Synthesis of Metal Nanoparticles Inside Living Human Cells Based on the Intracellular Formation Process

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Developing nanoparticles that target the cell's nucleus is a promising approach in biological research because of the genetic information inside the nucleus. However, nuclear targeting is difficult to achieve because nanoparticles have to first pass into the cytoplasm and then cross the nuclear membrane. Here, the ability of the intracellular and extracellular formation of metal nanoparticles based on the reduction of metal salts was investigated in different cell lines. Moreover, the cells were fixed by metal ion solution during the metal nanoparticle synthesis process. Atomic force microscopy (AFM), transmission electron microscopy (TEM), scanning electron microscopy (SEM), energy dispersive X-ray spectroscopy (EDX) and UV-Vis absorption were utilized to identify the formation of metal nanoparticles inside the cells as well as in the incubation solution. In addition, the potential of using these nanoparticles to enhance the Raman signals from the cell was examined.

Synthesis of metallic nanoparticles (NPs) with different shapes and sizes is currently in high demand and is a challenging issue in various fields including nanotechnology and nanobiotechnology,[1] due to their unique physicochemical and optoelectronic properties.[2] The unique properties of gold (Au) NPs compared to other metals NPs (chemical, physical, optical, easy preparation, efficient bioconjugation, potential noncytotoxicity, tunable, enhanced scattering and absorption properties, etc.), provide Au NPs great potential applications in several fields such as optoelectronic devices, ultrasensitive biochemical sensors,[3] medicinal therapeutics,[4] catalysis,[5] and cancer applications, as efficient bioconjugation, potential noncytotoxicity, tunable, enhanced scattering and absorption properties, etc.), provide Au NPs great potential applications in several fields such as optoelectronic devices, ultrasensitive biochemical sensors,[3] medicinal therapeutics,[4] catalysis,[5] and cancer applications, and selective photo-thermal therapy. Moreover, Au NPs were used in several biomedicine purposes such as leukemia therapy,[6] biomolecular immobilization,[7] as anti-angiogenesis, anti-malaria and anti-arthritic agents.[8]

Surface-enhanced Raman Scattering (SERS) is a rapid, highly sensitive, reagent-free and non-destructive technique that has important implications in biological research in terms of analyzing the chemical composition within a single living cell at unprecedented resolution.[9] Recently, many studies have utilized colloidal suspension of metallic NPs mainly Au and silver (Ag) to monitor cellular processes and events that take place inside living cells.[10] The delivery of Au NPs inside human cells based on a passive uptake mechanism was recently reported,[11] however, this approach has several drawbacks, such as lack of control over aggregation, non–homogeneous distribution of NPs and a very poor translocation efficiency of NPs to the cell nucleus, which results in a relatively low reliability and reproducibility in the SERS analysis. The cell nucleus is the most important cell organelle because it encompasses the genetic information that plays a critical role in most cell functions i.e. cell growth, proliferation, and cell apoptosis.[12]

Targeting the nucleus with NPs is a promising approach in biological research due to its role in different cell functions. However, nuclear targeting is difficult to achieve because the NPs must pass into the cytoplasm and then cross the nuclear membrane.[13] In addition, NP targeting of cancer cell nuclei has been reported to influence cellular function, causing cytokinesis arrest, DNA damage, and programmed cell death, which leads to failed cell division, thereby resulting in apoptosis.[14] Therefore, many studies have attempted to develop methods of forming metal NPs inside the human cell nucleus.

Recently, certain microorganisms such as bacteria, fungus, yeast and actinomycetes were reported to have the ability to synthesize different metallic NPs from their ions.[15] Furthermore, Anshup et al.[16] and Shamsaie et al.[17] reported the extracellular and intracellular syntheses of Au NPs based on the reduction of auric chloride. Here, we aimed to explore the potential of forming NPs (Au and Ag) intracellular and extracellular in different human living cell lines (cancer and healthy cells) through the reduction of ions. In addition, we examined effects of these metal ions (auric chloride or silver nitrate) on cell viability as well as cell morphology in different living cell lines.

Our results demonstrated that treatment of different cell lines with metal ions resulted in the cell fixation. While, AFM, TEM, EDX, SEM and UV-Vis absorption techniques were demonstrated the formation of metal NPs inside the cell’s nucleus as well as larger particles of different sizes and shapes in the incubation solution. In addition, the treated cells shown strong Raman signals compared with weak and noisy Raman
peaks from control cells, which indicated the potential role of these NPs to enhance the Raman sensitivity.

We have follow the previously reported method for intracellular formation of Au NPs inside the cancer HeLa and MCF-7 cell lines and healthy HEK293T cell line. Briefly, different cell lines were incubated with 1 mM solution of auric chloride in phosphate buffered saline (PBS). Figure 1 shows the cell morphology changes before and after treatment for different periods with PBS solution or auric chloride solution under identical conditions. Figure 1a shows that the incubation solution (auric chloride solution) became intensely purple after 4 days of incubation, which indicated the formation of colloidal Au NPs. Figure 1b displayed the photomicrograph of control HeLa cells that exhibited elongated shape; while HeLa cells that treated with PBS solution only as a negative control for 4 hrs were demonstrated changes of their morphology to round shape with some shrunken cells and detached from the culture well (Figure 1c); on the other hand, HeLa cells that have been treated with auric chloride solution shown that the cells were stained (Figure 1d). Moreover, Figure 1e displayed the optical image of controls HeLa cells after 14 hrs, which demonstrated that cells have formed a confluent monolayer. In contrast, HeLa cells after treated with PBS for 14 hrs were completely shrunken and detached from the culture well as shown in Figure 1f. While, HeLa cells that have been treated with auric chloride solution for 14 hrs shown that the cell’s color become darker (Figure 1g). Moreover, HeLa cells treated for 4 and 8 days were shown in Figures 1h and i respectively, which indicated increase the color depth with incubation time, but no images for the control cells after these incubation time, because the cells were dead and detached from the culture well. The same behaviors were observed for treated HEK293T cells with PBS solution or auric chloride solution as shown in Figure S1. These results confirmed successful intracellular synthesis of Au NPs inside the cells and their quantity were increased with the incubation time.

The intracellular formation of Au NPs inside the cells was also investigated by using negatively stain technique. TEM images of a negatively stained cross thin sections from the treated HeLa, MCF-7 and HEK293T cell was shown in Figures 2a, d and g, respectively which clearly exhibited the presence of Au NPs inside the cell’s nuclei and plasma. TEM images of the Au NPs inside the cell’s nucleus of HeLa, MCF-7 and HEK293T cells was shown in Figures 2c, f, and i that demonstrated the formation of Au NPs of irregular morphology (10–20 nm in diameter). Moreover, energy dispersive X-ray spectroscopy (EDX) technique was used to validate the formation of Au NPs inside the cells. Figure 3a showed the EDX spectrum of control HEK293T cells, which didn’t show any peak for Au. While, Figures 3b and c showed the EDX spectra for HEK293T cells after treatment with auric chloride for 4 and 8 days, respectively; that demonstrated the formation of different characteristic peaks for Au, which indicate the presence of Au metals inside the cells and their quantity was increased as incubation time increased. Contrastively, fixed dead cells treated with 4% paraformaldehyde and incubated in auric chloride for 4 days didn’t show any peak for Au, which meant that NP could not be synthesized (Figure S2).
and UV-Vis techniques. Figures S3a and b showed the AFM images of Au NPs formed inside the cells and in the incubation solution respectively, that clearly demonstrated the formation of spherical NPs of different sizes. On the other hand, UV–Vis spectrum of the supernatant liquid from the cells lysed had a higher absorbance intensity and larger width than the spectrum of the incubation solution; furthermore, the absorbance band at 540 nm continued to increase for all samples over a period of 4 days, which might be related to the formation of Au NPs in the incubation solution (Figures S3c–e). In addition, the effect of different concentrations of Au³⁺ (from 0.1 mM to 5 mM) was examined. These experiments demonstrated that the cells were stained and fixed at the different Au³⁺ concentrations. Moreover, the intracellular formation of metal NPs were confirmed

To investigate the effects of incubation of cells with 1 mM of Au³⁺ solution for long time, the cells were incubated with Au³⁺ solution for different incubation periods (4, 8, 14, 21 and 28 days). Typical SEM images of selected Au particles that formed in the incubation solution after different incubation times were shown in Figure 4. Under these conditions, Au particles of different sizes were formed (from nano– to micrometer sizes). Although, the size of the Au particles increased with incubation time, the cell number didn’t increase, which indicates that the cells didn’t proliferate. Therefore, the presence of these metal ions resulted in cell fixation.

The intracellular formation of Au NPs was confirmed by study the morphologies of Au NPs, which were formed inside the cells as well as in the incubation solution by using AFM and UV-Vis techniques. Figures S3a and b showed the AFM images of Au NPs formed inside the cells and in the incubation solution respectively, that clearly demonstrated the formation of spherical NPs of different sizes. On the other hand, UV–Vis spectrum of the supernatant liquid from the cells lysed had a higher absorbance intensity and larger width than the spectrum of the incubation solution; furthermore, the absorbance band at 540 nm continued to increase for all samples over a period of 4 days, which might be related to the formation of Au NPs in the incubation solution (Figures S3c–e). In addition, the effect of different concentrations of Au³⁺ (from 0.1 mM to 5 mM) was examined. These experiments demonstrated that the cells were stained and fixed at the different Au³⁺ concentrations. Moreover, the intracellular formation of metal NPs were confirmed

Figure 2. TEM images of negatively stained thin sections from treated HeLa cells (a-c), treated MCF-7 cells (d-f) and treated HEK293T cells (g-i) with 1 mM of auric chloride solution for 4 days. The red and blue arrows indicated the spread of black dots across the cell’s nucleus and cytoplasm respectively, correspond to gold nanoparticles that are 10–20 nm in diameter (c, f and i).
It is interesting to note that, the cells proteins lysis could be used for fabrication of small Au particles (only nano-size), while the whole cells could be used for fabrication of large Au particles (micro-size) in the incubation solution.

using other cell lines, including liver cancer HepG2 cell line, breast cancer MCF–7 cell line and PC12 cell line as test models, which indicated that the cells were stained and fixed during the formation process.

Figure 3. EDX spectrum of (a) control HEK293T cells, (b) HEK293T cells incubated with auric chloride solution for 4 days, and (c) HEK293T cells incubated with auric chloride solution for 8 days.
including NADH dehydrogenase (ubiquinine) flavoprotein 2, quinone oxidoreductase-like (QOH-1). These redox enzymes might have some contribution in the biosynthesis of metal NPs. To investigate this role, the total cell proteins and two sub-cellular fractionations (cytoplasmic and nuclear fractions) from MCF-7 cells were extracted; then these three cell lysates were mixed with the 1 mM of Au $^{3+}$ solution.

Figure 5a demonstrated that the cytoplasmic cell lysate fraction didn’t show any color change after mixing with Au $^{3+}$ solution for several days, while the color of Au $^{3+}$ was converted into red and violet color after mixing with total cells lysed and nuclear cell lysate fractions within 1 min, respectively, which indicated the formation of Au NPs. Moreover, Figure 5b showed the absorbance spectrum of cytoplasmic cell lysate fraction didn’t show any absorption peak, but the mixture of Au $^{3+}$ solution with the nuclear cell lysate and total cell lysate solution shows absorption peak at 530 and 550 nm, respectively. The morphologies of Au NPs formed by mixing 1 mM Au $^{3+}$ solution with total or nuclear cells lysates were investigated by using TEM technique. TEM images of Au NPs formed in the incubation solution with HeLa cells after incubation with gold chloride solution for 4 days (a,b), 8 days (c,d), 14 days (e,f), 21 days (g,h), and 28 days (i,j).

In addition to that, we used the same method to synthesize Ag NPs based on the bio–reduction of silver nitrate instead of Au salt. It is worth noting that, the Ag nitrate solution could not be used in PBS. Because the Ag nitrate was converted to insoluble salts (silver chloride); therefore we used an aqueous solution of AgNO$_3$. As in the case of the Au solution, Ag NPs were formed and the cells were stained (Figures S4a and b).

The mechanism of Au$^{3+}$ ions reduction into Au metal NPs has not yet been systematically evaluated. However, several cell’s components including carbohydrates and redox enzymes of the cell–membrane and within the cytoplasm could play a role in the intracellular synthesis of the Au NPs.$^{[16,18]}$ Because the NPs were formed inside cells and the larger particles were formed in incubation solution, we believe that the mechanism of synthesis of the NPs involves electrostatic interactions between the positively charged Au$^{3+}$ ions and negatively charged groups in the enzymes, carbohydrates and other redox active components that present in the cell wall.$^{[19]}$ Thereafter, the Au$^{3+}$ ions were reduced into Au atoms that could be aggregated to Au NPs. Thus The NPs formed were then transported to the solution through the cell-membrane and the rest of the metal NPs aggregated, resulting in the formation of large particles.

Previously, we reported the extraction of some redox enzymes from cancer HeLa cells based on 2D electrophoresis techniques, including NADH dehydrogenase (ubiquinine) flavoprotein 2, quinone oxidoreductase-like (QOH-1).$^{[20]}$ These redox enzymes might have some contribution in the biosynthesis of metal NPs. To investigate this role, the total cell proteins and two sub-cellular fractionations (cytoplasmic and nuclear fractions) from MCF-7 cells were extracted; then these three cell lysates were mixed with the 1 mM of Au$^{3+}$ solution. Figure 5a demonstrated that the cytoplasmic cell lysate fraction didn’t show any color change after mixing with Au$^{3+}$ solution for several days, while the color of Au$^{3+}$ was converted into red and violet color after mixing with total cells lysed and nuclear cell lysate fractions within 1 min, respectively, which indicated the formation of Au NPs. Moreover, Figure 5b showed the absorbance spectrum of cytoplasmic cell lysate fraction mixed with Au$^{3+}$ solution didn’t show any absorption peak, but the mixture of Au$^{3+}$ solution with the nuclear cell lysate and total cell lysate solution shows absorption peak at 530 and 550 nm, respectively. The morphologies of Au NPs formed by mixing 1 mM Au$^{3+}$ solution with total or nuclear cells lysates for one week were investigated by using TEM technique. TEM images of Au NPs formed in the nuclear cells lysate fraction clearly demonstrated the formation of spherical NPs of different sizes as shown in Figures 5c-g. But, the Au NPs formed in the total cell lysate displayed the formation of spherical and rods NPs (Figures 5h-m). These results
To investigate the role of NADH and QOH-1 enzymes in the intracellular NPs synthesis, we have treated the cells with rotenone and 5-Methoxy-1,2-dimethyl-3-[(4-nitrophenoxy)methyl]-1H-Indole-4,7-dione (ES936) as enzyme inhibitors to inhibit NADH and QOH-1, respectively. Here, we investigated the effects of 100 nM enzyme inhibitors on MCF-7 cells for two hours (Figure 6). According to reference,\cite{21} 100 nM of these enzyme inhibitors treatments on MCF-7 and HeLa cells for two hrs could stop more than 95% of those enzymes but without any effect on the cell viability. Then, the total protein of cells lysis was extracted from the control and treated cells, and their ability to reduce the Au\(^{3+}\) ions into Au metal were studied. Figure 6a indicates that the treated Au NPs solutions have different colors. Figure 6b demonstrated that the absorbance of Au NPs solution, which results from the total protein of cells lysis of control cells, which have the higher absorbance intensity, this intensity was decreased after treatment of the cells with ES936, rotenone and mixture of them, respectively. The morphologies of the treated Au NPs were investigated by TEM. Figures 6c-f indicated the formation of solid spherical Au NPs, which results from total protein of control cells lysis; while the Au NPs in total protein of cells lysis which were treated with enzymes inhibitors demonstrated the formation of smaller particles coated with another material that could be proteins or surfactants (Figures 6g-r). These results confirmed that not only these two enzymes could play a role in the NPs synthesis. In addition, Figure S5 showed the TEM images of a negatively stained cross sections from control and treated HEK293T cells with enzymes inhibitors after incubation with 1 mM of auric chloride for 4 days, which indicated the formation of Au NPs inside all the cells samples before and after treatment with different enzyme inhibitors (as marked by arrows). Based on all the above, NADH and QOH-1 enzymes could have some contributions in the intracellular formation of Au NPs.
In order to measure the efficiency of the intercellular formation of Au NPs, we measured Raman spectra from HEK293T cells before and after Au NPs formation (4 days) using a laser with wavelength of 785 nm. The Raman spectra from control cells were noisy and individual Raman peaks could not be distinguished from the noise (Figure S6a). However, the Raman spectra of HEK293T cells after intracellular formation of Au NPs contained a series of bands that corresponded to all biopolymers found in the cells (Figure S6b). The main peaks for the nucleic acids were found at 1085−1060 cm⁻¹ (PO₂⁻), 788 cm⁻¹ (PO₂⁻ in DNA), and 811 cm⁻¹ (PO₂⁻ in RNA). The spectra of proteins were dominated by peaks corresponding to amide III vibration (1220 cm⁻¹). Amino acids were observed in peaks that corresponded mainly to phenyl groups, such as phenylalanine (1005 cm⁻¹), tyrosine (854 cm⁻¹) and tryptophan (760 cm⁻¹) as well as C–H vibrations (1448−1460 cm⁻¹). The strongest Raman peaks for lipids were present at 1448−1460 cm⁻¹ (C = C stretching and vibrations of the hydrocarbon chains). Also, carbohydrates were identified by their characteristic Raman peaks for C−O−C of the sugar rings (800−1500 cm⁻¹).

Interestingly, the intensity of the Raman peak at 1,005 cm⁻¹ was very week, which is a typical characteristic of fixed cells. In summary, unlike previous studies, our results demonstrated that treatment of different human cells (cancer and normal cells) with various metal ions solution resulted in cell fixation. In addition, the whole cells could be used as a green synthesis method for production of different metal NPs (Ag and Au) inside the cells and to larger particles (from nano- to micrometer sizes) outside the cells with different shapes; while, the cell lysis could be used as a green synthesis method for production of stable metal NPs. The results indicated that the living cells are able to synthesize metal NPs based on intracellular formation process but, during the synthesis process the cells will be fixed; while, the fixed cells couldn’t be able to synthesize metal NPs.
the metal NPs. Therefore, living cells initiate the reduction of the metal salts into metal NPs. Furthermore, the formed Au NPs inside cells were found to give enhanced Raman spectra with laser of large wavelength, that could minimizes fluorescence interference and photo-toxicity. The size of the formed metal NPs could be controlled by adjusting the concentrations of the supplied metal ions and the incubation time. Therefore, this method could be readily used as a cost effective and green method, which does not require the use of any reducing agents or capping agents, for the development of new metal NPs with unique optical, electrical, chemical, and magnetic properties.

**Experimental Section**

Auric chloride trihydrate (HAuCl₄·3H₂O, 99.9+%), silver nitrate (AgNO₃), phosphate buffered saline (PBS) (pH 7.4), Rotenone and ES936 were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals that are used in this study were obtained commercially as reagent grade.

Human cervical cancer cell line (HeLa), human embryonic kidney cells (HEK293T) and Human liver carcinoma cell line (HepG2) were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA), and a 1% concentration of antibiotics (Gibco). While, PC12 (pheochromocytoma) and human breast adenocarcinoma (MCF-7) cell lines were cultured in PRMI1640 (Invitrogen, Carlsbad, CA, USA) with 10% heat-inactivated fetal bovine serum and 2% antibiotics (streptomycin + penicillin). All cell lines were maintained under standard cell culture conditions at 37 °C in an atmosphere of 5% CO₂.

The medium was changed every two days. The intracellular formation of Au NPs was carried out according to the reported methods. Typically, cells were cultured in the suitable media and allowed to grow for 3 days. Then the growth medium was rinsed out and the cells were washed twice with phosphate buffered saline (PBS). Finally cells were immersed with 5 mL of 1 mM HAuCl₄ solution prepared in PBS (pH 7.4). In addition, the formation of Ag NPs based on intracellular process was carried out by flooding the cells with 5 mL of 1 mM AgNO₃ in deionized water instead of Au solution in PBS. Then, the treated cells were kept in an incubator for 4 days at 37 °C and 5% CO₂ atmosphere.

Cell lysis was carried out to ascertain the presence of NPs inside the cell, and the NPs formed inside the cell was compared with those formed in the solution based on the UV-visible results of the solution obtained before and after lysis. Moreover, the protein cell lysate of control cells was used for biosynthesis of Au NPs.

Typically, the cells were allowed to grow for 2 days before devoted to the preparation of the cell lysate. The growth medium was removed. Then, the cells were washed with PBS and were scrapped off the well surface using a cell scraper gently to prevent foaming; then, the cell suspension in the well was transferred into a centrifuge tube and was spin at 5000 rpm for 5 min at 4 °C. Then, any remaining buffer was removed; 0.25 mL ice cold lysis buffer was added and kept in ice for 20 min. This cell lysate was centrifuged at 5000 rpm for 5 min at 4 °C, and the supernatant liquid was separated.

Subcellular fractionation, the total and fractionated cell lysates were prepared as previously described. Cells (1 × 10⁶) were cultured in 100 mm plates, and on the next day, cells were collected and washed in cold PBS and lysed in 300 μL of fractionation buffer (10 mM Tris-HCl, 1 mM EDTA, 0.5% NP-40, and protease inhibitor cocktail). After incubation on ice for 30 min, cells were centrifuged at 600 rcf for 10 min. The supernatants and dissolved pellets were used as cytoplasmic and nuclear fractions, respectively. The total and fractionated cell lysates were prepared in RIBA buffer.

The morphologies of metal particles that formed outside the cells were analyzed by SEM (ISI DS-130C, Akashi Co., Tokyo, Japan). On the other hand, the intracellular synthesis of metal NPs was confirmed by analysis the NPs in the cell’s nucleus by using TEM. Ultrathin sections (70 nm thick) were cut using a diamond knife (Diatome) in Leica Ultracut UCT microtome and were taken up on 300 mesh copper grids. Thereafter, the grids with the sections were stained with uranyl acetate and lead citrate and were viewed with a TEM (JEM 1010, JOEL, Japan) at an accelerating voltage of 80 kV. Raman assay. HEK293T cells were examined before and after NPs formation by detecting SERS spectra using NTEGRA spectra (NT-MDT, Moscow, Russia) equipped with a liquid nitrogen-cooled CCD detector. The maximum scan-range, X/YZ was 100 μm × 100 μm × 6 μm, and the resolutions of the spectrometer in the XY-plane and Z-axis were 200 nm and 300 nm, respectively. SERS spectra was obtained using infrared laser emitting light at a 785 nm wavelength with an irradiation laser power of 3 mW on the sample plane. Five scans of 10 s from 500–1600 cm⁻¹ were recorded and the mean curve was used as SERS signals.

The morphology of the Au NPs that formed in the cells lysis or in the supernatant solution was analyzed by AFM (NTEGRA spectra, NT-MDT, Moscow, Russia), equipped with an inverted optical microscope, in semi–contact mode. The maximum X/YZ scan range of the system was 100 μm ×100 μm × 6 μm. The silicon cantilevers used in this study had a resonant frequency in the range of 115–190 kHz and a force constant of 2.5–10 Nm⁻¹. The scan rate was 0.5 Hz.

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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