論文の内容の要旨

Experimental Evaluation of Gold Nanoparticles for Future Application in Medical Imaging

(医用画像応用に向けた金ナノ粒子の実験的評価)

トロノ　ジェイド　ドゥンガオ
(ドゥンガオ　ジェイド　ルーバス)

１．Introduction

The introduction and development of gold nanoparticles has led to studies dealing with its potential for medical imaging and diagnostic applications. Gold is considered a high atomic number Z material (Z=79) and absorbs x-rays as shown in Figure 1. In theory, the dose delivered to a tumour can be enhanced by loading high Z-material into the tumour. This results in greater photoelectric absorption within tumour than in surrounding tissues.

Most anti-cancer agents do not greatly differentiate between cancerous and normal cells, leading to systemic toxicity and adverse effects [1]. This greatly limits the maximum allowable dose of the drug. In addition, rapid elimination and widespread distribution into targeted organs and tissues requires the administration of the drug in large quantities, which is not economical and often results in undesirable toxicity.

Comparatively, gold nanoparticles significant unique features as follows: low toxicity, low osmolality even at high concentrations, low viscosity (easy to inject even into small vessels) enhances radiotherapy dose, which makes it a good candidate either as a contrast agent or as radio enhancer. Gold nanoparticles have been actively investigated in a wide variety of biomedical applications for several reasons: it has the advantage of small size which is highly tunable (1-100nm) [2], capable of evading the immune system [2], easy to characterize by UV-vis spectrophometry, ICP-AES or ICP-MS, and TEM[3]. The size effect provides a continuous range of nanoparticles for simultaneous detection by using multiple cancer biomarkers. The size tunability also provides large surface area which affects the density and types of functional groups that can be linked for multiple diagnostic and therapeutic applications. It has also been shown that the size range 10-100nm accumulate preferentially at tumor sites through an effect called enhanced permeability and retention (EPR) effect.
Several studies have been conducted evaluating factors that will potentially determine the uptake and uptake mechanism of nanoparticles and/or modified nanoparticles by the HeLa cell line [3-5]. Chithrani et. al [3] investigated the intracellular uptake of different sized and shaped colloidal gold nanoparticles. It was determined that kinetics and saturation concentrations are highly dependent upon the physical dimensions of the nanoparticles. The same group determined that transferrin-coated Au NP entered the cells via clathrin-mediated endocytosis pathway and exocytosed out of the cells in a linear relationship to size. We have also reported gold nanoparticle uptake of normal and cancer cell lines [6].

We propose that gold nanoparticles be developed and designed to target pancreas cancer in order to improve the current systems used in managing this disease. Shirato’s group demonstrated that 2mm gold can be used as internal fiducial marker of tumors in their real time tumor tracking radiation therapy [7]. One reports that although implantation of the fiducial was successful, it is not without technical issues. One is the size of the fiducial itself, requiring it to be transported via large needle, which itself poses danger to blood vessel. The size of the fiducial also makes it difficult to penetrate hard tissue, such as pancreatic tumor [8].
Our particular interest on using gold lies on its size tunability and its potential use either as radiosensitizer/dose enhancer in the treatment of cancer or as contrast agent for diagnostic

imaging of cancer using X-ray DDS(Drug Delivery System)[9]. Our group is continuously developing pinpoint keV/MeV X-ray source for X-ray DDS [10-12]. The X-ray DDS (Figure 2) uses advanced nano-scaled polymers which contain and deliver drug or contrast agent to cancers. This new modality combining physical energy (via pinpoint X-ray source) and drug (via DDS) in one compact system is very important for inspection and therapy of cancer.

The in-vitro evaluation on the combination of gold nanoparticles and physical energies via X-ray DDS is necessary for the development of a safe and effective treatment and/or imaging modality. To develop successful cell-based therapies and to monitor cell trafficking in vivo, it is important to assess the fate and distribution of cells non-invasively. Biocompatibility, toxicity and the ability to penetrate the cells are three critical factors that will determine the utility of nanoparticles in clinical application [5]

For improved uptake of gold nanoparticles, the preparations need to be optimized or modified. Fundamental studies on uptake of nanoparticles, investigating factors such as size, incubation time and concentration play an important role in the design of safe nanoparticles for diagnostic and therapeutic applications. For efficient internalization, surface modification is suggested.

Here, we present result on the uptake of human pancreas cancer cell line, taking into account factors such as gold nanoparticle size, incubation time and concentration. We aim to compare how the uptake of gold nanoparticles is affected by these factors. Eventually, by combining these parameters, uptake of gold nanoparticles by cancer cells can be confirmed and optimized. A preliminary result of in-vivo experiments is also reported.

II. Materials and Method

Reagents and chemicals

Gold nanoparticles (5, 10, 20, 30, 40, 50nm) were supplied from British Biocell International. Commercial gold chloride concentration was 0.01% and the mean diameter variation is less than 10%.

2.1 Characterization of gold nanoparticles

Prior to use in cell experiments, gold nanoparticles used were initially characterized using AAS (atomic absorption spectroscopy), UV-vis spectrometry and TEM.
The gold content of samples was determined using AAnalyst 800 atomic absorption spectrometer (Perkin-Elmer) with THGA graphite furnace, gold hollow-cathode lamp and AS-800 autosampler. All analyses were performed at wavelength of 242.8nm using 0.7nm slit width. New graphite tubes were conditioned by heating and the machine is recalibrated prior to use. For the evaluation of peak profiles, the calculation of peak area and permanent storage peak profiles, AAWinlabTM software was used. Argon was used as the inert gas.

2.2 Cell culture

We used human cancer cells for these experiments. PK-1, PK-45, and Panc-1 were purchased from Riken cell bank (RIKEN, Tsukuba). PK-1, PK-45, Panc-1 are all human pancreas cancer cells. Initial experiments were also conducted to check for aggregation of 20nm gold nanoparticles using different media with serum, checking for aggregation at several intervals from 1-48 hours.

The cell medium for all cell lines used is RPMI-1640 (GIBCO, 11875, RPMI-1640), supplemented with 10% serum (HYCLONE, SH30396.03, Fetal Bovine Serum). Cells were

continuously subcultured in T-25 flask (BD Falcon, 353108) and prior to achieving confluency, cells were seeded into 60mm dishes (BD Falcon, 353002, T-25) for gold treatment. While the cells were on the exponential phase, gold colloid was diluted with media (2.5mL) to achieve the desired concentration. Dishes were stored in a humidified atmosphere at 37°C, 5% CO_2 in air, except when specified. Treatment duration time depends on the condition to be tested.
After gold treatment, cells were washed with 1mL Dulbecco Phosphate Buffered Saline (SIGMA, D8537, DPBS) thrice. Cells were collected from the dish by trysinization, using trypsin-EDTA solution (0.05% trypsin, 0.02% EDTA, T3924, SIGMA), counted (Nucleocounter) and transferred to 1.5mL eppendorf tubes. The cell solution was homogenized using sonicator (Tomy HandSonic).

2.3 Cytotoxicity analysis

Colony assay and MTT assay was used to measure the viability of cells at the parameters considered. In colony assay, cells were incubated with gold nanoparticles at different conditions, washed and subcultured into 3 dishes. After 10days, colonies are dyed and counted. In MTT assay, Roche Cell Proliferation Kit I (Cat. No. 11 465 007 001) was used. This is quantified by using a scanning multiwall spectrometer multiplate reader (Thermo Appliscan and ScanIt software). The absorbance value is correlated to the cell number, and to viability of cells.

2.4 Determination of uptake by AAS

The concentration of gold nanoparticles used was verified using AAS. In AAS analysis, five-point calibration curve using 1000ppm Au standard diluted to maximum of 100ppb was used. The gold content of samples was determined by AAS as previously described [13] with minor modification. Samples were diluted 1 to 50 fold by mixing 0.5% HCl. Using an autosampler, 20uL of diluted sample and 5uL of matrix modifier (0.1% palladium and 0.06% magnesium nitrate in 1.5% HNO3) were injected in the graphite furnace to measurement the gold content of the sample. Two replicates were taken for each sample analyzed.

2.5 Biodistribution studies and uCT experiment

For the in-vivo experiment, commercial 20nm gold nanoparticles will be concentrated and injected to CDF1 mice following the animal experiment protocol. Prior to imaging, the mouse will be sedated. Images at different time intervals will be compared and checked for contrast. CT images will be taken at 10min, 30min, 1hr, 3hrs, 6 hrs and 24 hours after injection of gold nanoparticles. After imaging experiment, organs will be harvested and gold content will be analyzed by AAS.

III. Results and Discussion

The effect of gold nanoparticles size, incubation time and concentration on the uptake of pancreas cancer cell lines used were investigated. For diagnostic and medical purposes, uptake data are presented in gold amount (nmol) per cell, and not as number of gold particles per cell. The concentration of gold detected in the sample using AAS is simply divided by the average number of cells present in the sample.

3.1 Characterization of gold nanoparticles

TEM of gold nanoparticles of different sizes are also shown in Figure 3: 10nm, 20nm, 30nm, 40nm, 50nm, 100nm.

3.2 Determination of uptake by AAS

3.2.1 Size

Cell samples were treated with 11.8μM gold nanoparticles colloid of various sizes mixed with media for 24 hours. In Figure 4, a plot of the gold content of cells versus size shows that the uptake is highly dependent on size. Among the gold nanoparticles used, 5 and 10nm gold nanoparticles have significantly lower uptake (at least 5x lower) as compared to 20nm. This trend is observed on all cells used in the experiment, with 20nm gold nanoparticles uptake still higher compared to 30, 40 and 50nm. Based on these results, we will be reporting uptake dependence using 20nm gold nanoparticles.

3.2.2. Incubation time

Cells were treated with 47.2 μM 20nm gold nanoparticles from 1 to 48 hours. Figure 5 shows the uptake of gold nanoparticles increases gradually with time. Also, plateau in uptake was observed indicating that cells can still uptake gold nanoparticles beyond 48 hours. Previous reported results indicated that HeLa cell approaches plateau within 4-7 hours of incubation[3].

3.2.3 Concentration

The effect of concentration of gold nanoparticles uptake of the cell was also investigated Figure 6. Cells were treated with 11.8, 23.6, 47.2 and 94.4 μM of 20nm gold nanoparticles for 24 hours. From [4], the uptake of gold nanoparticles increased as the concentration was increased.

In previous experiments using another cell line, it was found that uptake is also dictated by the amount of gold nanoparticles present in the solution. For lower concentrations, increasing the incubation time will not increase the gold nanoparticles uptake of the cell. If there are fewer gold nanoparticles in the sample, we expect that the gold nanoparticles have a small probability of getting near the receptors; hence the uptake on lower concentrations is low. However, for the case when there are abundant gold nanoparticles present in the solution, receptors will be able to take-in nanoparticles faster and easier. As such, approaching the plateau of uptake could mean two things, one is that there are few nanoparticles in the solution (receptors have nothing to reach), second is that the cells are already saturated with gold nanoparticles that some are released back into the solution.

The mechanism of uptake can be explained further by serum adsorbed on the nanoparticles surface. Gold nanoparticles itself is hydrophobic and the protein serum in the media is adsorbed on the surface of the gold nanoparticles. The adsorption of serum proteins on the surface is very important in the internalization of gold nanoparticles by cells. It was hypothesized in [3] that the uptake mechanism is via receptor-mediated endocytosis (RME). RME is an endocytotic mechanism in which specific molecules enter the cell. Cells have a limited number of receptors on the membrane, and through these receptors the gold nanoparticles enter the cell. The protein serum-coated gold nanoparticles aids in RME mechanism of the cell.

3.3 Biodistribution studies and uCT experiment

CT images will be taken at 10min, 30min, 1hr, 3hrs, 6 hrs and 24 hours after injection of gold nanoparticles. Shown in Figure 8 is the images produced 2 min and 6 hours after

injection of gold nanoparticle.

IV Summary

We aim to develop gold nanoparticles DDS for non-invasive imaging for future dynamic tracking radiation therapy and X-ray DDS. To achieve it, we analyzed the uptake of gold nanoparticles into pancreas cancer cells. Gold nanoparticles intracellular uptake was investigated using pancreas cancer human cells PK-1, PK-45H and Panc-1. Cellular uptake using different gold nanoparticles sizes was analyzed using Atomic Absorption Spectrometry (AAS). It was found that 20nm gold nanoparticles uptake is higher compared to other gold nanoparticles sizes. The dependence on incubation time and concentration was also investigated. Accumulation of gold nanoparticles can be increased further thru longer incubation and higher concentration. From our present results, we were able determine optimum conditions for the uptake of gold nanoparticles by pancreas cancer cells.

We will extend this further by conducting the experiments in-vivo using modified gold nanoparticles. The findings of this study will help in the design and optimization of the nanoparticles uptake for therapeutic and diagnostic applications of X-ray Drug Delivery System.

Further investigations will be conducted using Compton monochromatic x-ray source at AIST and The University of Tokyo, combining gold nanoparticles and polymers in order to develop X-ray DDS for verification of safe and effective use of gold nanoparticle-based agents in the near future.

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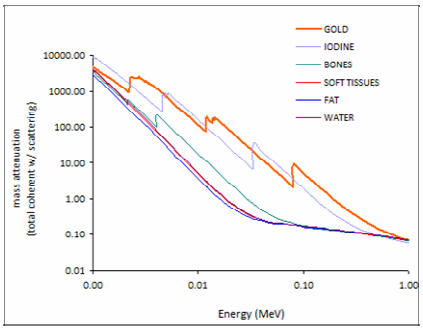

Figure 1. Attenuation of gold in comparison with iodine, bones, soft tissues, fat and water.

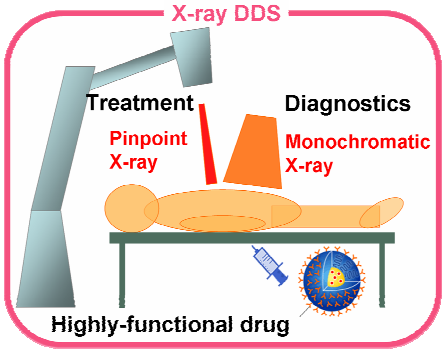

Figure 2. Xray Drug Delivery System

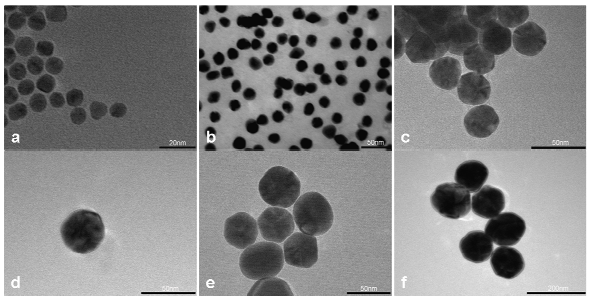

Figure 3. TEM of gold nanoparticles a) 10nm, b) 20nm, c) 30nm, d) 40nm, e) 50nm, f)100nm

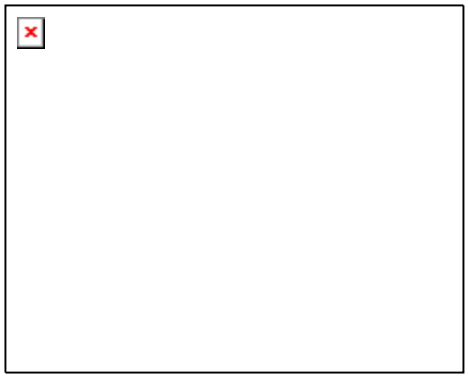

Figure 4. AAS uptake for different cell lines. Maximum uptake was observed using 20nm gold nanoparticles.

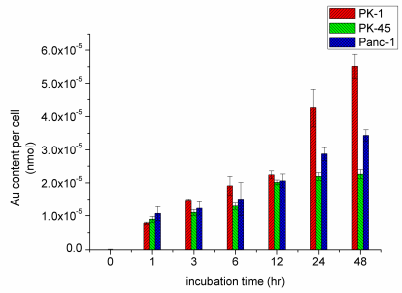

Figure 5. AAS dependence on incubation time. Continuous increase in uptake. Plateau not reached for the incubation times considered.

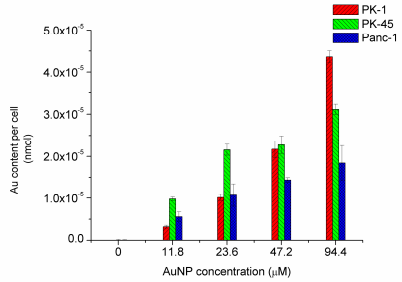

Figure 6. Uptake dependence on gold nanoparticles concentration.

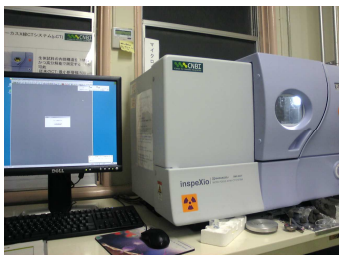

Figure 7 inspeXio SMX-90CT microCT

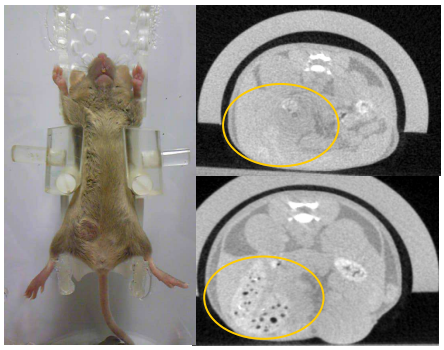

Figure 8. Preliminary result of uCT experiment. a) mouse immobilized by jig. b and c) transverse plane of mouse showing liver after 2min and 6 hours respectively.