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*Original Article*

# Apigenin stabilized gold nanoparticles increased radiation therapy efficiency in lung cancer cells

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**Abstract:** One of the recent trends in cancer treatment is the combination of different therapies (i.e. chemotherapy, radiation or immunotherapy etc.). This work described a combination of chemotherapy and enhanced radiotherapy for *in vitro* treatment of lung cancer by using gold nanoparticles stabilized by apigenin-a bioactive gradient that has been investigated for cancer treatments. The gold nanoparticles (AuNPs) were synthesized via reducing gold salts with apigenin, and had an average diameter of 20 nm to 30 nm and a zeta potential of -20 mV. Upon interacting with lung cancer cells, the nanoparticles were able to induce cell apoptosis, inhibit cell proliferation and arrest cancer cells in G0/G1 phases in a dose dependent manner. When using X-rays and the nanoparticles together, an additive anti-cancer effect was generated from chemotherapeutic functions of apigenin and the enhanced radiation killing effect produced by nanoparticles & X-ray interactions. This study may provide a promising therapeutic strategy for cancer treatment which integrates the advantages of both radiation and chemotherapies.

**Keywords:** Apigenin, gold nanoparticles (AuNPs), chemotherapy, radiation therapy, lung cancer

## Introduction

Numerous therapies have been developed for the treatment of cancers, e.g. radiotherapy, surgery, chemotherapy, and immunotherapy [1-3]. While each of these therapies has its own advantage, and shows efficacy to a certain extent, recurrence of lung cancer occurs frequently due to the limitations of each therapy. Radiotherapy of cancer relies on using powerful X-ray or γ-ray that can kill cancer cells effectively [4]. A disadvantage of this therapy is that while X-ray can kill cancer cells effectively, it also destroys normal proliferating cells, such as hematopoietic cells. A possible solution of reducing the side effects of radiation is using radiation sensitizers, such as gold or bismuth nanoparticles, which can amplify radiation efficiency by generating auger electrons or free ions that can damage DNAs within cancer cells thus kill cancer cells. Nanoparticles of gold have been widely used as radiation enhancers [5-7]. Previous studies found that the nanoparticles have shown great potency in the radiation treatment of cancer cells as a radio-sensitizer by inducing cancer DNA fragmentation and apoptosis [8-11]. On the other hand, chemotherapies with different anticancer drugs still remain the primary choice for lung cancer treatment [12, 13]. For example, platinum based drugs have been the first line of treatment since they were discovered. Although the efficacy of these drugs has been proved by different studies, numerous undesirable side effects are generated from drug treatment. Therefore, it is necessary to search for other anti-cancer therapies that may be effective as well as have fewer side effects.

Apigenin (4', 5, 7-trihydroxyflavone) is a bioactive gradient that can be extracted from different plants (e.g. parsley, chamomile etc.). Studies in recent years found that apigenin possessed remarkable therapeutic functions such as anti-inflammation, anti-oxidation and anti-carcinogenic functions in mammalian systems [14]. For instance, studies reported the apigenin could inhibit the inflammation induced by TNFα or lipopolysaccharide by reducing production of pro-inflammatory cytokines such as IL-8 and IL-6 [15-17]. Anti-cancer research found that apigenin could inhibit the proliferation of

pancreas cancer cells by activating p53 gene, a key tumor suppressor gene [18, 19]. Interestingly, apigenin has been employed to generate gold nanoparticles (AuNPs) through the reduction of $HAuCl_4$ salts. When applied in *in vitro* experiments, these nanoparticles showed dramatic anti-carcinogenic effect by inhibiting cancer cell progression and promoting apoptosis [20].

In this work, gold nanoparticles are synthesized by reducing the gold salt with apigenin as reported in literature [20]. The nanoparticles are characterized with their physio-chemical properties (e.g. size, zeta potential, UV-Vis etc.) and applied in a lung cancer cell line (A549 cell). We found that apigenin stabilized nanoparticles were able to inhibit cell proliferation and induce cell apoptosis, and arrest cells in G0/G1 phases. Moreover, these nanoparticles act together with X-ray irradiation to generate an additive anti-cancer effect. Flow cytometry analysis showed that an increased ratio of cells arrested in G0/G1 phases; Immunochemical staining revealed the combination of chemotherapy and radiotherapy induced an increased expression of free radicals that could promote cancer cell death.

## Materials and methods

### *Materials*

Antibodies for cleaved PARP and actin were purchased from Santa Cruz Biotechnology. The live/dead kit was obtained from Invitrogen. RPMI 1640 medium, Penicillin, streptomycin, fetal bovine serum (FBS), Dulbecco's phosphate-buffered saline (D-PBS) were from Sigma-Aldrich (St. Louis, MO). Ultrapure water (18.2 MΩ·cm) from a Nanopure System (Barnstead, Kirkland, WA) was used throughout our experiments. 4, 6-diamidino-2-phenylindole (DAPI) was purchased from Sigma-Aldrich. Apigenin was from Sigma-Aldrich. $K_2CO_3$ was from VWR. A549 cells were from American type culture collection (ATCC, Manassas, VA). A Mini-X Portable X-ray tube (Amptek, Bedford, MA) was employed for generating the radiation. The radiation is operated at 40 kV and 100 mA at a distance of 4 to 6 cm upright the cells. Dosage of the X-ray imposed to cells is calculate according the manufacturer's instructions and followed what reported in literature [4, 21].

*Nanoparticles synthesis:* Nanoparticles are produced according to the instruction reported in literature and slightly modified. Briefly, Apigenin was dissolved in DMSO at a concentration of 10 mM. pH of apigenin solution (0.3 mM) is adjusted with buffer ($K_2CO_3$ aqueous solution, 0.3 M). $HAuCl_4$ was added dropwise in to boiled apigenin solution. The solution was stirred at room temperature for 72 to 96 hours, with nanoparticles collected by centrifugation at 15000 g for 15 min and imaged under transmission electron microscopes (TEM, Hitachi H-8100 TEM).

*Cell culture and proliferation assay:* Cells were cultured in RMPI 1640 medium (supplemented with penicillin (100 U $ml^{-1}$), streptomycin (100 mg $ml^{-1}$)), and 10% FBS) and kept in 5% $CO_2$ at 37°C. Cell viability was tested according to the instructions from the kit producer. Briefly, cells were seeded in a 96-well plate ($10^5$ cells per mL), followed by adding the nanoparticles at differentiation (0.5, 1, 3 and 5 nM) of apigenin modified nanoparticles. Proliferation of cells was determined by using live/dead assay. The cells were added with 100 μl of D-PBS, followed by adding 100 μl of dual fluorescence calcine AM/EthD-1 assay reagent. The cells were incubated in dark for 30 min, followed by reading with a microplate reader with a Synergy HT multimode microplate reader (Winooski, VT) at a wavelength of 530 and 630 nm, respectively. The wells with loaded with PBS were used as control and background.

*Nanoparticles and cells interactions:* The amounts of nanoparticles up-taken by cells were quantified with inductively coupled plasma mass spectrometry (ICP-MS) (Complete Analysis Laboratories Inc., Parsippany, NJ). Briefly, cells at a density of $2 \times 10^5$ cells per well were cultured in a 6-well plate, with the nanoparticles added into the wells and incubated for 48 h. The cells were then washed gently with PBS (1 ×) for 3 times to remove free nanoparticles. Trypsin-EDTA was then added into the wells for 2 min to detach the cells. Number of cells was counted with a cell counter. The cells were collected by centrifugation 3000 rpm for 10 min. The cell pellets were digested with 50 ml of aqua Regia for 20 min, and gold ion concentrations were measured by ICP-MS. Number of gold nanoparticles up-taken by the dividing the mass of a single nanoparticles with the total mass of gold uptake by the cell. For X-ray irradiation, the following X-ray condition is used: 40 kV, 100 mA, and 80 mGy $min^{-1}$ for 5 min.

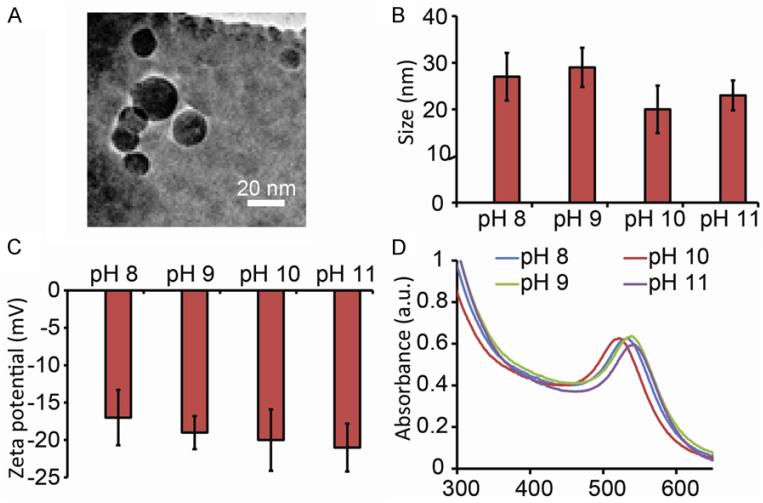


**Figure 1.** Characterization of gold nanoparticles stabilized by apigenin. A. Transmission electron microscope image of AuNPs synthesized at pH = 10. B. Diameter of nanoparticles synthesized under different pH conditions. The diameter was assessed with dynamic light scattering. C. Zeta potential of nanoparticles synthesized under different pH. The zeta potential was slightly decreased with the increase of pH conditions. D. UV-Vis absorbance of nanoparticles synthesized under different pH.

*Western blot*

Whole cells were lysed in lysis buffer [2% SDS, 10% glycerol, 10 mM Tris (pH 6.8), and 100 mM DTT], boiled for 10 min, and then subjected to immunoblotting by corresponding antibodies.

*Flow cytometry*

Accuri C6 cytometer (BD Bioscience Inc.) was employed for flow cytometry assessment. The cytometer was equipped with an air-cooled laser (20 mW, wavelengths: 488 and 640 nm). To analyze life cycles of cells with different treatments, the cells were first treated with ethanol for 1 h. DNAs within the cells were then stained with PI in PBS (added with 0.15% Triton X-100). The cells were then washed with PBS for twice, followed with flow cytometry assessment.

*Free radical assessment*

Production of free radicals in cells with different treatments was with ROS detection kit (Image-iT™ LIVE Green Reactive Oxygen Species). The detection kits had a fluorescence markers (5-(-6)-carboxy-2,7-di-chlorodihydro-fluorescein diacetate, carboxyl H2 DCFDA), that can permeate live cells. H2 DCFDA can be deacetylated by non-specific intracellular esterase or free radicals, showing green color under a fluorescence microscope.

*Statistical analysis*

Results were presented as average ± SD. *P* values were determined by two-tailed Students t-test with Excel software.

## Results

*Nanoparticles were synthesized and characterized*

The work started by synthesizing gold nanoparticles using apigenin and gold salt. The nanoparticles were imaged under transmission electron microscopy (TEM), where the nanoparticles are spherical in shape (**Figure 1A**). We assessed the impact of pH on nanoparticle size and the size of nanoparticles synthesized under different pH were listed in **Figure 1B**. Nanoparticles synthesized at pH 8 and pH 9 had a size around 30 nm. The nanoparticles synthesized at pH 10 and pH 11 had a size around 25 nm (**Figure 1B**). We also studied the impact of synthesis pH to nanoparticle zeta potential. All the nanoparticles had a zeta potential around -19 mV (**Figure 1C**). UV-Vis was also used to assess the absorbance, where the absorbance peak is around 520 to 530 nm for particles synthesized from pH 8 to pH 10 (**Figure 1D**).

*Uptake of apigenin modified nanoparticles by cancer cells*

The Apigenin modified nanoparticles at different concentration were then incubated with A549 lung cancer cells for 48 hours. Cell viability was observed under different concentration of AuNPs by microscope (**Figure 2A**). We found that cell death was obviously induced when AuNPs concentration was higher than 5 nM. Therefore, we use 3 nM of AuNPs for the following experiments performed. Then cells were counted before being lysed to analyze all com-

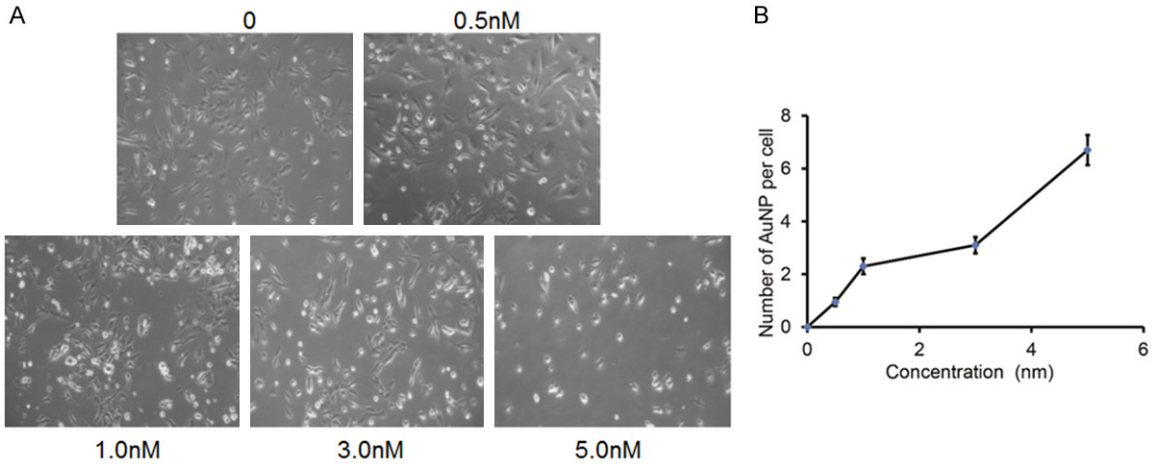


**Figure 2.** The number of apigenin modified nanoparticles internalized into A549 cells. The cells were incubated with AuNPs for 48 hours and then harvested for analysis. A. Cell viability under different concentration of AuNPs was observed by microscope. B. Cells were first washed with PBS twice and were counted before being lysed. All the lysate was collected for ICP-MS analysis, which determined the total amount of gold components up-taken by the cells. The number of particles uptake into each cell was estimated via dividing the mass of each gold nanoparticle and the total number of cells by the total amount of gold components determined by ICP-MS.

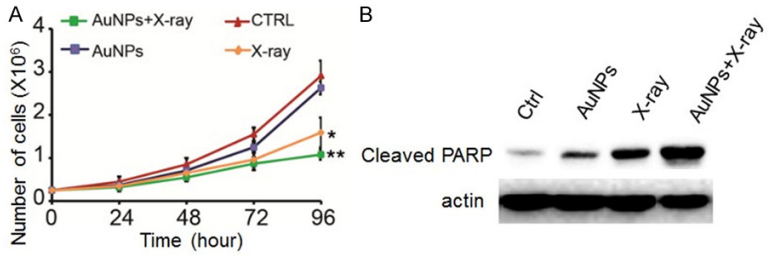


**Figure 3.** Using apigenin stabilized nanoparticles (i.e. AuNPs) and X-rays together reduced cancer cell proliferation rate and cell viability. A. A combinational use of AuNPs and X-rays inhibited cell proliferation rate compared to using each therapy alone. Using AuNPs or X-ray alone also reduced cell proliferation rate compared to cells with no treatment (CTRL). B. Using AuNPs and X-rays together induced apoptosis more dramatically than using each therapy alone as determined by cleaved PARP, a marker for apoptosis. The use of AuNPs in both cases was 3 nM and the X-ray is dose is indicated in the method section.

ponents with inductively coupled plasma mass spectrometry (ICP-MS) [21]. We calculated the number of nanoparticles up-taken by cells according to the following estimation: the nanoparticles had an average diameter of 25 nm, and Au density equals 19.30 g $cm^{-3}$. The weight of single nanoparticle therefore was around $1.25 \times 10^{-18}$ g; the number of nanoparticles up-taken by cells can therefore be estimated by dividing the mass of single nanoparticle with the value tested by ICP-MS. Then the number of nanoparticles up-taken by single cells could be obtained by dividing the number of cells with the total number of particles by them. Our study found that cells uptake nanoparticles in a dose dependent manner. When the cells were added with a low concentration of nanoparticles (e.g. 1 nM), there were around 2 nanoparticles per cell. This number went up when the nanoparticles concentration was increased (**Figure 2B**).

*Combination of X-ray radiation and nanoparticles generated an additive effect on inhibiting cells proliferation and promoting apoptosis*

The nanoparticles as well as X-ray were employed to test the impact on cell proliferation and apoptosis. The apigenin stabilized nanoparticles inhibited cell proliferation rate compared to control group, which can be attributed to the therapeutic effect of apigenin that stabilizes the nanoparticles. Since gold nanoparticle is an ideal radio-sensitizer that can enhance the radiation effect. The use of X-ray alone also reduced the proliferation rate of cancer cells compared to CTRL (**Figure 3A**). Dramatically, using the nanoparticles and X-ray together imposed an even more potent inhibition over cancer cell proliferation than the sum

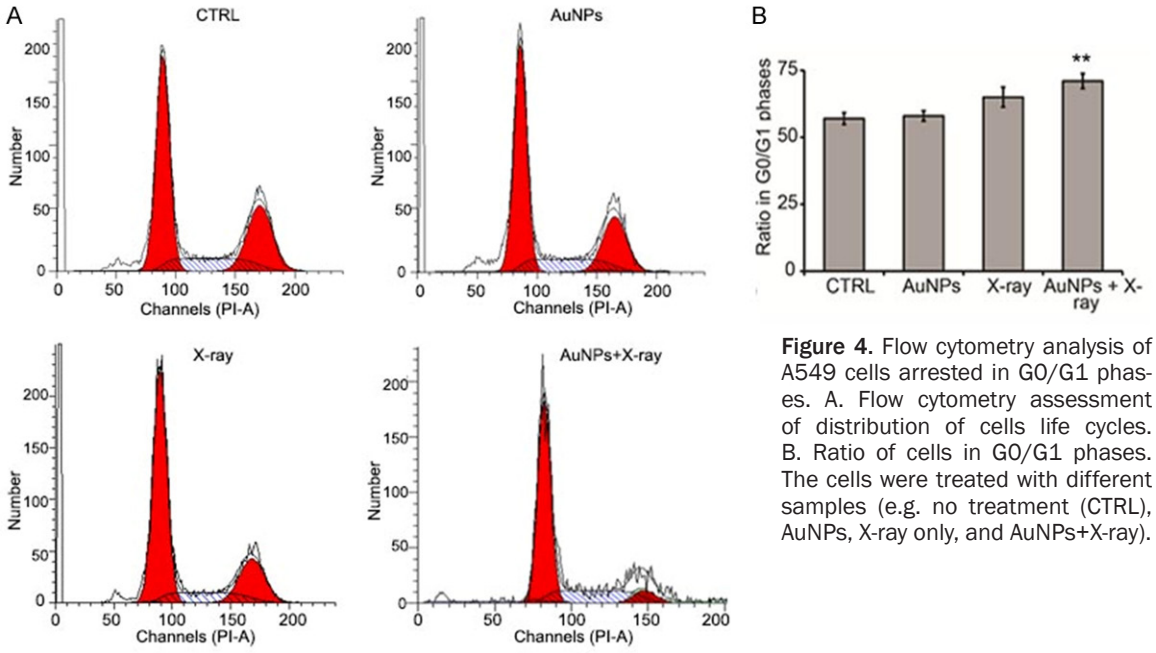


**Figure 4.** Flow cytometry analysis of A549 cells arrested in G0/G1 phases. A. Flow cytometry assessment of distribution of cells life cycles. B. Ratio of cells in G0/G1 phases. The cells were treated with different samples (e.g. no treatment (CTRL), AuNPs, X-ray only, and AuNPs+X-ray).

of the individual effect, indicating the benefit from the therapeutic role of apigenin and the radio-sensitization function of gold nanoparticles. Cell apoptosis was analyzed by detecting cleaved PARP, an apoptosis marker, by western blot. Consistent with the cell proliferation data, the use of X-ray or the nanoparticles alone could lead to apoptosis, a combinational use of AuNPs and X-ray resulted in even more induction of apoptosis, again confirming the additive effect (**Figure 3B**).

*A combination of chemotherapy and radiotherapy increased the ratio of cells arrested in G0/G1 phases*

Flow cytometry was employed to analyze the impact of different treatments on cell cycle. Apigenin stabilized nanoparticles arrested more cells in G0/G1 phases compared to control cells (cells with no treatment). On the other hand, the conventional citric acid stabilized AuNPs (no apigenin) did not affect the number of cells arrested in G0/G1 phases (**Figure 4A** and **4B**). Since X-ray could induce DNA damage and active the check point pathway, using X-ray alone also increased the ratio of cells arrested in G0/G1 phases (**Figure 4A** and **4B**). The use of X-ray and apigenin stabilized nanoparticles together arrested even more cells in G0/G1 phases compared to the individual effect of X-ray or the nanoparticles (**Figure 4B**), indicating an additive effect was generated during this process.

*The use of apigenin stabilized nanoparticles and X-ray radiation together enhanced the production of free radicals in lung cancer cells*

To test the production of free radicals, the cells were treated with AuNPs, X-ray, or AuNPs+X-ray, followed by staining with carboxyl H2 DCFDA-a dye that will show fluorescence when encounters free radicals. The results were assessed with fluorescence microscope and flow cytometry. **Figure 5A** showed the fluorescence image of control samples (cells with no treatment). Blue color indicated the nucleus stained with DAPI. The use of apigenin stabilized gold alone didn't promote free radicals' production very much (**Figure 5C**) but X-ray alone did (**Figure 5D**). Apigenin stabilized nanoparticles and X-ray together yielded an enhanced production of free radicals, as indicated by the green color (**Figure 5B**). Flow cytometry assessment of fluorescence intensity showed X-ray+AuNPs yielded the strongest fluorescence intensity compared to other samples (i.e. AuNPs, X-ray, and CTRL). Cells treated with X-ray also showed strong intensity because X-ray promoted free radical productions by interacting with the water molecules resided within cells. The use

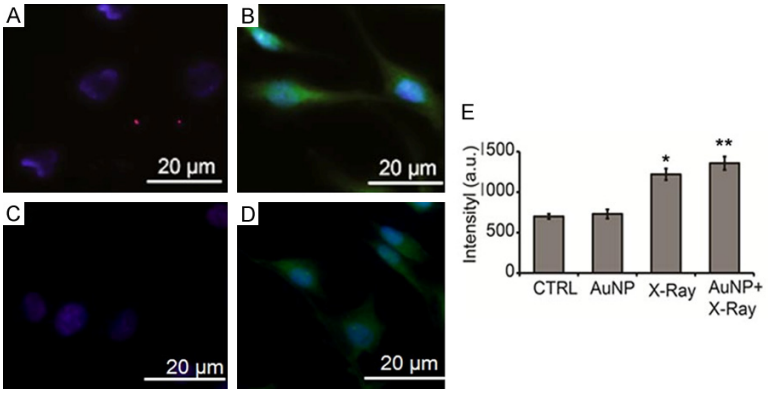


**Figure 5.** Assessment of free radical production in cells with different treatments by fluorescence staining of free radicals' production using a commercial detection kit. A. Control cells; B. Cells treated with AuNPs+X-ray; C. Cells treated with AuNPs; D. Cells treated with X-ray. After the treatments, the cells were assessed with the detection kits for 25 min, followed by washing with PBS twice and imaged under fluorescence microscopy. E. Quantification of fluorescence intensity showing the production of free radicals in cells treated with different samples. A stronger intensity indicating more production of free radicals in cells.

of apigenin stabilized AuNPs alone also lead to a slightly increase of free radical production compared to control group (**Figure 5E**).

## Discussions

A number of therapies have been developed to combat cancers, including chemotherapies, radiations, surgery and thermal therapies. Since none of the existing therapies is effective individually in all cancers, a promising direction is to combine different therapies-a strategy that can integrate the advantages of different therapies for an improved efficacy in cancer treatment. In this study, apigenin-a bioactive gradient that has been widely studied for cancer treatment, was used to reduce gold salt of nanoparticles. The nanoparticles stabilized with apigenin thus possesses chemotherapeutic potency of apigenin for cancer treatment. In addition, considering gold nanoparticle was a natural radio-sensitizer-a substance that can enhance X-ray absorbance for cancer killing, X-ray radiation was used in this work to explore the possibility of integrating two chemotherapy and radiation therapy together for cancer treatment.

We synthesized the apigenin stabilized nanoparticles and then size and zeta potential assessments were determined that the nanoparticles had diameters ranging from 20 nm 30 nm, and were slightly negative charged on surface (**Figure 1A** and **1B**). We found the pH value of the environment affected the nanoparticle size. A pH 10 environment produced nanoparticles with a diameter of 20 nm and this size was employed in the whole *in vitro* studies (**Figure 1B**). As for surface zeta potential, we found that while all the nanoparticles were negatively charged on surface, the intensity of charge was slightly affected by the pH conditions (**Figure 1C**). Since the difference in surface charges was not dramatic, this parameter was not further studied in the *in vitro* assays.

Followed by the characterization, the nanoparticles were employed to interact with A549 cells. Through ICP-MS, the number of nanoparticles up-taken by cancer cells was investigated. The results showed that the uptake of nanoparticles by cancer cells was dose dependent, where a higher dose of nanoparticles yielded higher uptake but also induced cell death if higher than 5 nM (**Figure 2**). It was noted nanoparticles uptake efficiency in this work was not as high as what reported by other researchers [3, 21]. This is probably because the nanoparticles used in this work were negatively charged, but other groups used positively charged nanoparticles, which can enhance the uptake of nanoparticles by cancer cells [3, 21]. A potential solution to this problem, if necessary, might be the use a positively charged materials (i.e. polyethylenimine or chitosan or cell penetrating peptides) to enhance the uptake efficiency.

Via a number of *in vitro* assays (i.e. growth curve, apoptosis, cell cycle analysis and free radical productions), nanoparticles together with X-rays were employed to test their impact on cancer cells. The gold nanoparticles are ideal radio-sensitizer for enhancing radiation therapy and apigenin has been indicated to treat different cancers [1, 19]. So, combination of these two strategies might generate a better effect. We found the use of apigenin modified nanopar-

ticles or X-ray alone could induce the apoptosis of the A549 cells and reduce cell proliferation rate compared to control cells (**Figure 3A** and **3B**). Strikingly, the use of X-rays and the nanoparticles together showed a potent additive effect in inhibiting cancer growth and promoting apoptosis (**Figure 3A** and **3B**). These results indicated that the chemotherapeutic effect of apigenin might contribute to the therapeutic effect of apigenin stabilized nanoparticles under X-ray radiation, thus generated an enhanced inhibitory effect on cancer cells. Therefore, these data indicated that the apigenin stabilized nanoparticles could be a useful agent for combing radiation therapy and chemotherapy. Cell cycle is an important part of cell life. Cancer cells are featured by rapid proliferation and therefore tend to have lower ratio of cells in G0/G1 phases [22]. By arresting cells in G0/G1 phases, the growth of cancer cell could be potentially controlled [22]. We therefore analyzed the effect of the combined therapy to cell cycle. We found that while the use of apigenin stabilized nanoparticles or X-ray alone could slightly increase the ratio of cells in G0/G1 phases, a combinational use of these two therapies greatly enhanced this ratio (**Figure 4A** and **4B**), again indicating the potent additive efficacy of this treatment. Since the use of radiation sensitizer can enhance the production of free radicals [4, 23], which can damage nuclear acids within the cancer cells. The production of free radicals in cancer cells with different treatments was therefore studied. The results showed that cells with no treatment had very low production of free radicals (**Figure 5A**) due to the fact that cells in normal state have the ability to clear some of the free radicals. Fluorescence imaging showed that the use of apigenin stabilized nanoparticles and X-rays together yielded a stronger production of free radicals compared to cells with no treatment (**Figure 5B**). Flow cytometry analysis quantitatively confirmed that a combinational use of X-rays with the nanoparticles lead to a higher level of free radicals compared to control group (**Figure 5E**). However, compared with using X-ray alone, the combinational therapy did not induce a very high level of free radicals, indicating apigenin did not play a significant role in the production of free radicals. This is probably because apigenin has anti-oxidant effect which may slightly down-regulate the level of free radicals. However, considering the facts that the apigenin are involved in anti-cancer process via multiple cellular pathways [24-26], this comprising effect in free radical production is acceptable.

## Conclusions

This work studied the use of apigenin stabilized nanoparticles in a combinational therapy with X-ray radiation to treat lung cancer cells in vitro. The combination of this two strategy benefits from the therapeutic effect of apigenin and the radio-sensitization function of gold nanoparticles. Proliferation rate of cancer cells were significantly reduced and apoptosis was promoted by the combination therapy. In addition, flow cytometry analysis showed the combined therapy arrested a higher ratio of cells in G0/G1 phases compared with using each therapy alone. The use of X-ray during the treatment promoted the production of free radicals that could help to kill cancer cells. This study may provide a new strategy for cancer treatment.

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## Disclosure of conflict of interest

None.

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