Gold nanoparticles (GNPs) and modified GNPs having two kinds of functional molecules, cysteamine (AET) and thioglucose (Glu), are synthesized. Cell uptake and radiation cytotoxicity enhancement in a breast-cancer cell line (MCF-7) versus a nonmalignant breast-cell line (MCF-10A) are studied. Transmission electron microscopy (TEM) results show that cancer cells take up functional Glu-GNPs significantly more than naked GNPs. The TEM results also indicate that AET-capped GNPs are mostly bound to the MCF-7 cell membrane, while Glu-GNPs enter the cells and are distributed in the cytoplasm. After MCF-7 cell uptake of Glu-GNPs, or binding of AET-GNPs, the in vitro cytotoxicity effects are observed at 24, 48, and 72 hours. The results show that these functional GNPs have little or no toxicity to these cells. To validate the enhanced killing effect on cancer cells, various forms of radiation are applied such as 200 kVp X-rays and $\gamma$-rays, to the cells, both with and without functional GNPs. By comparison with irradiation alone, the results show that GNPs significantly enhance cancer killing.

1. Introduction

We are facing tremendous opportunities and challenges in combining emerging nanotechnology with cellular and molecular techniques to develop better cancer diagnosis and therapeutic designs. Therapy combined with metallic nanoparticles is a new way to treat cancer, in which gold nanoparticles (GNPs) are injected and bound to tumor sites. When an external X-ray source hits these nanoparticles, particles can subsequently generate radicals that damage cancer cells and induce cell apoptosis. Results have shown improvement in the treatment effects on cancer cells with little or no increase in harm to normal surrounding tissues in mice models.\textsuperscript{[1–3]} In a translation study, GNPs were used to enhance cancer apoptosis by radiotherapy.\textsuperscript{[4,5]} GNPs with a 1.9-nm diameter were intravenously injected into mice bearing subcutaneous EMT-6 mammary carcinomas. Afterwards, 250 kVp X-ray therapy was applied to the mice. The results showed a significant increase in survival rate in the group with the treatment by both GNPs and X-rays compared to the control and the group that received irradiation only.\textsuperscript{[5]} The utilization of focused X-rays precisely kills tumors located in various body organs.\textsuperscript{[6]} Although nonfunctionalized gold nanoparticles have shown a reasonable level of appetency to cancer tissue, novel gold nanoparticles with stronger affinity for and specificity to cancer cells are needed to achieve a more effective diagnosis and treatment.

In our previous work,\textsuperscript{[7]} we developed a series of functional GNPs that were synthesized with various active biological reagents to modify their surface properties. In this paper, we
report that localized uptake and binding of GNPs to cancer cells can be achieved by modifying the surface properties of GNPs. We also evaluate how the changes in GNP surface properties can affect their biological activities on breast-cancer cells. Glucose-capped GNPs are designed to take advantage of an increased cancer-cell requirement for glucose in order to target the cell cytoplasm. Cysteamine (AET)-capped GNPs were synthesized by hybridizing GNPs with AET, which made the GNPs strongly positive. This type of nanoparticle can be selectively bound onto the cell’s surface. It is expected that modifying the surface properties of both GNPs can significantly enhance cell uptake and radiation cytotoxicity.

2. Results

2.1. Characteristics of GNPs

Figure 1 shows the schematic diagram of both AET-GNPs and Glu-GNPs. Figure 2 shows transmission electron microscopy (TEM) images of the GNPs. The diameters of the GNPs were measured with TEM and the average diameter of GNPs was 10.8 nm. The same size GNPs were used for all experiments in this study. To identify the binding of GNPs with either AET or thioglucose, the nanoparticles were characterized using X-ray photoelectron spectroscopy (XPS). The average numbers of biomolecules (around $2 \times 10^4$ for AET and $2.5 \times 10^4$ for thioglucose) on one nanoparticle were calculated by measuring the gold-to-sulfur atom ratio acquired with XPS (Figure 3). The results are listed in Table 1.

2.2. Distribution of GNPs in Targeted Cells

The typical distribution of GNPs in MCF-7 cells can be observed by TEM. Figure 4A indicates the appearance of normal cells. Figure 4C is the micrograph of the cells treated with 15 nm Glu-GNPs. The Figure shows that most of GNPs were distributed in the cytoplasm. The appearance of MCF-7 cells treated with 15 nm AET-GNPs is shown in Figure 4B, in which we can see that most GNPs were bound to the cell membrane in this case.

2.3. GNPs Bound to or Internalized by Target Cells

The number of GNPs that bound to or were taken up by MCF-7 cells in cell lysate was quantified with inductively coupled plasma mass spectrometry (ICP-MS). After the cells were exposed to 15 nm of naked GNPs, AET-GNPs, or Glu-GNPs for two hours, the average number of GNPs associated with each MCF-7 cell was $7.34 \times 10^4$ for naked GNPs, $2.96 \times 10^4$ for Glu-GNPs, and $1.187 \times 10^5$ for AET-GNPs per cell (Figure 5). Exposure to AET-GNPs resulted in a factor of a three- to fourfold increase of GNPs compared to Glu-GNPs in each target cell. When 15 nm Glu-GNPs and 3.85 nm AET-GNPs were used to treat the cells, the targeted cells have the same level of nanoparticles for each type of GNPs in the targeted cells, or about $2.96 \times 10^4$ GNPs per cell. We later use these samples in the experiments of cytotoxicity assay and irradiation studies.

2.4. Cytotoxicity of GNPs

The cytotoxicity of either AET-GNPs or Glu-GNPs on MCF-7 cells that were treated for 24, 48, or 72 hours, respectively, was measured with the MTT assay. The data was analyzed with a t-test and no significant difference was noticed between the control and the AET-GNP-treated cells or the Glu-GNP-treated cells ($p < 0.05$), as shown in Figure 6. By using the percentage to measure the viability, no significant changes in the cytotoxicity were seen as incubation time increased. Therefore, we can conclude that neither the AET-GNPs nor the Glu-GNPs induced remarkable cytotoxicity in MCF-7 cells.

2.5. Irradiation

2.5.1. Enhancement of Irradiation by GNPs

The target cells were treated with GNPs for two hours. Both the treated and untreated cells were then irradiated with 200 kVp X-rays with a dose of 10 Gy. We detected the induced cytotoxicity with the MTT assay 48 hours after the irradiation. The responses of the MCF-7 cell line with and without X-ray treatment are shown in Figure 7. After irradiation, 200 kVp stimulated cancer-cell growth and induced a cell viability of 114.8% after 48 hours (Figure 7A). Glu-GNPs (15 nm) and AET-GNPs (3.85 nm) induced about a 63.5% and 31.7% increase in radiation cytotoxicity, respectively, when compared to irradiation alone.

In clonogenic survival assay, 200 kVp X-rays induced a significant decrease in cell survival (Figure 7B) after five days. However, 43.2% of cancer cells still survived after five days and 13.8% of cells survived after fourteen days. For the cells exposed to Glu-GNPs, the irradiation of 200 kVp X-rays resulted in complete cancer-cell death after five days and this trend continued.
2.5.2. Comparison of Radiation Sensitivity in Cancer or Nonmalignant Cells

We selected MCF-10A as a nonmalignant breast-cancer cell line to provide evidence to prove the efficiency of the functional GNPs in cancer killing. After exposing to Glu-GNPs, both MCF-7 and MCF-10A cells uptook the same level of GNPs because both types of cell line have the same growth rates and need the same amount of glucose for metabolism (results not shown). Figure 8A shows that a 200 kVp X-ray induced a 20% cell viability decrease in the MCF-10A cells but not in the MCF-7 cells. After incubation with Glu-GNPs for two hours, the cell viability of MCF-7 cells decreased to 40% after irradiation. However, no significant changes in radiation sensitivity were shown in MCF-10A cells that were treated either with or without Glu-GNPs \( p < 0.05 \). These results indicate that Glu-GNPs only enhance the radiation sensitivity in cancer cells but not in nonmalignant breast-cancer cells. These results also show our nanoparticles can be used for targeted cancer treatment.

2.5.3. Radiation Cytotoxicity Resulting from Various Types of Irradiation

The capability of GNPs to enhance the cytotoxicity induced in the MCF-7 breast-cancer line by various types of irradiation (X-ray and \( \gamma \)-ray) was evaluated. Compared to 200 kVp X-rays (30% cell death for AET-GNPs and 60% cell death for Glu-GNPs), \(^{137}\text{Cs} \ \gamma\)-rays and \(^{60}\text{Co} \ \gamma\)-rays have smaller enhancement on cell killing (12.7% cell death for \(^{137}\text{Cs} \ \gamma\)-rays and 13.1% for \(^{60}\text{Co} \ \gamma\)-rays; see Figure 8B).

3. Discussion

One major concern in applying nanoparticles for cancer diagnosis and therapy is how to deliver these nanoparticles to targeted cancer tissue. Various approaches for targeted delivery have been reported.\(^{10,11}\) In this study, we report that the localized uptake and binding of GNPs at selected locations in cancer cells can be achieved by modifying the surface properties of GNPs.

We developed two types of GNPs, Glu-GNPs and AET-GNPs, to enhance localized uptake and binding to the cancer cell. In AET-GNPs, the cysteamine molecules were bound to the colloidal gold surface via thiol groups, resulting in a net positive charge to the particles due to amino groups. Strong attractive electrostatic interaction between the positively charged gold nanoparticles and negatively charged biomolecules enhanced...
the binding of nanoparticles with cells. When AET-GNPs were added into cell culture, they quickly bound to negatively charged cell membranes. The TEM images presented in Figure 4B provide clear evidence to support the conclusion that AET-GNPs selectively bind on the cell membrane.

In Glu-GNPs, we exploited the role of glucose as a basic nutrient in cellular energy metabolism where it is taken up by glucose-metabolizing cells such as brain, kidney, and cancer cells. Glucose is a widely used targeting molecule, as exemplified by the widespread usage of (18F) fluorodeoxyglucose (FDG) in clinical oncology as a positron emission tomography (PET) tracer.[12] In this study, thioglucose was bound to GNPs. Glucose-metabolizing cells, such as MCF-7 breast-cancer cells, took up the glucose along with the GNP it was attached to. The Glu-GNPs were selectively distributed in the cytoplasm (Figure 4C).

By using various biomolecule reagents to modify the surface properties of GNPs, we achieved access of GNPs to desired locations at the subcellular level. The naked GNPs used in current biological research are neutral and passively bind to cells. However, the biomolecule-modified GNPs reported in this paper were shown to selectively target locations at the subcellular level. With this property, the GNPs can be brought to desired cellular locations that are biologically significant. For example, there are at least three benefits for cancer treatment: 1) active and specific binding will significantly increase the local concentration of GNPs (Figure 4), 2) GNPs in the cytoplasm kill cancer cells more efficiently than those on

Figure 4. TEM images of MCF-7 cell uptakes for GNPs; the targeted cells were treated with or without GNPs for two hours: A) control cell, B) AET gold nanoparticles, and C) glucose gold nanoparticles. Bottom-left image shows an enlarged part of (C).

Figure 5. Cell uptakes of GNPs bound with either AET or thioglucose.

Figure 6. Toxicity test of AET-GNP, Glu-GNP, and control in MCF-7 cells by MTT assay.
the cell membrane and are a better choice for X-ray radiotherapy (see Figure 7A), and 3) the results in Figure 7B indicate that Glu-GNPs enhance the radiation sensitivity in cancer cells but not in nonmalignant breast cells, though both cell lines uptook the same level of Glu-GNPs. The results will be very useful in our future clinical trials.

Radiotherapy using X-rays, γ-rays, or α-particles has been used for more than half a century.[13–15] However, toxic effects on surrounding tissue and side effects on various organs limit the amount of radiation used for cancer radiotherapy. Metallic nanoparticles, such as gold nanoparticles, have been reported to enhance the toxicity of radiation on cancer cells and decrease side effects on surrounding normal tissue. In an animal test, GNPs significantly increased the survival rate in mice bearing subcutaneous EMT-6 mammary carcinomas and receiving 250 kVp X-ray irradiation.[5] The data obtained from this study clearly showed that the efficiency of 200 kVp X-ray was significantly increased when cells uptook Glu-GNPs or AET-GNPs. With the same dosage of 200 kVp X-rays, cytotoxicity increased by about 63.5% for Glu-GNPs and 31.7% for AET-GNPs compared to irradiation alone. This phenomenon indicates that the location of GNPs in the cells is an important factor in the increase of radiation cytotoxicity induced by GNPs. When this numerical difference was taken into account, the GNPs in the cytoplasm increased radiation cytotoxicity more than those on the cell membrane. The GNPs on the cell membrane are useful for other purposes, for example, we can use AET-coated fluorescent quantum dots as tracers to bind to cells to investigate the features of the cell membrane.

Both MCF-7 breast-cancer cells and MCF-10A normal cells uptook the same amount of GNPs and similar cytotoxicity to both cell lines is expected after irradiation. However, after irradiation, the viability of the cancer cells decreased significantly (about 40%) but no significant changes were observed in normal cells (Fig. 8A). This result indicates that the radiation-sensitization effects induced by GNPs in cancer cells is not only due to a simple physical interaction between GNPs and radiation but also involves a complicated cellular mechanism. Turner et al. reported that metal may arrest cells at the G2/M phase, the most radiosensitive phase of

Figure 7. A) The cytotoxicity on MCF-7 breast-cancer cells induced by 200 kVp X-ray irradiation with or without gold nanoparticles at 48 hours. B) Cell survival rate determined by clonogenic survival assay (at 5 days and 14 days).

Figure 8. A) Comparison of radiation sensitivity in cancers and nonmalignant cells. B) Comparison of the cytotoxicity induced in MCF-7 cell line by various types of irradiation with gold nanoparticles (48 hours after irradiation).
the cell cycle and thus enhance the sensitivity of cancer cells but not nontransformed cells to ionizing radiation. However, the mechanism explaining how the GNP s enhance the sensitivity of breast-cancer cells to radiation but not nonmalignant cells needs to be investigated further.

The results shown in our study (Figure 8B) indicate that GNP s had no significant effect on cytotoxicity induced by both Co γ-rays (average 1.25 MeV) and Cs γ-rays (662 keV) at 48 hours. On the other hand, GNPs increased the cytotoxicity of low-energy X-rays by 35–40%. The results suggest that the photoelectric effect might be a major mechanism in GNP-induced increase of cytotoxicity by the interaction between low-energy X-rays and high-Z materials generate free radicals. These radicals generate reactive oxygen species (ROS) that can damage proteins and genes, resulting in cytotoxicity. However, the molecular mechanism behind low-energy X-ray-induced cytotoxicity should be further investigated.

4. Conclusions

We have developed functional nanoparticles with modified surface properties to achieve targeted delivery at the subcellular level and have demonstrated selective binding with the cell membrane or localization to the cytoplasm. Our results showed that GNPs significantly increased the cytotoxicity of 200 kVp X-rays. There are at least three benefits to such an approach: 1) compared to naked GNPs, localized delivery produces a higher local concentration of GNPs in target locations; 2) GNPs can increase the cytotoxicity of radiation. Thus, lower doses of radiation can be used, avoiding the risk of side effects; 3) local damage to normal tissue surrounding the cancer is decreased. Our future work will focus on binding GNPs with tumor-specific biomarkers to achieve targeted delivery. The molecular mechanisms of enhancement of radiation cytotoxicity will be investigated and the functionalized GNPs will be tested in animal models. Targeted GNPs have the potential to be used as an agent for cancer therapy and, if appropriately labelled with a radiotracer, as an aid to diagnosis and disease management.

5. Experimental Section

Materials: All chemicals were obtained from Sigma–Aldrich (Milwaukee, WI). MTT cell proliferation assay kit was purchased from Invitrogen (Burlington, Ontario).

Synthesis of gold nanoparticles: The general synthesis method for making gold nanoparticles follows three steps: i) 2 mL of 25 mM HAuCl₄ solution was added into 25 mL of deionized water in an ice bath with moderate stirring, ii) 2 mL of 30 mM NaBH₄ was then added as a reductant to obtain GNPs without any capping agents. iii) To functionalize the GNPs, 4 mL of 25 mM thioglu cose or AET was added into the previous gold solution to obtain functional GNPs. Considering the GNPs in step (ii) are easy to aggregate, we added sodium citrate (TGS) to cap them as naked GNPs.

The purpose for using the same GNP solution was to make sure the resulting functionalized nanoparticles had identical sizes. Both AET-capped gold nanoparticles (AET-GNPs) and thioglucose-capped gold nanoparticles (Glu-GNPs) were dialysed for two days before cell uptake and irradiation experiments. The average sizes of three types of GNP s (naked GNPs, AET-GNPs, and Glu-GNPs) as measured by dynamic light scattering (DLS) were about 10.8 nm. The surface of AET-GNPs and Glu-GNPs were characterized by using XPS (Kratos Analytical).

Cell culture and culture conditions: Human breast-cancer adenocarcinoma line MCF-7 was used in all the experiments. MCF-7 cell line was purchased from American Type Culture Collection (Manassas, VA, USA). MCF-7 cells were maintained in Dulbecco’s modified Eagle medium (DMEM, Invitrogen) containing 10% fetal bovine serum (FBS) (Invitrogen), 100 UI penicillin G, and 100 µg mL⁻¹ streptomycin (Sigma). The cells were incubated at 37°C in a humidified 5% CO₂ atmosphere.

Cellular morphology with TEM: The cell cultures treated with and without GNPs for two hours were centrifuged and the supernatants were removed. The pellets with 2 phosphate-buffered saline (PBS) washes were fixed in 4% (v/v) formaldehyde in 0.1 M phosphate buffer (pH 7.2) for two hours at 4°C. After being washed in the same buffer, the cells were resuspended in 1% OsO₄ for one hour at room temperature. They were then washed twice by centrifugation and resuspension in distilled water. The final pellet was resuspended in a small volume of warm 2% (w/v) agarose, poured onto a glass slide, and allowed to cool. Once set, small pieces of gel containing the cells were cut out and dehydrated through a graded series of ethanol solutions. The pieces were then embedded in epoxy resin and thin sections were cut with an ultramicrotome, stained with uranyl acetate followed by lead citrate, and examined in a Philips EM301 electron microscope operating at 80 kV.

Determination of GNPs bound to or uptaken by target cells: A 5 mL MCF-7 cell suspension containing 5×10⁵ cells was added to 6-cm dishes and cultured overnight. When the cells reached 70% confluence, the target cells were exposed to 5 mL of fresh medium with 15 nm of GNPs (final concentration). After two hours of incubation, the medium with GNPs was removed and cells were washed twice with 5 mL PBS buffer. The cells were detached with trypsin-EDTA. After centrifugation and removal of the supernatant, the cells were resuspended into PBS to a final volume of 5 mL. The number of cells in suspension was counted with a hemocytometer. 5 mL of 20% HNO₃ was added into each sample to lyse the cells. The gold mass in the lysis solution was measured by ICP-MS. We can calculate the number of gold nanoparticles via the gold mass, and the number of GNPs in the lysis solution divided by the number of cells provided a quantitative measure of GNP cell uptake.

MTT assay and clonogenic survival assay: The cytotoxicity induced by GNPs with and without radiation was assessed by an MTT assay in 96-well plates and clonogenic survival assay. MCF-7 cells were tested. The starting number of cells was 3000 per well with 150 µL of medium. The cells were allowed to grow on the 96-well plates overnight. After 70% confluence was reached, the old medium was replaced with 150 µL of freshly prepared medium containing GNPs at the desired concentrations. After two hours of
incubation and the cell uptakes reached about $2.96 \times 10^4$ GNP per cell, the medium was replaced with fresh medium. The cells were treated with or without irradiation (see above). Cell response to the GNPs with and without irradiation was monitored by the MTT assay following the manufacturer’s instructions and clonogenic survival assay. For clonogenic survival assay, the cells either with or without treatment were incubated for 2–3 weeks. Cells were then fixed with 3:1 ethanol to acetic acid solution and stained with crystal violet. Colonies were counted for the control and experimental groups, with each experiment performed in triplicate.

Cell irradiation: Human breast-cancer MCF-7 cells and nonmalignant breast cell line (MCF-10A) were used in these studies. 200 kVp X-ray irradiation with the PANTAK Therapax3 series, γ- ray irradiation with the Shepherd Mark I-68A $^{137}$Cs Irradiator (L. Shepherd & Associates, San Fernando, CA), and the $^{60}$Co irradiator (Atomic Energy of Canada Ltd) were used in this experiment. The targeted cells were exposed to hybrid gold nanoparticles (Glu-GNPs or AET-GNPs) and then followed by either i) 200 kVp X-ray irradiation at 1.19 Gy min$^{-1}$, ii) γ-ray irradiation with $^{137}$Cs at $\approx$1.57 Gy min$^{-1}$, or iii) with $^{60}$Co irradiator at $\approx$4.76 Gy min$^{-1}$, each with a total dose of 10 Gy at room temperature. The control experiments were performed with the same procedures as above except replacing hybrid nanoparticles with nonlabeled naked GNPs. For all the experiments, after irradiation, the cells were incubated for 48 h before measuring cell viability with the MTT assay. The cells growing in plain medium without nanoparticles or irradiation were used as a control.

Statistical analysis: Experimental values were determined in triplicate. All values regarding measurement and percentage of gold content were expressed as means and standard errors (SE). The one-way analysis of variance (ANOVA) and Tukey multiple comparison post-test were used. Differences less than 0.05 ($p < 0.05$) were considered statistically in significant.

Acknowledgements

The authors thank Dr. Michael Brett and Dr. Tarek El-Bialy for providing the lab space and facilities to carry out some of our research. We also thank Ms. Jane Lee and Ms. Patricia Lassin for help with the irradiation tests, Dr. Mary Hitt for providing breast normal cells, Mr. Rakesh Bhatnagar for help with TEM measurements, Dr. Guangzhao Zhang for DLS measurements, and Dr. Shihong Xu and Dr. Guangcheng Chen for XPS and ICP-MS. The authors acknowledge the funding support of the Canadian Breast Cancer Foundation.


Received: September 1, 2007
Revised: June 9, 2008