Growth of Gold Nanoparticles in Human Cells

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Received July 16, 2005. In Final Form: October 9, 2005

Gold nanoparticles of 20-100 nm diameter were synthesized within HEK-293 (human embryonic kidney), HeLa (human cervical cancer), SiHa (human cervical cancer), and SKNSH (human neuroblastoma) cells. Incubation of 1 mM tetrachloroaurate solution, prepared in phosphate buffered saline (PBS), pH 7.4, with human cells grown to \sim 80% confluency yielded systematic growth of nanoparticles over a period of 96 h. The cells, stained due to nanoparticle growth, were adherent to the bottom of the wells of the tissue culture plates, with their morphology preserved, indicating that the cell membrane was intact. Transmission electron microscopy of ultrathin sections showed the presence of nanoparticles within the cytoplasm and in the nucleus, the latter being much smaller in dimension. Scanning near field microscopic images confirmed the growth of large particles within the cytoplasm. Normal cells gave UV-visible signatures of higher intensity than the cancer cells. Differences in the cellular metabolism of cancer and noncancer cells were manifested, presumably in their ability to carry out the reduction process.

Introduction

With significant developments in the understanding of nanosystems, research efforts are being focused on integrating them with biology.¹ Broadly, this is being attempted in areas such as biosynthesis of nanoparticles, use of nanosystems for therapeutics and diagnostics, biosensing, understanding the principles of biosystems, etc. Biosynthesis acquires special importance due to its inherent environment friendly nature. Intracellular and extracellular syntheses of nanoparticles have been reported. Klaus-Joerger and co-workers have shown that the bacterium Pseudomonas stutzeri AG259, isolated from a silver mine, is capable of reducing Ag⁺ to nanoparticles.² Sastry and co-workers have reported extracellular synthesis of silver and gold silver nanoparticles by the fungus Fusarium oxysporum and the actinomycete Thermomonospora sp., respectively.^{3,4} The same group has reported intracellular synthesis for gold and silver nanoparticles by fungus Verticillium sp. as well.⁵ Nair and Pradeep

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reported the growth of nanocrystals and nanoalloys using Lactobacillus.6

Interactions of transition metals with cells systems have been widely studied. Certain transition metal ions are essential for the function of most proteins involved in redox reactions. Additionally, metal ions also catalyze cytotoxic reactions within the cell system. Metal ion transporters act in concert with various organelles and cellular membranes to maintain the correct concentrations of various ions in different cellular compartments.^{7,8}

Cellular reduction has implications to nanoparticle synthesis,^{9–11} detoxification, and metal recovery.^{12–13} Here we report an approach to synthesize nanoparticles intracellularly using human cells. This study is the first of its kind and is relevant from the context of metal ionhuman cell interaction. More importantly, to our expectation, we also observed that there exists a significant difference between the response of cancer and noncancer cells toward the bio-reduction process, which may be attributed to differences in the cell metabolism and the kinetics of nanoparticle formation in the cells investigated. The differential ability with which nanoparticles are synthesized by cancer and normal cells can have implications to cancer diagnostics.

Experimental Details

Cell Culture. The experiments were conducted with following types of cell lines: HEK-293 (human embryonic kidney), HeLa (human cervical cancer), SiHa (human cervical cancer), and

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Figure 1. UV-visible spectra of the supernatant solution during incubation (200 μ L diluted to 1 mL). (a) HEK 293, (b) HeLa, (c) SiHa, and (d) SKNSH cells, all with 1 mM chloroaurate ion solution. (e) 1 mL solution from various cell lines after 96 h incubation. (f) HEK 293 cells with 10^{-4} M chloroaurate ion solution.

SKNSH (human neuroblastoma). HEK-293 cell is a nonmalignant kidney cell line, HeLa and SiHa cells are malignant cervical epithelial cell lines and SKNSH cell is a malignant neuroblastoma cell line.

Cells were grown to ~80% confluency starting with 3×10^5 cells per well in 6-well tissue culture plates (2 wells for each cell line) in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and incubated at 37°C in humidified atmosphere containing 5% CO₂.

Incubation with HAuCl₄ Solution. One of the important constituents of the growth medium is the pH indicator phenol red which imparts pink-red color to the growth medium and changes to yellow when growth medium gets acidified due to cellular metabolism. On day 0, growth medium was removed and the cells were washed twice with phosphate buffered saline (PBS). This was necessary as phenol red gives strong peak in the UV–visible spectrum at 560 nm which interferes with the characteristic nanoparticle signature. Also various chemical reagents such as salts and glucose present in the growth medium may cause reduction of chloroaurate ions. A total of 3 mL of 10^{-3} M HAuCl₄ solution prepared in PBS, pH 7.4 and filter sterilized using MILLEX GV Durapore PVDF membrane filter (pore size: 0.22 μ m), was added to each well. The cells were then kept in an incubator at 37 °C and 5% CO₂ atmosphere. The biotransfor-

mation was observed by periodic sampling of aliquots (200 μ L diluted to 1 mL) of the aqueous component and measuring the UV–visible spectrum of the solution using a Perkin-Elmer Lambda 25 UV–visible spectrophotometer. The absorbance values were compared over a period of 96 h. Optical images of the cells were acquired at regular intervals using an inverted Nikon Eclipse TE 300 microscope.

Cell Lysis. At the final hour (96 h) of the experiment, one well was devoted to the preparation of the cell lysate. After the incubation medium was removed, the cells were washed with PBS and were scrapped off the well surface using a cell scraper. Thereafter, the cell suspension in the well was transferred into a centrifuge tube and was sonicated using a Branson Sonifier 450 at a duty cycle of 50% and in output mode 1 by giving 3 pulses for 10 s duration, each pulse being separated by 20 s of ice cooling. This cell lysate was centrifuged at 500 g for 5 min at 4 °C, and the supernatant liquid was separated. Cell lysis was carried out to ascertain qualitatively the difference in number of nanoparticles present inside the cytoplasm and in the solution. UV-visible spectra of the solution obtained before and after lysis were compared.

Transmission Electron Microscopy. Cells of the other well were prepared simultaneously for transmission electron microscopy (TEM). Cells were detached with 0.05% trypsin-0.03%



Figure 2. (a) Photograph of HEK 293 cells before addition of gold ions (day 0, $10 \times$). (b) Photograph of HEK 293 cells with gold ions (day 2, $20 \times$).

EDTA followed by scraping with a cell scraper and were fixed in 1% glutaraldehyde in PBS. After fixation in 1% osmium tetroxide, cells were dehydrated in graded series of acetone and infiltrated and embedded in Araldite 502 and polymerized at 60 °C for 3 days. 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 transmission electron microscope (H 600) at an accelerating voltage of 75 kV.

X-ray diffraction data were obtained after lysing the cells (96 h since the start of the process) and plating the lysate on a glass slide. X-ray powder diffractograms were measured with a Shimadzu diffractometer with Cu K α radiation.

The possibility of reduction being done by various salts present in PBS was discounted by blank measurements, which involved monitoring the UV–visible spectra of chloroaurate ions mixed with PBS over 96 h.

Scanning near field optical microscopic (SNOM) images of ultramicrotome cuts (70 nm thick) were taken in the transmission mode with a Witec Alpha-SNOM instrument using 514.5 nm excitation through an aperture of <100 nm diameter.

Results and Discussion

The bioreduction of chloroaurate ions and subsequent growth of gold nanoparticles was monitored by periodic measuring of absorbance using a UV-visible spectrophotometer. Figure 1a-d demonstrates the steady increase in the absorbance at \sim 550 nm for all of the samples over a period of 96 h, and they suggest that the reduction process is slow, as expected for a biological event. The surface plasmon resonance band of gold appears in the range of 520-600 nm depending on the dielectric constant of the medium, particle size, type of capping agent, etc. To confirm the intracellular synthesis mechanism, cells were lysed after 96 h. The UV-visible spectrum of the supernatant liquid showed 4-5 times increase in absorbance (Figure 1a-d, topmost curve in all). A possible



Figure 3. X-ray diffractograms of lysed cells after treatment with gold ion solution for 96 h; (a) HEK 293 and (b) HeLa.

explanation for continuous growth in the UV-visible signatures is that nanoparticles, after synthesis, are transported from cytoplasm to the solution. It is possible that a part of the ion reduction process occurs on the membrane surface. It may be noted that the width of the UV-visible feature after lysis is much larger than that of the solution. As the particle size increases, the resonance broadens and shifts toward longer wavelengths in the large particle-size regime.¹⁴ It appears that larger particles are present inside the cells than in solution and are seen in UV-visible only after lysis. This phenomenon is more prominent in the case of malignant cell lines. Note that the cells are adherent on the well surface during the nanoparticle growth and cannot contribute to the data during the incubation.

The differences in response of the malignant and normal cell lines in terms of nanoparticle transport is evident in the spectra of 1 mL solutions, taken from each cell well, presented in Figure 1e. This essentially means that, from a quantitative perspective, normal cells transport more nanoparticles across the cell membrane, compared to the malignant ones. Additionally, the concentration dependence of the ionic reduction process is confirmed by negligible presence of gold nanoparticles in the well containing low ionic concentrations of 10^{-4} (Figure 1f) and 10^{-5} M (data not shown). The formation of nanoparticles within the cell is clear even at reduced concentration as the trace of the lysed cells shows distinct plasmon absorption. Results of blank measurements (data not shown) established that reduction is indeed a biological process. Additionally, no free Au³⁺ ions were detected in the medium after the end of 96 h.

The solution at the 96th hour became intensely purple in color, a characteristic of colloidal gold. Optical images

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Figure 4. Electron micrographs of growth of gold nanoparticles in HEK 293 (a–c), HeLa (d), SiHa (e), and SKNSH (f) cells. The region in the box of (b) is enlarged in (c). The black dots spread across the cytoplasm of the cells are gold nanoparticles in the range of 50-100 nm. Smaller particles are seen in the nucleus (e).

of the cells incubated with chloroaurate ion solution showed staining of the cells (Figure 2). Some cells had started floating in the solution possibly due to cell death. It is certainly unusual for cells to be viable without growth medium for such long durations. It may be noted that staining makes cell contour clearly observable which is an indication of intracellular synthesis of nanoparticles (Figure 2b).

Figure 3, panels a and b, shows the XRD patterns for HEK 293 and HeLa cell samples, obtained after lysing the cells (96 h since the start of the process) and plating the lysate on glass slides. They show the three prominent peaks which are characteristic of the gold lattice, namely (111), (200), and (220).

TEM images of the microtomed specimens clearly indicate the presence of nanoparticles within the cell (Figure 4a-f). To present features of the cell, only low magnification images are presented. Particles of irregular morphology, as large as 100 nm, are observed. Some of the large particles appear as aggregates. Aggregation of nanoparticles inside the cell could be effected by the presence of various salts inside the cytoplasm. Note that larger particles are seen in the UV-visible spectrum of the lysed cells. It is seen that the nanoparticles are present all over the cytoplasm. Nucleation of particles around the membranes of some of the cell bodies is observed (Figure 4c). It is important to see that particle growth is seen also in the nucleus. While the particles in the cytoplasm are



Figure 5. SNOM images of microtomed samples of SKNSH cells in (a) two and (b) three-dimensional views; (c) and (d) are the corresponding images for HEK cells. Scan areas were $20 \times 20 \ \mu m$ (SKNSH) and $35 \times 35 \ \mu m$ (HEK).

large, the particles within the nucleus are much smaller. This is especially noticeable in the SiHa cell (Figure 4e). Other cells also show the presence of nanoparticles in the nuclei at larger magnifications.

Scanning near-field optical micrographs of ultramicrotome cuts also revealed the presence of gold particles in the cytoplasm of the cells. Representative images of SKNSH and HEK-293 cells after 96 h incubation with gold ions are shown in Figure 5. The gold particles are seen as yellow dots distributed throughout the cytoplasm. The nucleus is seen to be featureless, without nanoparticles, primarily because the particle size is too small, below the resolution limit of our measurements (~50 nm).

There exists a difference in the UV-visible feature for the cancer and noncancer cells. This is noticeable in the spectra of the lysed cells and in the supernatant fluid. Further, the spectra for cancerous cells have significantly broadened after lysis, which suggests that the nature of the nanoparticles present inside and outside the cells are different. This may mean difference in morphology and particle size. This could be an indication of the difference in the nucleation rate between cancer and noncancer cells. It may also be noted that nucleation process occurs so as to decrease the exposed surface area of the nanoparticles.⁶

The exact mechanisms involved in the process of gold ion transport, reduction, formation of nanoparticles, and their subsequent growth are yet to be studied thoroughly. It is well-known that sugars and enzymes present on the cell membrane and within the cytoplasm can participate in the reduction process.¹⁵ It is well-known that all cells have a complex set of ion channels. This enables a cell to control the flow of ions in order to perform specific functions. Various anions carriers have been known to play a prominent role in transporting anions across the cell membrane.¹⁶ In this case, the transfer of ions across the membrane is either accomplished by diffusion via ion channel or by active transport. The reduced nanoparticle size in the nucleus may be due to the reduced concentration of the ions in the nucleus due to the longer diffusion length involved. A difference in ion transport mechanism across the nuclear membrane can also contribute to the reduced concentration.

Although the mechanism of reduction is not investigated in this study, we note that there are differences in the reduction process between cancer and noncancer cells. We attribute two possible reasons for this behavior. First, it is known that there are differences in the cell-surface carbohydrate distributions for the two kinds of cells.¹⁶ The difference in carbohydrate distribution is the explanation for the fact that normal cells stop growing when they contact each other in the process called contact inhibition, whereas cancer cells continue to grow even after touching each other (malignancy).¹⁷ Second, cancer cells acquire resistance to hypoxia¹⁸ which selects highly malignant cells during the progression of tumor. Under hypoxia, hypoxia inducible factor (HIF)-1 is stabilized and transactivates hundreds of genes including growth factor and their receptors as well as glycolytic enzymes. The increased glycolytic activity within the tumor cell compared to normal cells often help the cancers to metabolize or reduce ectopically introduced toxic compounds. The increased expression of glyoxalase-1 enzyme activity in most solid tumor cells compared to normal cells support

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this hypothesis.^{19,20} It may be noted that predominant surface reduction for malignant cells is unlikely because transport across cell membrane bears strong dependence on pore size of the transporting channel.

Summary and Conclusions

Our experiments show the intracellular growth of gold nanoparticles in human cells from chloroaurate ions. Nanoparticles were distributed throughout the cytoplasm and their dimensions vary up to 100 nm. There appears to be a difference in the reduction process between the cancer and noncancer cells investigated in this study. The mechanism of reduction is not elucidated yet, but it is believed that it happens as a result of cell metabolism. The diminished cellular response to low concentrations indicates that ion transport occurs through diffusion mechanism. Establishing mechanism needs further research which will help in understanding the kinetics of the cellular reduction process as well. Nanoparticles are also found in the nucleus, albeit they are much smaller in dimension. It is noteworthy to point out that once conclusive differences between cancer and noncancer cells with regard to metal ion reduction process is established for a broader set of cancer and noncancer cells, the difference in the growth characteristics of nanoparticles between the cancer and noncancer cells could be developed into a diagnostic tool.

Acknowledgment. We thank the Department of Science and Technology, Government of India for constantly supporting our research program on nanomaterials. C.S. thanks the Council of Scientific and Industrial Research (CSIR) for a research fellowship.

LA0519249

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