, the synthesis of functionalized AuNPs still presents a major challenge, particularly in the case when it is desired to fabricate AuNPs conjugated with multiple types of ligands at a defined ratio to form multifunctional conjugates. This is due to the fact that an excess amount of ligands is typically required to be added with free ligands in solution helping to ensure colloidal stability of AuNPs during the conjugation, especially if AuNPs stabilized by the charges from the adsorbed surfactants. In contrast to surfactant-stabilized gold nanoparticles, the i-

colloid Au produced by femtosecond laser ablation, which has surfactant-free and naturally negative charged surface^[16]. The conjugation of functional ligands, such as Poly (ethylene glycol) (PEG), to these nanoparticles could be carried out with surface coverage tunable between 0 and 100%, thereby without unreacted free ligands left in the gold nanocolloids, and meantime, the suspension stability of i-colloid Au is maintained^[16]. By taking advantage of the unique property regarding surface functionalization mentioned above, i-colloid Au allows versatile and controllable conjugation to construct a mixed monolayer composed of multiple biologically important molecules at a certain ratio onto surface of AuNPs^[29]. The quantitative bioevaluation of this novel surfactant-free gold nanocolloids regarding detailed surface chemistry has not been achieved so far. There are many questions which still need to be addressed before these AuNPs can be widely adapted into the clinical setting. In this study the novel surfactant-free gold nanocolloids have conjugated quantitatively with RGD peptides which have been proven to specifically target tumor cells^[30]. We examined, using our newly developed radioactivity analysis method, cellular uptakes of AuNPs as a function of the RGD density on the AuNP surface, as well as a function of time.

2. Materials and methods

2.1. Production of RGD-PEG-AuNPs

Femtosecond pulses delivered from a commercially available Ytterbium-doped femtosecond fiber laser (FCPA μ Jewel D-1000, IMRA America) at a repetition rate of 100 kHz with 10 μ J pulse energy and 700 femtosecond pulse width centered at a wavelength of 1.045 μ m were focused onto a spot of about 50 μ m on a gold metal plate (99.99% purity) which was placed on the bottom of a glass vessel filled with 20 ml deionized water (18 M Ω cm). The procedure of sequential conjugation comprises: (i) Thiolated PEG 5000 molecules are first mixed with suspension of colloidal AuNPs with amount high enough to permit stability of the nanoparticles in an electrolyte solution while still less than the amount that provides for 100 % surface coverage of the nanoparticles so that there is still sufficient room on the surface of the GNPs to allow for subsequent conjugation of RGD peptides. After mixing, the solution is kept undisturbed for at least 2 hours at room temperature to provide a sufficient amount of time for the PEG molecules to be conjugated onto the surface of the GNPs via Au-thiol bonding; (ii) A defined number of RGD peptides are added and the resulting mixture is kept undisturbed for additional 24 hours at room temperature to ensure complete conjugation of RGD peptides via binding to unoccupied sites on the surface of GNPs; (iii) PEGylated cysteine (RGD)₄ conjugated gold nanoparticles are collected by centrifuge at 15000 g for 30 minutes, removing the supernatant, and then redispersing into phosphate buffered saline (PBS) with a PH of 7.4.

2.2. Iodine Conjugation

Sodium Iodide-125 solution in concentrations of 50-100 μ Ci/mL was combined with the conjugated AuNPs and gently mixed for 10 minutes at room temperature. The mixtures were then centrifuged at 6900 rpm for 9 minutes to remove unbound iodide and washed twice with Milli-Q water. The mixtures were diluted to 25-50 μ Ci/mL in Milli-Q water. The absorption spectroscopy at 520nm was performed on the final mixtures.

2.3. Cell Culture

The HeLa 229 cell line was obtained from the American Type Culture Collection (ATCC) and maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with fetal bovine serum (10%). The cells were cultured at 37°C in humidified 5% CO₂ in air. Round plates 9 cm in diameter were grown to 50% confluency at approximately 4.2 million cells per plate for all experiments.

2.4. In vitro experiment

Cell plates were aspirated and 3mL of DMEM supplemented with 10% FBS solution was added to each plate. GNP mixtures were added to the plates in 100-600μL volumes. The cells were then incubated at 37°C in a humidified atmosphere in 5% CO₂ air. After specific periods, the DMEM nanoparticle mixtures were removed and the cells were washed with 3mL of phosphate buffered saline (PBS). The PBS solution was removed and the cells were extracted using 3mL of 0.05% Trypsin-EDTA (1×) solution for 10 minutes. The radioactivities of the cells and of each removed solution were then measured using an Auto-Gamma counter 5000. The percentage of radioactivity in cells over the total radioactivity for each plate was calculated.

3. Results and Discussion

Gold nanoparticles used in these experiments were synthesized using the method of femtosecond laser ablation of a metal target immersed in a liquid. AuNPs obtained with this method have an average diameter of 20 nm and a virgin surface^[16]. This allows the versatile and controllable construction of mixed monolayers composed of multiple types of biologically functional ligands onto the AuNP surfaces to form multifunctional composite nanoparticles.

Using both molecular weight 5000 g/mol thiolated mPEG (PEG 5000) molecule and cysteine-modified (RGD)₄ peptide as model compounds, we fabricated a series of colloidal suspensions of i-colloid Au conjugated to these two functional molecules at different ratios via novel methodology of "Sequential Conjugation" as illustrated in Figure 1. PEG was chosen because it is a biocompatible material that can improve colloidal stability of AuNPs in biological media of high ionic strength (greater than or equal to 50 mM NaCl) and minimize nonspecific interactions of AuNPs with biomolecules, cells, and tissues. The RGD peptide was selected because of its demonstrated ability to induce receptor-mediated endocytosis which results in cellular uptake of AuNPs into a variety of cell lines.

A total of four colloidal suspensions of i-colloid Au conjugated with both PEG and RGD peptides were prepared by adding a fixed amount of PEG 5000 molecules and different amounts of RGD molecules in sequence. The final molar ratio between the number of PEG 5000 molecules and the number of Au nanoparticles in the mixed solution (PEG/gold nanoparticle) has been fixed at 200, and the final molar ratios between the number of RGD molecules and the number of Au nanoparticles in the mixed solution (RGD/gold nanoparticle) has been selected to be 0, 200, 400, and 800, respectively, to adjust the surface density of targeting ligands as desired. To ensure the prepared i-colloid Au nanoconjugates

were functional, their expected abilities to interact with membrane receptors, which result in their internalization into cells, were tested using HeLa cells. The optical microscopy images of cells were shown in Figure 2, the black color was due to AuNPs internalized within cells. The nanoparticles incubated in cell culture medium contained equal concentration (120 pM) of four samples of i-colloid Au nanoconjugates only differing in the ratio of RGD/GNP. The images confirmed not only AuNP internalization into HeLa cells but also the significant enhancement in the cellular uptakes of i-colloid Au nanoconjugates by increasing the surface density of RGD targeting ligands, indicating that the method of “sequential conjugation” generates fully functional gold nanoconjugates.

To further characterize the dependence of cellular uptakes of AuNPs as a function of surface density of RGD targeting ligands, we quantified and compared the amount of internalized AuNPs in HeLa cells via radioactive analysis of gold nanoconjugates labeled with radioisotope iodine-125. The same method reported for radiolabeling of conventional chemically prepared AuNPs has been used^[26]. ¹²⁵I-AuNP-RGD conjugates were incubated with HeLa cells for about 24 hours, then cells were separated from media and rinsed with PBS. The percentage of radioactivity which had been bound to the cells for each observation was calculated, as the results summarized in Figure 3 and Figure 4.

It was observed that the binding of ¹²⁵I-labeled RGD-PEG-AuNP conjugates to the cells increased when more RGD molecules were present on the GNP surfaces. With 200 or less RGD molecules per each AuNP, the binding was equivalent to PEG-AuNP. This further proved that ¹²⁵I-labeled RGD-PEG-AuNP conjugates specifically bind to human pancreatic cancer cells, and hold potential for specifically targeting of tumors *in vivo*.

For each specific RGD density, as more total AuNPs were added by adding more volume at constant concentration, the binding to cell ratios remained constant (Figure 3). This finding was agreed with previously reported trends of endocytosis/exocytosis of GNPs^[31, 32]. There are several suggested mechanisms to explain and calculate the dynamic and kinetic of GNP entry/exit of cells^[31-34]. The constant uptake, as indicated by the flat curves in Figure 3, suggests the balance of GNP endocytosis and exocytosis.

Among all the GNPs, RGD 1600 showed the strongest binding affinity. However, for GNPs with RGD density of 1600 on the surface, disassociation and precipitation were also observed during the experiments. Therefore, GNPs with 800 RGD showing both excellent specific binding and stability were chosen for a further study of time-dependent cellular uptake.

For better presenting of the cellular uptake as a function of the RGD density, the finding in Figure 3 was shown in another way in Figure 4, where each data point is an average over different concentrations of GNPs in cell culture media ranging from 80 pM to 400 pM. We can see directly that the cellular uptake by HeLa cells increased with the loading ratio of RGD to 20 nm GNPs up to 800 per nanoparticle. The observed very clear dependence of intracellular delivery of GNPs on surface density of targeting ligands reveals that the method of sequential conjugation, which allows precisely tuning of the number of functional ligands

bound onto the surface of nanoparticles, holds promise for optimized design of nanoparticles for biomedical applications.

Binding assays at different time points have also been studied, as shown in Figure 5. During the first 6 hours, the uptake was increased sharply, suggesting that rate of GNPs entering cells was faster than the rate of GNPs exiting cells during this period. After 6 hours until around 17 hours, there was no significant change in the cellular uptake, suggesting that balance of GNP endocytosis and exocytosis has been reached after 6 hour. The decline in cellular uptake of GNPs when beyond 17 hours is believed to be due to the unhealthy environment due to the over-growth of cells in the culture.

4. Conclusion

This is the first report of quantitative bioevaluation of novel laser fabricated AuNP surface chemistry using newly developed radioactivity analysis method. ^{125}I -labeled AuNP conjugates with different RGD densities have been evaluated. The binding of ^{125}I -labeled RGD-PEG-AuNP conjugates to the cells increased when more RGD molecules were present on the AuNP surfaces. With 200 or less RGD molecules per each AuNP, there was no specific binding comparing to PEG-AuNP. AuNPs with 800 RGD showed both excellent specific binding and good stability and, therefore, have been chosen for a further study of time-dependent cellular uptake. The novel laser fabricated AuNP with clean surface and the method of sequential conjugation allow precisely tuning of the number of functional ligands bound onto the surface of nanoparticles. This unique advantage over conventional method should facilitate flexible and optimized designs of AuNPs for various biomedical applications.

The methodology developed here can be used to analyze, evaluate, and optimize multifunctional AuNP conjugates for various medical applications. Both thiolated PEG and RGD peptide used in the experiments just serve as examples to testify that the method of sequential conjugation could produce colloidal GNPs bearing defined number of functional molecules. The discussed strategies and results of conjugation are of general nature and can be applied in the same way to the binding of other ligand molecules, including DNA, aptamer, antibodies, and enzymes. In addition, the method of sequential conjugation allows fabrication of biomolecule-NPs conjugates without having previously required large excess of biomolecules, which makes the process very attractive for use with very rare or expensive ligand molecules.

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Figure 1.
Schematic diagram of “Sequential Conjugation” of PEG and RGD to AuNPs.

Figure 2.

(a) Optical microscopy images of HeLa cells incubated for 12 hours at room temperature in cell culture medium containing equal concentration (120 pM) of four samples of i-colloid Au nanoconjugates differing only in the ratio of RGD/AuNP varying from 0 to 800. The black color in the images, which is due to AuNPs internalized within cells, indicates the significant enhancement in the cellular uptake of gold nanoconjugates by increasing surface density of RGD targeting ligands. (b) TEM image confirming the uptake of AuNPs by HeLa cells.

Figure 3.
 ^{125}I -labeled RGD-PEG-AuNPs binding to tumor cells at various concentrations

Figure 4.
 ^{125}I -labeled RGD-PEG-AuNPs binding to tumor cells at various RGD densities

Figure 5.

Kinetics of AuNPs conjugated with both PEG 5000 and (RGD)₄ peptides by HeLa cells as a function of incubation time. The loading ratios of cysteine (RGD)₄ and PEG 5000 to AuNPs are 800 and 200 per nanoparticle, respectively. The concentration of GNPs in cell culture media is 400 pM.