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Effect of Size of Gold Nanoparticles (GNP) on Intracellular Uptake and Cytotoxicity in Breast Cancer Cells

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Abstract—**We investigated the effects of intracellular uptake of different sizes of gold nanoparticles (GNP) ranging from 10 nm to 100 nm on breast cancer cells (MDA-MB-231). The experimental results showed the cell cytotoxicity is high dose- and dimension- dependent. We further investigated the effect of intracellular uptake of GNP on cell morphology, including cell area, perimeter, and aspect ratio. We showed GNP with a diameter of 20 nm enhanced cell proliferation with a low concentration of GNP (2 μg/mL, 4 μg/mL, and 10 μg/mL). To evaluate the specific sizes of GNP affection in wound healing, we investigated the cell migration ability after GNP uptake. Also, we detect the Dll4 expression when the GNP works as a probe, in which the 10 nm GNP-LNA complex reveals significant signal intensity.**

Keywords— gold nanoparticles, intracellular affection, cytotoxicity, dll4 expression

I. INTRODUCTION

Gold nanoparticles (GNPs) have been investigated and demonstrated to be an effective tool as therapeutic delivery vectors, intracellular imaging agents due to their favorable chemical and optical properties in the field of developmental biology and clinical study[1]. The capability of endocytosis of GNPs enables it to be applied in the field of drug delivery, gene delivery, and nanobiosensor. For example, previous studies have shown the combination of Locked Nucleic Acid (LNA) probes and gold nanorods (GNRs) or GNPs can be applied to detect mRNA gene expression dynamics at the single cell level[2]. Although several groups have investigated the effects of GNP uptake on cell cytotoxicity, the effects of GNP uptake on cell morphology and motility have not been adequately addressed[3]. Here, we investigated the effects of different sizes of GNP on the breast cancer cell line (MDA-MB-231). First, we evaluated the cell viability after endocytic uptake of GNP with a concentration of 2 μg/mL, 4 μg/mL, 10 μg/mL, and 20 μg/mL. Next, we further investigated the cell morphology change after the intracellular uptake of GNP with the incubation time of 72 hours and 168 hours. Our results indicated that the cytotoxicity of GNP uptake is dose- and size-dependent. The cell area, perimeter, and aspect ratio after GNP uptake is dependent on the dimension, concentration, and incubation duration.

To show how MDA-MB-231 cells migrate and test the difference between healthy MDA-MB-231 cells and the cells after scratching, we detected Dll4 expression in the cells. By previous studies, the leader cells usually show higher expression with Dll4, so in our research, the cells leading to cover the scratched area can show different genetic expression with other cells[4]. The double-stranded locked nucleic acid (dsLNA) complex is used to monitor the dynamic expression of target mRNA near the border as a probe. A donor probe composed of a nucleic acid sequence complementary to the target mRNA is labeled with a 6-FAM fluorophore at the 5' end. In its complementary sequence, we placed a quencher (Iowa Black RQ) on the 3' end. By using Dll4 dsLNA donate - quencher (DQ) complex probes, we can detect the Dll4 mRNA expression in a single cell. Also, the MDA-MB-231 cells with scratching and without scratching behave different migration tendency, the cells after scratch will be more involved in the migration, with the mRNA expression proved this hypothesis.

As we concern, we choose 10 nm and 20 nm GNP with (11-Mercaptoundecyl)- N, N, N-trimethylammonium bromide (MUTAB) coated in the concentration of 2 μg/ml and 4 μg/ml. Based on these particles, we found they have less cytotoxic and small affection with cell morphology changes. Then we test the cell migration ability on wound healing assay by using DQ probe. Due to the toxicity of the lipofectamine-2000 with the cells during the transfection process, due to the property of MUTAB coated GNP, we use GNP as a vector to reduce the toxicity when transfection[5]. Meanwhile, GNP has a greater surface ratio. Thus, it brings a higher amount of Dll4 sequence into the cells, and we can get an apparent signal intensity.

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II. Methods and Materials

A. Cell Culture and Reagents

Double-Stranded locked nucleic acid (dsLNA) probes, and synthetic targets for calibration were synthesized by Integrated DNA Technologies (San Diego, CA). 10 nm, 20 nm, 50 nm, 100 nm gold nanoparticles were purchased from Sigma (Saint Louis, MO). (11-Mercaptoundecyl)- N, N, N-trimethylammonium bromide (MUTAB) coated 10nm, 20 nm gold nanoparticles were purchased form Nanopartz (No. C11-10-TMU-DIH-50-1 and No.C11-20-TMU-DIH-20-1, Loveland, CO). Other chemicals and reagents were also purchased from Sigma (San Diego, CA).

Human breast cancer cells (MDA-MB-231) were obtained from ATCC (Manassas, VA). Roswell Park Memorial Institute (RPMI) 1640 medium (Thermo Fisher, Waltham, MA) was used to maintain the cells and supplemented with 10% fetal bovine serum, 5% penicillin/streptomycin. Cells were plated either in the 6-well plate or 96-well plate under the 37 °C with 95% humidity and 5% CO_2 incubation environment.

B. Gold Nanoparticles Uptake

Seed the cells with the number of 3.2×10^4 in 96-well plate, then add 10 nm, 20 nm, 50 nm, 100nm gold nanoparticles (GNP) with the concentration of 2 μg/mL, 4 μg/mL, 10 μg/mL, 20 μg/mL. Also, there is a control group without GNP was also prepared.

C. Cell Morphology and Cell Viability Detect

To prepare the PI/Hoechst dyes, 3 mM of Propidium Iodide solution was diluted into medium, and 0.0% Hoechst was added. The cells were stained at 72 hours, 120 hours, and 168 hours with 100 μL PI/Hoechst dyes in each well, respectively. After staining for 30 minutes, the images were taken under the fluorescence microscope.

After uptaking the GNP, the cells were culture for 72 hours, then checked under the microscope. The cell viability was determined by Cell Counting Kit-8 (CCK-8). A microscope reader (BioTek, Winooski, VT) read the results with 450 nm wavelength.

D. LNA Probe Complex Synthesis

The dsLNA (donor) and Dll4 quencher were prepared at 100 nM in 1× Tris–EDTA buffer. Mix 20 μL donor with 40 μL quencher, and heated up to 95 °C for 5 minutes, then cool down to 70 °C for 90 minutes. The Donor-Quencher (DQ) dsLNA probe complexes were then cooled down to room temperature over 2 hours before they were ready for the experiment.

E. GNP-LNA Probe complex Synthesis

The gold nanoparticles (GNP) were 10 nm and 20 nm in diameter and were modified with (11-Mercaptoundecyl)-N, N, N-trimethylammonium bromide (MUTAB). The dsLNA was prepared at 100 nM in 1× Tris–EDTA buffer. 20 μL donor heated to 95 °C for 5 minutes, then cool down to 70 °C for 1 hour. Then add 40 μL 1.5mg/mL GNP and warmed at 70 °C for 1 hour. After the gold nanoparticles – dsLNA probe complex (GNP-LNA probe) cooled down to room temperature, they were ready for the experiment, and do not store in the fridge, or it will inactivate.

F. Probes Uptake

Seeding the cells with a concentration of 0.96×10^5 cells/mL in the 6-well plate, after overnight as the cells attached, add 10 nm and 20 nm MUTAB coated GNP (0 ug/ml, 2 μg/ml and 4 μg/ml) to the cells. After 12 hours, do the scratches on each group of the cells then wash the cells twice by 1× PBS. Add the DQ probe into the cells, then check the fluorescence signals and wound size after 16 hours and 20 hours and 24 hours.

Also, we take another 6-well plate with confluence cells, scratch the cells with 1mL pipette, wash the cells twice by 1X PBS. Then we add 4 μL 10 nm and 20 nm GNP-LNA probe in 100 μL Opti-MEM incubate for 10 minutes, mix with 1.9 mL fresh medium to replace the washing PBS, culture the cells for 12 hours.

G. Image and Statistic Analysis

Images of MDA-MB-231 were captured using an inverted fluorescence microscope (Bio-Rad, Hercules, CA). Data collection and wound migration measurements were supported by using ImageJ software.

III. Results

A. Gold nanoparticles will affect the morphology and viability of breast cancer cells

We first characterized and compared the UV-vis-NIR absorption spectrum of GNP with different sizes, as shown in Figure 1. One wavelength peak was observed from the absorption spectrum of GNP at about 525 nm for GNP with a diameter of 10 nm, 20 nm, and 50 nm. The absorbance peak for 100 nm GNP was observed at 560 nm, indicating the absorbance peak of GNP is size-dependent.

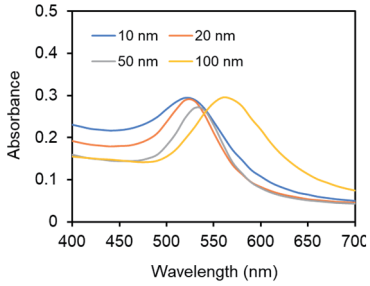

Figure 1. UV-vis-NIR absorption spectrum of gold nanoparticles with the size of 10 nm, 20 nm, 50 nm, and

The viability of MDA-MB-231 cells with different treatments of GNP was measured, with higher GNP concentration, the cell shows lower viability. At 20 μg/mL, the number of cells surviving in the same field of view significantly reduced. It was shown that all GNP with different sizes showed high cytotoxicity to the cells with about 50% cell viability when the Au concentration is as high as 20 μg/mL. The GNP with a concentration of 2 μg/mL, 4 μg/mL, and 10 μg/mL showed low cytotoxicity with about

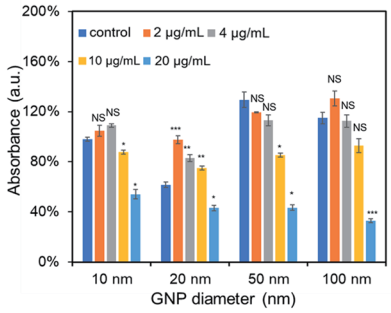

Figure 2. Cell viability analysis using cck-8 assay after intracellular uptake of GNP with different sizes (10 nm, 20 nm, 50 nm, and 100 nm) at different concentration. MDA-MB-231 cells were incubated for 72 hours. Data are expressed as mean ± s.e.m.

90% cell viability. Another interesting phenomenon was observed that with the same amount of gold, GNP with a diameter of 20 nm showed increased cell viability compared to control, indicating the GNP diameter may be one of the factors influencing cell proliferation.

As shown in Figure 3, for all four different sized GNP, increasing GNP concentration to 20 μg/mL significantly decreased the cell number, indicating the cell cytotoxicity. The intracellular uptake of GNP at the level lower than 10 μg/mL did not show a significant change. Next, the effects of intracellular uptake of GNP with different sizes on MDA-MB-231 cell morphology were investigated by evaluating the cell area, cell perimeter (data not shown), aspect ratio after GNP incubation. As shown in Figure 5, for the GNP with the sizes of 20 nm, 50 nm, and 100 nm, the cell area did not show a significant difference after seven days of incubation even with a high concentration of 20 μg/mL. Interestingly, for 10 nm GNP, the cell area was increased significantly. However, the aspect ratio of the GNP did not show significant change for 10 nm GNP. These results indicate that the size of the cell gets larger without shape change. For the 50 nm and 100 nm GNPs, the cell area did not change. However, the cell aspect ratio was decreased with increased GNP concentration. All these results indicate the complexity of endocytic uptake of GNPs in MDA-MB-231 cells. Thus, the 10 nm and 20 nm GNP is the best option for the detect Dll4 expression on GNP intracellular affection.

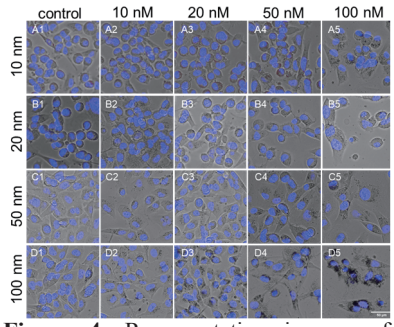

Figure 4. Representative images of MDA-MB-231 cells after intracellular uptake of GNP with the size of 10 nm (A), 20 nm (B), 50 nm (C) and 100 nm (D) at different concentration for 72 hours. Blue color indicate nucleus. Scale bar: 50 μm.

B. Gold nanoparticles can inhibit wound healing

Comparing the cells in the scratch assay and the normal cells without scratches, it shows that when the cells begin to proliferate, they will migrate following with the leader cells to generate an expansion effect. When the cells tend to be saturated, the expression of Dll4 will decrease. It can also be shown the cells at both ends of the scratches that these cells have higher Dll4 expression, proving their ability to lead the migration of other cells.

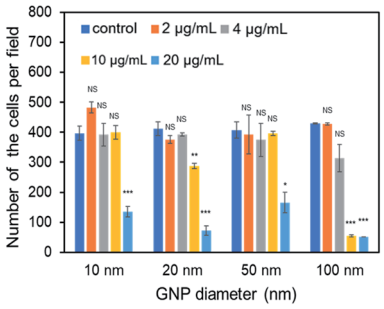

Figure 3. Comparison of the number of cells per field after GNP uptake for 7 days. MDA-MB-231 were incubated with GNP of different sizes and different concentrations. Data are expressed as mean ± s.e.m.

We can verify the effect of GNP on cell migration and wound healing through the wound healing assay. We can see that, compared with the control group, cell migration rate, and cell migration acceleration rate are inhibited and gradually decrease with time. Among them, under the higher concentration of 10nm GNP, the cell migration rate was slightly higher than other sizes and concentrations of GNP. At the same time, the expression of Dll4 can show changes in β-actin activity.

C. 20 nm gold nanoparticle is more suitable for the GNP-LNA probe

After scratching the MDA-MB-231, the cells uptake the GNP-LNA probe in 12 hours. The signal shows in the cells on the sides of the wound. Compared with 10 nm and 20 nm GNP-LNA probe, as the fluorescence signal indicates, the probe uptake rate was similar. The GNP-LNA probe intensity demonstrates that the 20 nm GNP-LNA probe has a higher dll4 signal, but does not show an apparent difference between 12 hours and 24 hours.

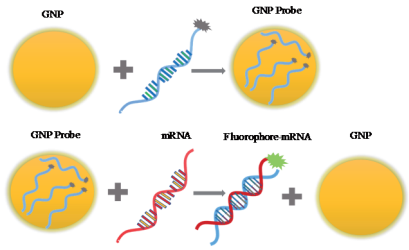

Figure 5. Schematic diagram of GNP-LNA probe synthesis.

Interestingly, the GNP probe does have Dll4 signals, after probe uptake 12 hours, we can observe the fluorescence signals from the cells. Comparing with 10 nm and 20 nm probe, it shows that a 10 nm probe has a higher uptake rate than the 20 nm probe. However, still, the average uptake rate of two sizes of probes is around 20%, which lower than the DQ probe we use in previous experiments.

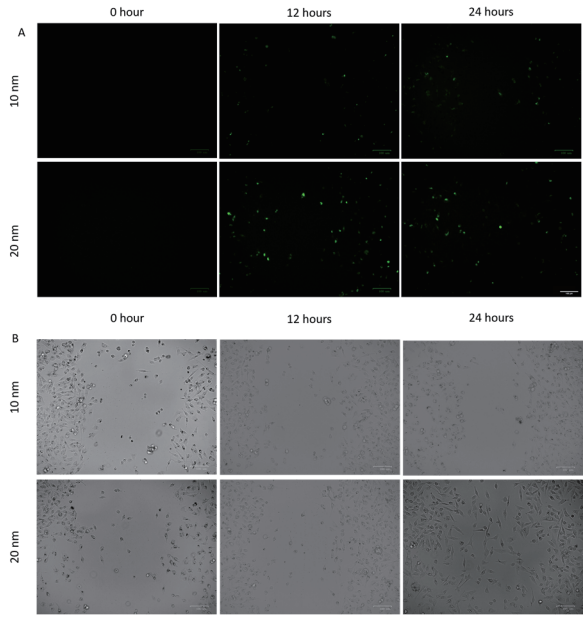

Figure 6. GNP-LNA probe uptake in 0, 12 and 24 hours, fluorescence images were show in A, and the bright field images were show in B. Scale bar: 100 μm.

Due to a higher specific surface area, 20 nm GNP has more bonding size available to form a more effective GNP-LNA complex while the uptake rates were similar in a different size, as shown in Figure 7. The quantified dll4 intensity supports this result.

IV. Conclusions and Discussions

The intrinsic uptake of GNPs by MDA-MB-231 cells is complicated. After MDA-MB-231 uptake different sizes and concentrations of GNP, the higher the density of GNP, the lower the cell viability, and the diameter of GNP may be one of the factors affecting cell proliferation, indicating that it has cytotoxicity to cells. When evaluating the effect of intracellular uptake of GNPs of different sizes on the morphology of MDA-MB-231 cells after GNP incubation, there was no significant difference in the cell area. Our results show that the size of the cells becomes larger without changing the shape, and the aspect ratio of the cells decreases with increasing GNP concentration.

GNP inhibits wound healing and reduces cell migration rate. Since the activity of actin is closely related to cell migration, a decrease in the rate when the wound heals can indicate that the activity of actin is correspondingly reduced. It can also be verified by Dll4 expression that when a concentration of GNP is endocytosed, it will affect the activity of actin and have a specific inhibitory effect on tumor cell migration and tissue formation.

The Dll4 signals can easily be observed after the cells treated with GNP-LNA probes demonstrate that Dll4 is a biomarker in breast cancer cells. 20 nm gold nanoparticles are more applied with dsLNA transfection compared with 10 nm, which displayed specific surface area will also affect signal intensity. In the selection of drugs for breast cancer cells, biomechanical research, and clinical treatment options, gold nanoparticles corresponding to physical and chemical effects can be selected. This work can be used as a pilot study for the gold nanoparticle-based vector or design of an LNA biomarker complex.

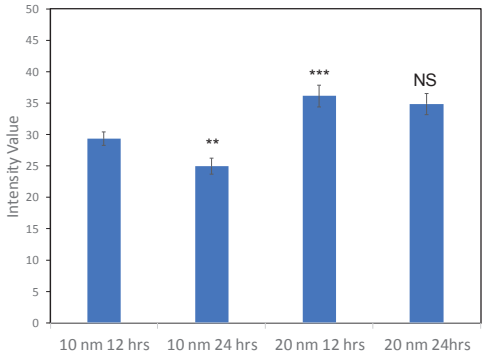

Figure 7. Dll4 signal intensity quantification in different groups start from 12 hours GNP-LNA probe uptake. Data are expressed as mean ± s.e.m.

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