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Enhanced x-ray irradiation-induced cancer cell damage by gold nanoparticles treated by a new synthesis method of polyethylene glycol modification

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Abstract

We explored a very interesting gold nanoparticle system—pegylated gold in colloidal solution—and analyzed its uptake by mice colorectal adenocarcinoma CT26 tumor cells and the impact on the cell's response to x-ray irradiation. We found that exposure to polyethylene glycol (PEG) modified ('pegylated') 4.7 ± 2.6 nm gold nanoparticles synthesized by a novel synchrotron-based method enhances the response of CT26 cells to x-ray irradiation. Transmission electron microscopy (TEM) and confocal microscopy revealed that substantial amounts of such nanoparticles are taken up and absorbed by the cells and this conclusion is supported by quantitative induced coupled plasma (ICP) results. Standard tests indicated that the internalized particles are highly biocompatible but strongly enhance the cell damage induced by x-ray irradiation. Synchrotron radiation Fourier transform infrared (SR-FTIR) spectromicroscopy analyzed the chemical aspects of this phenomenon: the appearance of C=O stretching bond spectral features could be used as a marker for cell damage and confirmed the enhancement of the radiation-induced toxicity for cells.

1. Introduction

A key objective in cancer radiation and x-ray irradiation therapy is to reduce the dose and the damage to healthy tissues and organs. Nanotechnology continuously produces new methods to improve the effectiveness and selectivity toward this objective [1, 2]. Gold nanoparticles are top candidates as drug carriers and radiotherapy enhancers due to the combination of small size and leaky vasculature

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of tumor tissues—the 'enhanced permeability and retention (EPR) effect' [3–5]—that facilitates highly selective particle localization in cancer cells and tissues. The effects of gold microparticles and nanoparticles on the cell's response to high-energy photon irradiation (x-rays and gamma rays) were previously demonstrated at the tissue and animal level [6, 7]. Most interesting was the recent discovery [7] of substantial dose enhancement without cancer drugs or labellers by pure gold nanoparticles—which in animal studies exhibited differential accumulation at the tumor site with 5–8-fold increase. Such interesting findings stimulated our present study that discovered new pathways to the therapy enhancement and produced new information on the corresponding mechanisms.

The nanoparticle system was obtained with a recently developed [8–10] synthesis procedure: irradiation by synchrotron x-rays produces colloidal solutions of gold nanoparticles with rather interesting properties compared to more conventional approaches [11, 12]. The process exploits the reduction due to the radiolytic radiolysis effects of synchrotron x-rays and requires no reductant and no stabilizer in the precursor solution—therefore, the particle surfaces are immune to possible contamination. The high-intensity synchrotron x-rays trigger a fast radiolytic radiolysis reaction and yield a large quantity of nanoparticles in a clean, well-dispersed colloidal solution, stable and without the common problem of agglomeration in cell culture media. This approach could be easily extended to the preparation of PEG-modified colloidal gold by simply adding the PEG to the precursor solutions and performing a one-solution x-ray irradiation synthesis. The obtained highly concentrated and stable pegylated gold colloidals enabled us to study the interaction of pegylated gold nanoparticles with cancer cells under realistic therapy-like concentrations and conditions.

2. Experimental procedure

2.1. Preparation and characterizations of the pegylated gold nanoparticle solution

Pegylated gold nanoparticles were synthesized by the synchrotron x-ray irradiation method reported elsewhere [8–10] with a few modifications. In short, to obtain well-dispersed pegylated gold colloidal solutions a mixed water solution of gold precursors (2 mM $HAuCl_4{\cdot}3H_2O$, Aldrich, MO, US) with appropriate NaOH (0.1 M, Showa Inc., Japan) and polyethylene glycol (PEG) (MW 6000, Showa Inc., Japan) were placed into polypropylene conical tubes (15 ml, Falcon®, Becton Dickinson, NJ) and transferred to the facility for x-ray irradiation.

The irradiation time was set to 5 min to guarantee the complete reduction of the gold precursor. The exposures were performed at the 'white light' x-ray microscopy beamline BL01A of the National Synchrotron Radiation Research Center (NSRRC), Hsinchu, Taiwan [13]. The energy distribution of the x-ray photons was centered at ∼12 keV, with a broad width of 8–15 keV [14].

The particle morphology, structure and size were analyzed with a JEOL JEM 2010 F Field Emission Gun Transmission Electron Microscope operating at 200 kV. The samples for TEM were prepared by placing a drying nanoparticle-containing solution on carbon-coated copper grids before transfer to the microscope. The pegylated gold nanosols were also characterized with a ultraviolet–visible (UV–vis) spectrometer (GBC Cintra 10e, GBC, Australia) to detect the optical absorption and with a zeta potential measurement system (Zetasizer 3000 HAS, Malvern Instruments Ltd, Malvern, Worcestershire, UK) for surface charge measurements.

2.2. Cell culture

Mice colorectal adenocarcinoma CT26 cells (CRL-2638, ATCC, Rockville, MD) were cultured in RPMI-1640 (Gibco, Invitrogen Corp., Carlsbad, CA) medium containing 10% fetal bovine serum (FBS) at 37 °C in a humidified 5% CO_2 incubator. The cells grew to 80% confluence and were detached by trypsin (0.5 g porcine trypsin and 0.2 g EDTA · 4Na per liter of Hanks' Balanced Salt Solution) (Sigma, Saint Louis, MO).

2.3. Transmission electron microscopy and confocal microscopy cell analysis

For the TEM sample preparation, 1 × 105 CT26 cells were seeded on a 100 mm culture dish. After 24 h, an appropriate volume of concentrated pegylated gold was added to the culture media to achieve a final colloidal concentration of 500 μm. After co-incubating for 48 h, the cells with gold nanoparticles were trypsinized, centrifuged and washed with PBS (phosphate buffered saline)/5% sucrose for at least three times to remove the remaining particles. Subsequently, the cells were fixed for 2 h in 2.5% glutaraldehyde and postfixed for 2 h in 1% osmium tetroxide. Dehydration was achieved by 25%, 50%, 75%, 95% and 100% ethanol. The samples were then infiltrated and embedded in 100% resin.

Ultrathin sections prepared by an ultramicrotome were placed on 200 mesh copper grids for TEM measurement. Cells grown on glass slides and fixed with 2% paraformaldehyde for 15 min were imaged by confocal microscopy (with an Olympus FV-1000 system): the cell nuclei, stained with a fluorescent dye (Hoechst 33258), were clearly observed.

For quantitative analysis of the cellular uptake of pegylated gold nanoparticles, CT26 cells were seeded in 6-well microplates at a density of 2 × 104 cell/well. After 24 h of cell attachment, the cells were treated with different concentrations of colloidal pegylated gold. We applied the methods reported in the literature [15] to quantitatively evaluate the gold uptake into the CT26 cells.

2.4. Cell viability

The cell viability and the possible cytotoxicity of pegylated gold nanoparticles were evaluated by examining the metabolic activities of CT26 cells using standard Alarma Blue (AB) assay. The AB assay was conducted according to the manufacturer's instructions. In short, control media or test exposures were removed; the cells were then rinsed with HBSS (Hank's Balanced Salt Solution) and 100 μl of AB/NR

(Nature Red) medium (5 vol/vol% solution of AB and 1.25 vol/vol% solution of NR dye) prepared in fresh media (without FBS (Fetal Bovine Serum) or supplements) was added to each well. Following 3 h incubation, the AB fluorescence was quantified at the excitation and emission wavelengths of 540 and 595 nm. Wells containing medium and AB without cells were used as blanks. The mean fluorescent units for the six replicate cultures were calculated for each exposure treatment and the mean blank value was subtracted from them.

2.5. Colonogenic cell survival measurement

Colonogenic cell survival tests was performed with a radio-oncology linear accelerator (Clinac IX, Varian Associates, Inc., Palo Alto, CA) operating at 6 MV and with a dose rate of 2.4 Gy min^{-1}. 150 CT26 cells/well were seeded and grown in a 6-well culture dish for 24 h. Pegylated gold nanoparticles in the colloid solution (500 μM) were then introduced and retained for 48 h, followed by irradiation by a 2 Gy dose of x-rays. The irradiated cells were further incubated for 14 days. Finally the cells were stained by 3% crystal violet and colonies were counted.

2.6. SR-FTIR analysis

For SR-FTIR analysis, CT26 cells were seeded and grown on non-toxic gold-coated glass plates in a 6-well culture dish at density of 5000 cells/well for 24 h. The 2 Gy x-ray irradiation was performed at the same linear accelerator after exposing the cells to the 500 μM colloidal solution of pegylated gold nanoparticles for 48 h. CT26 cells samples were then critical point dried (CPD) in CO_2 leaving them in a well-preserved state. Infrared spectral line scans were collected using the SR-FTIR spectromicroscopy facility at the NSRRC BL14A beamline.

The spectra were recorded in reflectance mode from individual cells with 4 cm^{-1} resolution, 5 μm step size and 64 co-added interferograms using a Thermo Nicolet Magna-IR spectrometer. The peak position, baseline corrections and smoothing were automatically performed by the OMNIC peak resolving software.

2.7. Statistical analysis

Statistical analysis was performed with the Microsoft Excel 2003 package. The significance level of the difference between the control and experimental groups was determined by Student's *t*-test. A difference was considered to be statistically significant when $p < 0.05$.

3. Results and discussion

We first investigated the uptake by CT26 cells of (4.7 ± 2.6 nm) pegylated gold nanoparticles in our high-density colloidal solution by TEM, laser scanning confocal microscopy and ICP analysis. The experimental evidence for the internalization of a large amount of pegylated gold nanoparticles was provided by confocal microscopy and TEM results like those of figures 1(A) and (B). We found that large amounts of nanoparticles are internalized in the cytoplasm. The nanoparticles can indeed be clearly seen inside vesicles within the cytoplasm. No nanoparticles could be detected inside the cell nucleus—and most nanoparticles were agglomerated. In figure 1(C) a quantitative analysis by ICP assay indicates that, after co-culturing for 48 h, the amount of cellular uptake depends on the concentration of the added gold: the uptake was about 1×10^5 at 250 μM, soared to more than 5×10^5 at 500 μM and reaching 1×10^6 nanoparticles per cell at 3000 μM. Standard cell viability tests, however, demonstrated that these particles are not toxic, as shown in figure 1(D).

The viability tests also revealed that the nanoparticles strongly enhance the effects of cell irradiation by x-rays. We examined the biological effects of the internalized nanoparticles by measuring the cell proliferation ability. Tests were conducted—before and after x-ray irradiation—both on unexposed (control) cells and on cells exposed to a 500 μM pegylated gold nanoparticle colloid. Prior to x-ray irradiation, the surviving fraction of the two kinds of cells was almost the same, as seen in figure 2: this indicates biocompatibility of the pegylated gold nanoparticles. The results of figure 2 also reveal the nanoparticle enhancement effect on the suppression of the cell proliferation rate by x-ray irradiation. For example, at an x-ray dose of 1 Gy the survival percentage was 92% for the control cells and 58% for the cells treated with the 500 μM pegylated gold nanoparticle colloid. At 2 Gy, the corresponding rates were 64% and 48%.

Nanoparticle internalization within cells is a potentially important factor in the enhancement of therapeutic effects. The presence of strongly absorbing elements in the cells can increase the production of photoelectrons or free radicals and lead to damage of organelles and/or nuclei.

In general terms, our experiments confirmed that differential accumulation of pegylated gold nanoparticles is not a peculiar phenomenon linked to a specific particle size: nanoparticles from a few nm to tens of nm all exhibited substantial uptake in animal studies. We also found that the pegylated nanoparticle uptake by cells is larger than for previously reported gold microparticles and nanoparticles [15]. These discoveries imply that the uptake pathway and therefore the mechanism of dose enhancement can be quite different for different types of gold particles.

Understanding the chemical modifications by the radiation and the degree of damage is quite important for potential therapeutical applications. We explored these issues with SR-FTIR, exploiting the exceptional quality of synchrotron radiation to enhance the spatial resolution and signal-to-noise level. Likewise, synchrotron radiation had already expanded the scope of FTIR spectromicroscopy to a variety of biological studies including investigation of cell membranes, proteins and nucleic acids, as well as tissue engineering [16–19].

The standard FTIR spectra of CT26 cells exhibit features corresponding to proteins amide A, lipid, proteins Amide I and proteins Amide II at 3300, 2936, 1640 and 1540 cm^{-1}. Previous studies indicated that the shape of the Amide I band can be influenced by the overall secondary structure of cellular proteins including α-helix, β-shift, turn and random

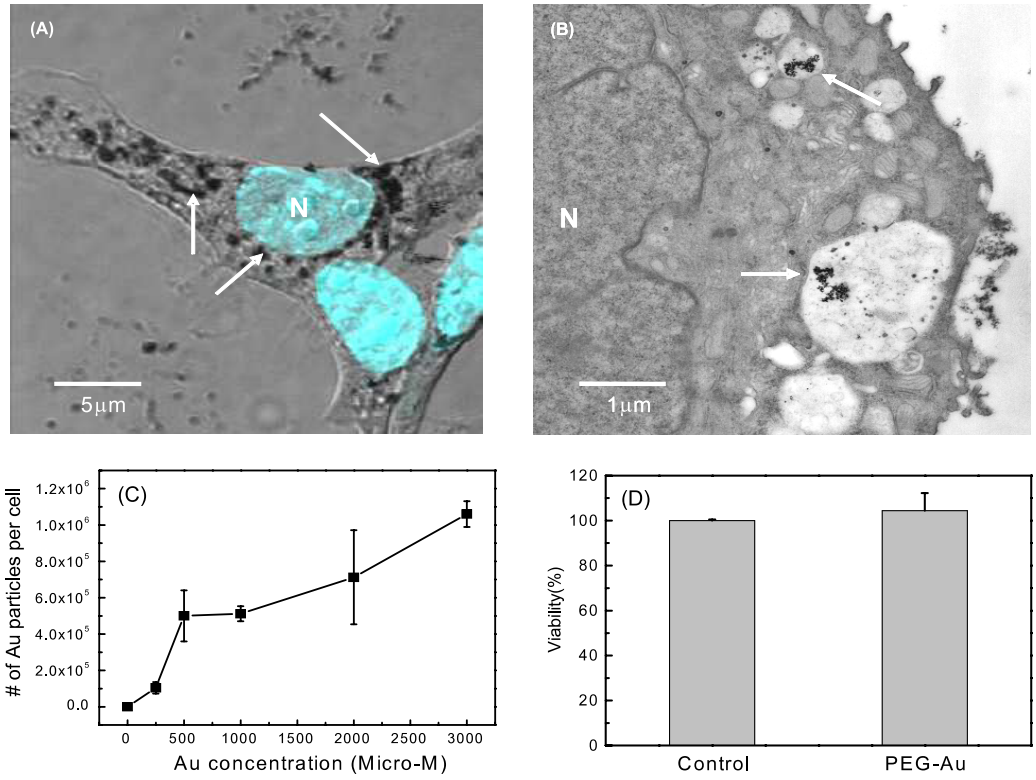

Figure 1. Results showing that large quantities of pegylated gold nanoparticles in high-density colloidal solutions are internalized within CT26 cells. However, the cell viability of cells is not affected: (A) confocal microscopy image; (B) TEM micrograph; (C) ICP analysis of cell uptake and (D) Alamar Blue assay. CT26 cells co-cultured with pegylated gold nanoparticles for 48 h. White arrows indicate clusters produced by particle aggregations; N is the nucleus.

(This figure is in colour only in the electronic version)

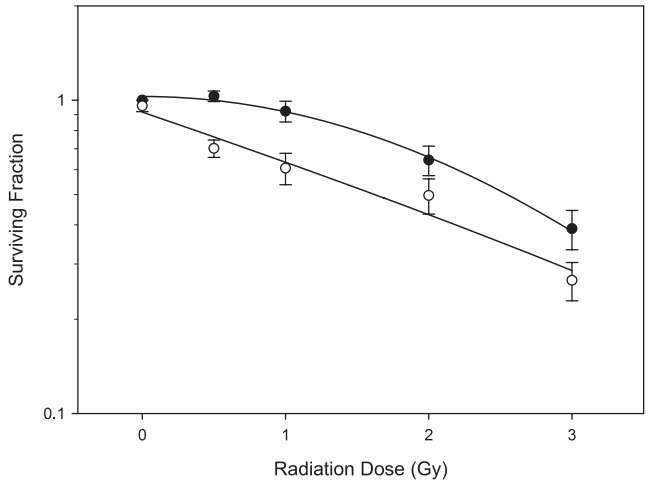

Figure 2. Radiation survival curves for CT26 cells. The full dots are results for CT26 cells co-cultured for 48 h, whereas the open circles refer to 48 h exposure to 500 μM pegylated gold nanoparticle colloids—before and after x-ray irradiation with the indicated dose. The data represent the mean $\pm$ a standard deviation of the results from four independent experiments.

coils [20]. We found no difference in this band between the control cells and the cells exposed to the nanoparticle colloid.

As shown in figure 3(A), the most striking x-ray-induced spectral variation is the appearance of a new peak at $\sim$1730 cm^{-1}, totally absent for non-irradiated specimens. This spectral feature was attributed to the formation of C=O bonds related to the lipid and protein endoperoxides [16, 18], the early state of apoptosis [21] or cell death [22]. The C=O bond peak intensity after x-ray irradiation is higher for cells treated with pegylated gold nanoparticles than for untreated cells, confirming the nanoparticle enhancement effect.

Gaudenzi *et al* argued that the additional C=O vibration can be attributed to cytotoxicity [20]. Our SR-FTIR results confirm therefore the cytotoxicity tests: pegylated gold nanoparticles are not toxic before x-ray irradiation. In order to take into account the effects of the individual cell thickness on the C=O peak strength, we derived the intrinsic absorbance by normalizing the peak intensity to that of the protein Amide II band (figure 3(B)). This ratio is increased by x-ray irradiation, from 0.05 ± 0.01 to 0.18 ± 0.01 for cells treated with pegylated gold nanoparticles. Considering the radiation survival results (figure 2), we can thus use this ratio as a useful indicator to index and evaluate the cell damage.

Our results cannot exclude the possibility of x-ray dose enhancement by intercellular nanoparticle accumulation. However, due to the large amount of nanoparticle uptake produced by our approach, we can conclude that this is a minor effect.

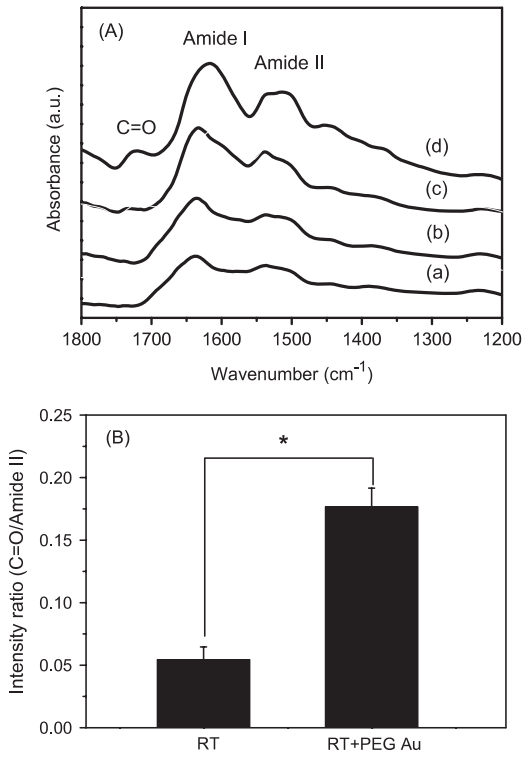

Figure 3. (A) Synchrotron radiation FTIR spectra of CT26 cells after different treatments. (a) Untreated cells; (b) cells after exposure to pegylated gold nanoparticles; (c) untreated CT26 cells after x-ray irradiation; (d) cells exposed to pegylated gold nanoparticles after x-ray irradiation. (B) Intensity ratio of C=O/Amide II for CT26 cells: the results are shown for the same cases, radiation-treated (RT) and radiation-treated with PEG Au (RT + PEG Au), as in the previous figure. The data represent the mean ± 1 standard deviation of the results from two independent experiments. *, $p < 0.05$, is considered to be statistically significant.

4. Conclusion

Our new synthesis approach for high-density, stable colloidal solutions of pegylated gold nanoparticles appears quite effective in enhancing the damage of cancer cells by x-ray irradiation. Substantial amounts of nanoparticles were found internalized in cell cytoplasm without exocytosis for a long period of time. Their internalization did not induce detectable toxicity. The enhancement of the x-ray irradiation cell damage by such nanoparticles was demonstrated both by toxicity tests and by SR-FTIR spectra. The FTIR carbonyl group/Amide II peak intensity ratio was found to strongly increase with the cell damage—and can be used to assess such damage. Conceivably, this ratio could be exploited to monitor the results of irradiation therapy as a valid alternative to the widely used pathology methods.

Our new approach could have a significant role in the development of cancer therapies based on nanoparticles—for example, to enhance the targeting and endocytosis. Nanoparticles taken up and absorbed by cells are indeed less likely to permeate back into the circulation system and reduce the dose enhancement effect. In general terms, our results show the importance of tailoring nanoparticle properties with suitable and innovative approaches to optimize their effects on cancer therapy.

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