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Study of in vitro toxicity of glucose capped gold nanoparticles in malignant and normal cell lines

Harminder Kaur[1], Geetanjali Pujari[1], Asitikantha Sarma[1], Yogendra Kumar Mishra[2], Mi Kyung Jin [3], Bikesh K. Nirala[4], Nivedita K Gohil[4], Rainer Adelung[2], Devesh Kumar Avasthi[1*]

[1] *Inter University Accelerator Center, Aruna Asaf Ali Marg, New Delhi, 110067, India*
[2]*Functional Nanomaterials, Institute for Materials Science, University of Kiel, Kaiserstr. 2, Kiel, 24143, Germany*
[3]*Division of Nephrology and Hypertension, University of Kiel, Schittenhelmstr. 12, Kiel, 24105, Germany*
[4]*Centre for Biomedical Engineering, Indian Institute of Technology, New Delhi, 110016, India*

[*]Corresponding author. Tel: (+91) 11 26893955; E-mail: dka@iuac.res.in

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ABSTRACT

Gold nanoparticles (AuNPs) are potential candidates for targeted drug delivery, imaging and early detection of cancer cells due to their ability to bind with cancer cells. To ensure their safe use in various possible biomedical applications, it is essential to examine the cytotoxicity and biocompatibility of AuNPs before use. The present work aims to study the cytotoxicity of glucose capped gold nanoparticles (Glu-AuNPs) in several cell lines (HeLa, A549, Jurkat, L929 and HUVEC). The synthesized Glu-AuNPs, using β-D glucose as reducing as well as capping agent, were characterized by SPR and TEM/EDAX analysis. Internalization of Glu-AuNPs in cells was studied by cross sectional TEM imaging. The cytotoxicity of Glu-AuNPs was evaluated by means of colony formation and MTT assays. The present study reveals that Glu-AuNPs (7±2 nm diameter) are non-toxic to the above-mentioned five cell lines, which are cancerous cell lines except HUVEC. Therefore Glu-AuNPs (around 7nm) can be explored for various bio-medical applications and can be of importance for therapeutic applications as evident from enhancement in radiosensitization from our previous work. Copyright © 2013 VBRI press.

Keywords: Glucose capped gold nanoparticles (Glu-AuNPs), Cytotoxicity, Colony forming assay, MTT assay.

Harminder Kaur obtained master degree in Biochemistry from Department of Biochemistry, Kurukshetra University (Kurukshetra) India in 2005. Her research work is focused on the biosynthesis and characterization of nanoparticles as well as evaluation of radiosensitization in AuNPs treatment treated cells after irradiation with heavy ions.

Asitikantha Sarma, born 26th March 1962, Ph.D. in experimental Nuclear Physics and Heading the High LET Radiation Biology programme of Inter University Accelerator Centre, New Delhi. His research area is focused on Molecular Radiation Biology.

Devesh Kumar Avasthi is Group Leader for materials science and radiation biology at Inter University Accelerator Centre, (formerly known as Nuclear Science Centre), New Delhi. He did Ph. D. from Panjab University Chandigarh in 1982. After serving in Defence Laboratory for about three years, he joined Nuclear Science Centre in 1986, an inter university accelerator based facility. He implemented elastic recoil detection analysis (ERDA) technique for light element depth profiling. Later a gaseous telescope detector was designed, fabricated and installed to enhance the capabilities of ERDA, which was used for electronic sputtering measurements. The facilities for thin film development and vacuum laboratory were developed to take care of need of accelerator. The most recent developments have been an atom beam sputtering set up for synthesis of nanocomposite thin films, in-situ ERDA, In-situ XRD, in-situ QMA in beam line. His main interest is ion beams for analysis, modification of materials, synthesis and engineering the nanostructures. His current research interest is on application of nanomaterials in radition biology. He had major research projects under 'Intensifying Research in High Priority Area' scheme and currently a project under 'Nano Mission' funded by Department of Science and Technology, Government of India.

Introduction

Gold nanoparticles (AuNPs) are promising candidates for various biomedical applications in diagnostic and therapeutics **[1-3]**. Employing AuNPs in combination with surface plasmon resonance (SPR) and surface enhanced Raman scattering (SERS) enable an imaging system with greater sensitivity and biosensing properties, respectively **[4-10]**. They are also being employed for therapeutic applications such as targeted drug delivery and radiosensitization in cancer therapy **[11-14]**. We recently showed enhancement in radiosensitization in Glu-AuNPs treated HeLa (human cervical cancer) cell line due to presence of AuNPs inside the HeLa cells **[15].**

One of the most attractive properties of AuNPs is their ability to conjugate with various biomolecules due to the presence of 6s free electrons in conduction band of nano-gold **[16]**. AuNPs can be synthesized by various approaches for being utilized in diverse applications **[17-20]**. AuNPs capped with suitable biomolecules facilitate their cellular uptake, thus mediate targeting certain cell population such as cancer cells, thereby becoming an attractive tool for detection and therapy of malignant diseases **[21-26]**. AuNPs have also been used to deliver anti-tumor agents such as tumor necrosis factor (TNF) or paclitaxel at the site of the tumour by the enhanced permeability and retention (EPR) effect **[27]**. Salem et al. and Thomas and Klibanov have explored the potential of AuNPs to act as a non-viral-based gene delivery system **[28-29]**.

The potential use of AuNPs in biomedical applications, raises an interest in risk assessment of these particles. Thus, it is essential to evaluate the cytotoxicity of AuNPs to ensure their safe use. There are conflicting reports on this particular issue. Several studies have demonstrated that the AuNPs are biologically inert and non-toxic **[30-32]**. Sadaukas et al. reported a nontoxic behaviour of AuNPs in mice **[33]**. Similarly Connor et al. also demonstrated a non-toxic effect of AuNPs on a leukemia cell line K562 **[34]**. On the other hand, Patra et al. observed the cytotoxicity of AuNPs in a human lung cancerous cell line A549 and reported the induction of concentration- dependent apoptosis **[35]**. It has also been observed that capping of sodium citrate on AuNPs triggers a loss of cell viability as well as alteration in the cell proliferation of alveolar cell lines **[36-37]**. Furthermore, 13 nm-sized gold nanoparticles coated with PEG induced an acute inflammation and apoptosis in the murine liver cells as studied by Cho et al **[38]**. The charge-dependent cytotoxicity of AuNPs has been studied by Goodman et al **[30]**. They reported the cationic gold nanospheres of 2 nm in diameter to be toxic while similar nanoparticles with negatively charged surface were non-toxic under the same concentrations for similar cell line because the cationic AuNPs interact with the negatively charged cellular membrane and results in its disruption. In summary, it has been established from the previous studies that the cytotoxicity induced by gold nanoparticles depends on size, shape, functional group, charge as well as on the method of cellular uptake **[39-41]**. This renders the cytotoxicity study of AuNPs as a burning field of investigation prior to their use in biomedical applications. Although various aspects of AuNPs have been studied, still significant efforts of experimental consideration are required for toxicological evaluation which has been unsatisfactory and insufficiently addressed.

From the previous studies, it is evident that capping of gold nanoparticles with biomolecules increases their uptake in cytoplasm of cells. As glucose is the main source of metabolic energy, its uptake by cancer cells increases as compared to normal cells. Further cancer cells are metabolically more active than normal cells, therefore larger number of glucose molecules are internalized via Glucose transporter (GLUT) receptors present on the cancer cell surface **[42, 22]**. The glucose tagging is likely to facilitate the entry of AuNPs into the cells. Keeping this in view, AuNPs capped with glucose were synthesized for internalization in the cells. From our previous work, it is evident that Glu-AuNPs resulted in enhancement in radiosensitization of HeLa cells following C^{6+} ion irradiation **[15]**. Thus they can be explored for therapeutic gain and other biological applications such as imaging and bio-sensing. Therefore, our objective was to study toxicity effect of Glu-AuNPs in various cells (normal and cancerous cells) for validating its use for various possible bio-medical and therapeutic applications. For this purpose we treated HeLa (human cervical cancer cell line), A549 (human alveolar cancer cell line) L929 (mouse connective tissue fibrosarcoma), Jurkat cells (human T cell lymphoma) and HUVEC (primary human umbilical vein endothelial cells) at various concentrations of Glu-AuNPs and evaluated their toxicity by colony forming and MTT assay.

Experimental

Materials

$HAuCl_4.3H_2O$, β-D glucose and sodium hydroxide were procured from SIGMA (US). HeLa and A549 were purchased from NCCS, Pune. While L929 cells were sourced from (GIBCO, 3rd passage). Non-adherent Jurkat cells (3rd passage) were cultured in RPMI 1640 (Invitrogen) supplemented with FCS (Hyclone, US). For HeLa, A549 and L929 cells, the cell culture medium was availed from DMEM (Invitrogen, Darmstadt, Germany) which was supplemented with fetal bovine serum (Hyclone, US) and penicillin-streptomycin (Hyclone, US). Primary HUVEC (2nd passage) were maintained in complete media (PromoCell, Germany) containing 20% FBS and growth supplements at 37ºC (5% CO_2) in a humidified CO_2 incubator.

Synthesis of Glu-AuNPs

Glucose capped AuNPs were synthesized by chemical route **[43]** using $HAuCl_4$ and β-D glucose. The aqueous solution of 0.05M $HAuCl_4 \cdot 3H_2O$ was added to β-D-glucose (0.03 M) and stirred for 30 minutes. Subsequently, 0.5 M sodium hydroxide (NaOH) was added for completing reduction of gold salt. This resulted in a red coloured solution of Glu-AuNPs. β-D glucose acted as both reducing as well as capping agent in the AuNP synthesis. Glucose capping of AuNPs was evaluated by Raman spectroscopic analysis using InVia Raman Microscope (Renishaw, UK). For removal of unbound glucose from the solution of Glu-AuNPs, it was centrifuged till Glu-AuNPs settled at the

bottom separated by liquid layer at top. The liquid was pippetted out without disturbing settled Glu-AuNPs. This was further resuspended in deionised water and this process of removing unbound glucose was repeated thrice. The Raman spectroscopy of such triply washed Glu-AuNPs solution on Si substrate was carried out to confirm the capping of glucose on AuNPs.

Characterization of Glu-AuNPs

The synthesis of AuNPs was confirmed by UV-visible absorption spectroscopy (Hitachi U-3300) which showed a surface plasmon resonance (SPR) absorbance peak around 540 nm. The red colour of the as prepared solution also indicated the presence of AuNPs. In order to evaluate the dimensions of AuNPs, transmission electron microscopic (TEM) measurements were performed using JEOL 2100F TEM microscope at Advanced Instrumentation Research Facility (JNU, New Delhi). The EDX analysis was performed to verify the presence of gold content in AuNPs.

Cell culture

HeLa (3^{rd} passage) and A549 (3^{rd} passage) cells were maintained in DMEM supplemented with fetal bovine serum and penicillin-streptomycin while L929 cells (3^{rd} passage) were cultured in DMEM supplemented with 10% foetal bovine serum and 1% penicillin-streptomycin. Non-adherent Jurkat cells (3^{rd} passage) were cultured in RPMI 1640 supplemented with FCS and penicillin-streptomycin. Primary HUVEC (2^{nd} passage) were maintained in complete media containing 20% FBS and growth supplements at 37ºC (5% CO_2) in a humidified CO_2 incubator.

Treatment of cells with Glu-AuNPs

A549, HeLa, HUVEC, L929 and Jurkat cells were treated with Glu-AuNPs at various concentrations of Glu-AuNPs ranging from 5.5µM to 30.0µM per ml of culture medium for 6 to 48 hours. Additionally the HUVEC cells (primary cells) were exposed to Glu-AuNPs for 48 hours to check for any chronic effect. We utilized autoclaved Glu-AuNPs throughout cell treatment procedure but without any centrifugation. After the designated time period of exposure, the medium with Glu-AuNPs was discarded and all cells were washed thrice with phosphate buffer saline (PBS) to remove free Glu-AuNPs.

TEM imaging of cell lines

To evaluate localization of Glu-AuNPs inside the cells, TEM characterization of ultra-thin section of control HeLa and A549 cells (without Glu-AuNPs treatment) along with Glu-AuNPs treated HeLa and A549 cells were performed using JEOL 2100F TEM apparatus at Advance Instrumentation Research Facility (AIRF), Jawaharlal Nehru University (New Delhi, India). Prior to TEM visualization of cells treated with Glu-AuNPs, the cell fixation was done in 2.5% glutaraldehyde in 0.1M Phosphate buffer (pH 7.2). The fixed cells were stained with 1% PTA (Phospho Tungstic Acid).

Colony forming and MTT assay to assess toxicity of Glu-AuNPs

Toxicity effect of Glu-AuNPs was studied using Colony forming for HeLa, A549 and L929 cells and MTT assay for HUVEC, HeLa, A549 and non-adherent Jurkat cells.

a. Colony forming assay: In order to evaluate the cytotoxicity of Glu-AuNP on HeLa, A549 and L929 cells, colony forming assay was performed. For this, these cell lines treated with different concentrations of Glu-AuNPs (5.5µM to 30.0µM/ml) were trypsinised. All the three Glu-AuNPs treated cell lines were counted using Countess automated cell counter (Invitrogen, USA) and 1000 cells were seeded in 25 cm^2 cell culture flasks. Untreated HeLa, A549 and L929 cells were included as negative control (treated with PBS). The cells were then incubated for 10-15 days to score visible colonies. Colonies were fixed with 0.25% methylene blue in 75% carbinol. Thereafter colonies were counted, with a criterion that a colony must have at least 50 cells. The plating efficiency (PE) is calculated as the percent number of cells which grow into colonies in control group for normalizing the colony counts from treated group. Surviving fraction (SF) is calculated as colonies counted divided by the number of colonies plated with a correction for the plating efficiency. The experiment was performed in triplicate for all three cell lines and average of data was included as final data with standard deviation as error bar.

b. MTT assay: MTT assay was performed to study the cell viability of HUVECs and non-adherent Jurkat cells in presence of Glu-AuNPs. Briefly HeLa, A549, HUVEC and Jurkat cells were seeded in 96 well plate at a density of 20000 cells per well prior to treatment with Glu-AuNPs at various concentrations (5.5µM to 30.0µM/ml). The Glu-AuNPs solution was used as positive control while complete culture medium was used as negative control. Then 25 µl of MTT reagent (stock solution - 5 mg/ml in PBS) was added in each well and incubated for 4 h minutes in incubator (37ºC, 5% CO_2 & 95 % humidity). Afterwards 10µl of solubilizing solution (50% (v/v) Dimethylformamide, 80% (v/v) acetic acid, 20% SDS and 0.025N HCl) was added and incubated for 4 h in incubator. Thereafter 180 µl of sample was transferred to another 96 well plate and absorbance was recorded using Biotek (EON, US) plate reader at 570 nm. During data recording, background was also calculated and subtracted from final reading. Experiments were performed in triplicate and average value for each data point was plotted with standard deviation as error bar.

Results

The Glu-AuNPs have been characterised by UV-Vis spectroscopy and spectrum is shown in **Fig. 1**(**a**). The strong absorbance peak is seen at 540 nm which is a characteristic of surface plasmon resonance of AuNPs and therefore it shows the presence of AuNPs.

During AuNP synthesis, β–D Glucose acts as reducing (Au^{3+} to Au^0) as well as capping agent in alkaline aqueous environment due to addition of NaOH. Glucose is bound to

surface of nanogold by hydrogen bonding of hydroxyl (–OH) group it.

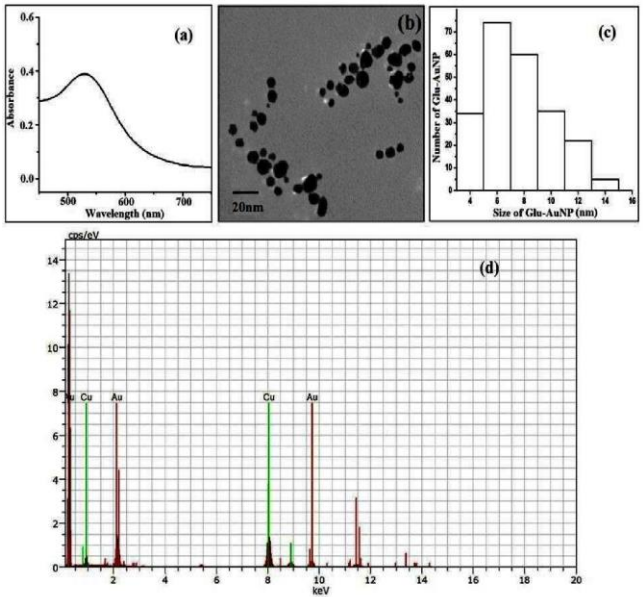

Fig. 1. (a) UV-visible absorption spectrum of Glu-AuNPs showing SPR peak at 540 nm. 1(b) shows the high magnification TEM image of a spherical gold nanoparticle. 1(c) size distribution curve respectively. 1(d) shows EDX measurements to evaluate presence of Au.

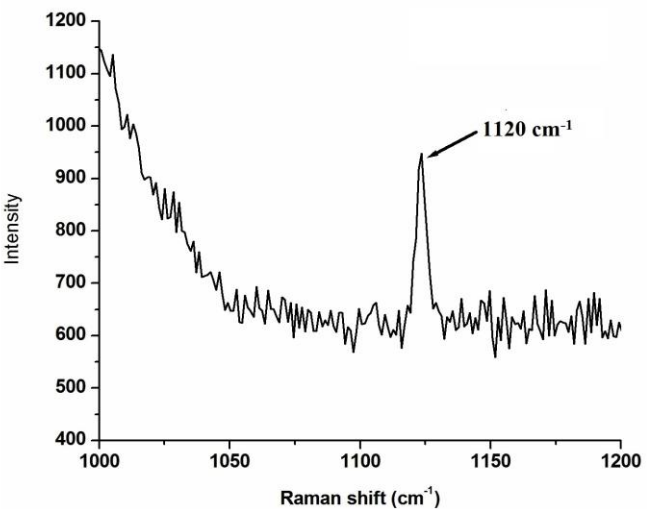

Fig. 2. Raman spectrum of Glu-AuNPs.

Transmission electron microscopy is employed to evaluate dimensions of Glu-AuNPs (**Fig. 1(b)**. TEM micrograph of dried suspension of the freshly prepared Glu-AuNPs on carbon coated copper grid is shown in **Fig. 1(b)**. From TEM micrograph, it could be clearly seen that Glu-AuNPs are spherical in shape and their size varies from around 4-15 nm. The maximum number of Glu-AuNPs are having size below 10nm and their average size is measured to be 7 ± 2 nm. The distribution of nanoparticle size is shown in **Fig. 1(c)**. To confirm the presence of Au in nanoparticle, EDAX measurement has also been performed and shown in **Fig. 1(d)**, which verifies that nanoparticles are made of Au. Glucose capping of AuNPs was ensured by Raman spectroscopic analysis of triply washed Glu-AuNPs (**Fig. 2**), reported recently by us **[15]**. Characteristic peak of glucose is seen around 1125 cm^{-1} in the Raman spectrum of Glu-AuNPs **[44]**. To study localization of Glu-AuNPs inside the cells, TEM analysis of ultrathin sections of HeLa and A549 cells treated with Glu-AuNPs has been carried out.

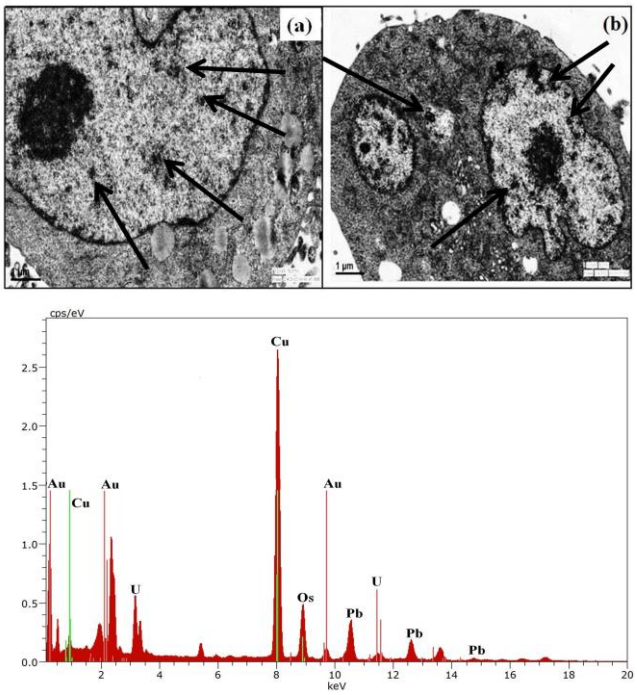

Fig. 3. TEM image of ultrathin sections of (a) HeLa cells exposed to Glu-AuNPs for 6 hours; (b) A549 cells exposed to Glu-AuNPs for 6 hours; **(c)** EDAX analysis.

Fig. 3 (a and **b)** shows TEM micrograph of HeLa and A549 cells after exposure to Glu-AuNPs for 6 hours. It is clearly evident from **Fig. 3** (**a** and **b**) that the Glu-AuNPs localize into the cytoplasm and nucleus of both cell lines. EDAX analysis (**Fig. 3**(**c**)) confirms the presence of Au in Glu-AuNPs seen as dark spots in **Fig. 3** (**a** and **b**). For the evaluation of toxicity of glucose capped AuNPs towards cells, the colony forming assay has been carried out.

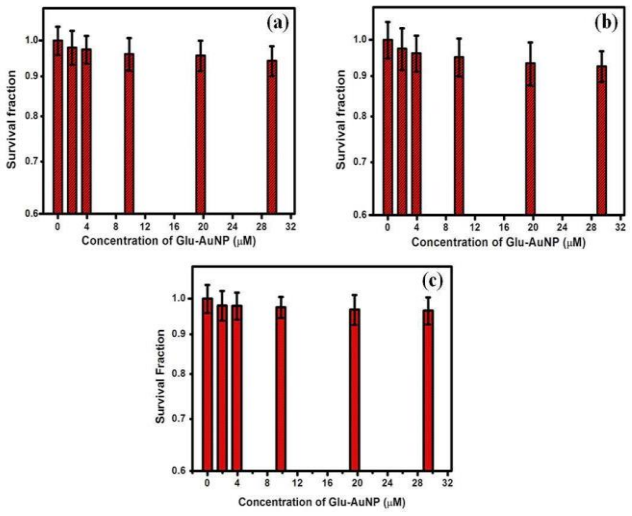

Fig. 4. Colony forming assay of (a) A549, (b) L929 and (c) HeLa cells exposed to Glu-AuNPs for 6 hours

Colony forming assay is one of the standard method in toxicological studies for verifying clonogenic survival ability of cells exposed to potential medicinal or chemical agent under study. Treatment of A549 and HeLa cells with Glu-AuNP for 6–48 hours upto 29.2μM/ml does not induce any toxicity as evident from the survival fraction values of 94.2, 96.2 and 92.6 respectively for these cell lines. Thus there is no significant loss of cell viability in A549, L929 and HeLa cell lines as shown in **Fig. 4** (**a, b** & **c**), as observed by colony forming assay. Furthermore cell viability as well as cytotoxicity evaluation in Jurkat cells (non-adherent cell line) along with HUVEC cells (primary endothelial cells) after Glu-AuNP treatment was carried out using MTT assay.

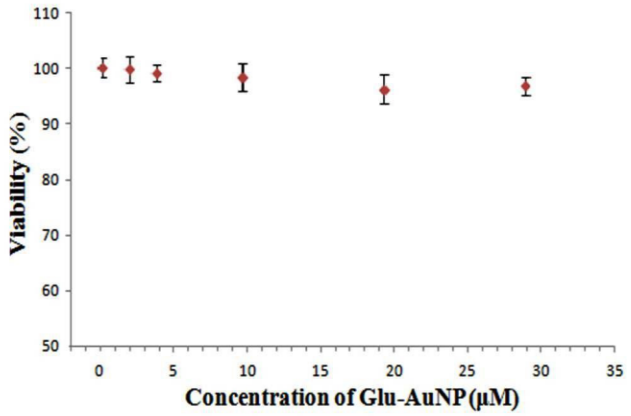

Fig. 5. MTT assay of Jurkat cells exposed to Glu-AuNPs for 6 hours.

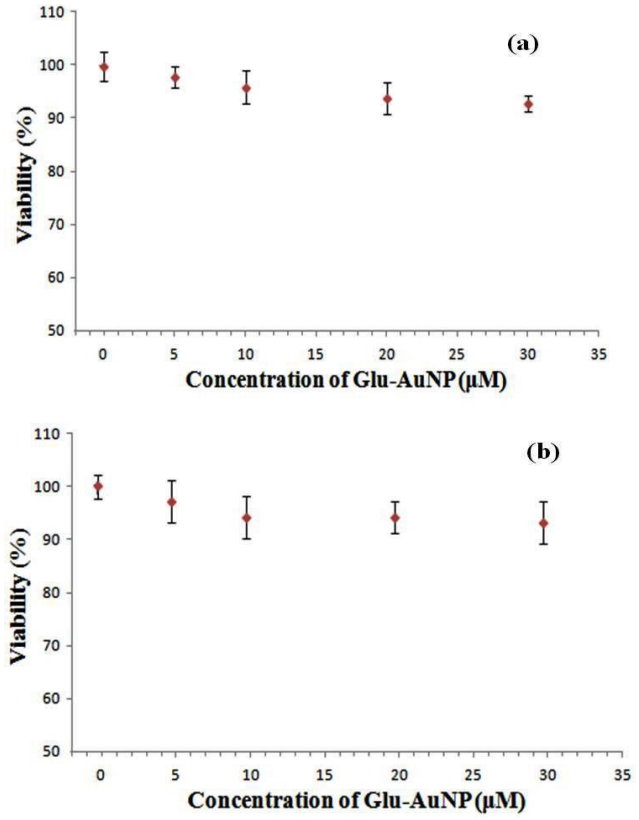

Fig. 6. MTT assay of HUVEC cells exposed to Glu-AuNPs for (a) 6 hours and (b) 48 hours.

The results from MTT assay show no cytotoxicity in Jurkat and HUVEC cells as given in **Fig. 5** and **6** respectively. From MTT assay measurements in Jurkat cells (**Fig. 5**) following Glu-AuNP treatment, cell viability is not affected even at higher concentration (29.4μM). Similarly HUVEC cells do not show much change in cell viability even after 48 hours as seen by MTT assay **(Fig 6** (**a** and **b**).

The time duration of 48 hours selected in case of HUVECs is based on the maximum residence time that the particles can have *in-vivo* when used as drug carriers for targeted delivery systems. It has been observed that with the highest concentration *viz.* 30μM of the Glu-AuNP, 92% and 91% cells remain viable after incubation for 6 and 48 hrs respectively while at 10μM of Glu-AuNP, 94% are viable. Further confirmation of non-cytotoxicity of Glu-AuNPs in HeLa and A549cell lines upto 48 hours of treatment was carried out using MTT assay. From **Fig. 7** and **8**, it is evident that Glu-AuNPs are compatible for HeLa and A549 cell lines even upto 48 hours. This coincides well with survival assay data (**Fig. 4** (**a** and **4c**)).

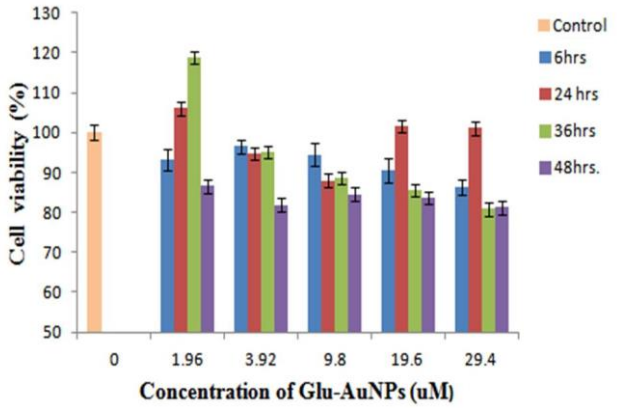

Fig. 7. MTT assay of A549 cells exposed to Glu-AuNPs for 6 - 48 hours.

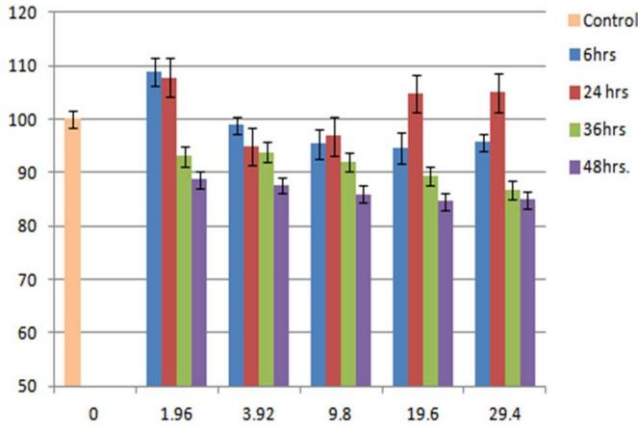

Fig. 8. MTT assay of HeLa cells exposed to Glu-AuNPs for 6 - 48 hours.

Discussion

It has been reported that the AuNP treatment of certain cells might result in reactive oxygen species (ROS) production, cytotoxicity, cytokinesis arrest, and apoptosis **[45-46]**. Patra et al. demonstrated the cell-selective cytotoxicity and apoptosis with 33 nm AuNPs has been

observed in A549 human lung cancer cell lines while being non-toxic in BHK 21 normal kidney cells. The induction of cell death in the human alveolar carcinoma cell line A549 has been shown to involve concentration dependence of the AuNPs **[35]**. Although such responses are not observed in certain cells e.g., human leukemia, it may not hold universal for all cells or cell types **[34]**. Kong et al. demonstrated accumulation of 10.8nm Glu-AuNPs in cytoplasm of MCF-7 (cancer cell) and MCF-10 (normal cell) without any sign of toxicity **[23]**. Similarly, Yang et al. also carried out Glu-AuNPs uptake studies in MCF-7. They demonstrated involvement of GLUT-1 mediated transportation of Glu-AuNPs and accumulation of AuNPs in the cytoplasm **[26]**. Furthermore, Nativo et al. observed the penetration of 16 nm citrate capped AuNPs in nucleus of human fibroblasts epithelial cells using cell penetrating peptides in combination with nuclear-localization-signal (NLS) peptides **[47]**.

Glu-AuNPs play pivotal role in targeting cancer cell that over-express of GLUT-1 receptors over their surface. In the present study, Glu-AuNPs treated HeLa and A549 cells show the accumulation of Glu-AuNPs in the cytoplasm and cell nucleus as shown in figure 3. It is revealed from study carried out by Patra et al. **[35]** that 33nm AuNPs exhibit cytotoxic behaviour in A549 cells whereas present work shows non-toxic behaviour of 5-9 nm Glu-AuNPs towards A549 cells even at higher concentrations (29μM).

We undertook study on the toxicity of AuNPs in endothelial cells especially for the following reason. Cancer drugs are mostly administered intravenously or in some cases intra-tumorally. When AuNPs are used as drug carriers for delivery of drugs intravenously, they go through the blood vessels and thus endothelial cells which lie at the blood-wall interface are directly exposed to all material passing through the vessel. In this context it is significant that at the concentration of AuNPs used, these should be non-toxic to the endothelial cells even after prolonged exposure. In the present study the Glu-AuNPs are found to be non-toxic and therefore, it is of relevance and importance for various applications. Huvec cells (2^{nd} passage) are primary cells which retain both *in-vivo* physiological and morphological characteristics. While on other hand, the cells derived from sub-culturing of established cell lines often undergo loss of cell characteristics and are more resistant. The toxicity studies in Glu-AuNPs treated HUVEC cells is of great importance as it is mandatory to observe toxicity in primary cells along with established cell lines prior to use of Glu-AuNPs for diagnostic and therapeutic applications.

The present study shows that Glu-AuNPs which are stable upto several months due to effective capping by glucose moieties. The strong bonding of glucose onto AuNP surface results from involvement of $-O^-$ group on Au surface and –OH group over glucose molecule. The alkaline environment created by addition of sodium hydroxide facilitates this reaction and reduction by excessive amount of glucose as compared to gold content generates small sized AuNPs. These Glu-AuNPs of 5-9 nm size are appropriate for imaging and therapeutic applications as they can be easily internalized inside the cells. It has been reported from previous studies that commonly employed reducing agents such as sodium citrate, PEG and sodium borohydate have shown toxicity to cells **[36-38]**. Use of glucose as reducing and capping agent eliminates use of other toxic chemicals for reduction of gold salt to gold nanoparticles. Therefore Glu-AuNPs used by us proved to be non-toxic to various cells of different origin (HeLa, A549, HUVEC, L929 and Jurkat cells) as evident from MTT assay and colony forming assay.

Conclusion

It can be concluded from the present study that the Glu-AuNPs of size 5-9 nm were non-toxic to both normal (HUVEC) as well as cancerous cells (A549, HeLa, Jurkat and L929 cells) upto 29μM concentration when treated for 6 to 48 hours. The absence of cyto-toxicity in our experiments may be explained by glucose capping which is a metabolic substrate for energy harvesting pathway in all cells under physiological conditions. The study validates that the Glu-AuNPs are suitable candidates for biomedical applications such as diagnostics and therapeutics.

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