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Determining the Size Dependence of Colloidal Gold Nanoparticle Uptake in a Tumor-like Interface (Hypoxic)

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ABSTRACT

Colloidal gold nanoparticles (GNPs) are being used as drug delivery vehicles and radiation dose enhancers in cancer therapy. Oxygen concentration in human tumours is highly heterogeneous with many regions at very low levels of oxygen (hypoxia). A majority of tumours contain regions with oxygen pressure values of less than 0.7% in the gas phase. The purpose of this study was to investigate how the size of the NPs affects their uptake process in a tumour-like hypoxic environment. We used GNPs of diameter 15, 50, and 74 nm, and carried out our experiment under 0.2% (hypoxic) and 21% (normoxic) oxygen levels using MCF-7 and HeLa cells. Our results showed that NPs of size 50 nm had the highest uptake following prolonged exposure to hypoxia. There was no significant toxicity introduced by NPs under hypoxic conditions. These findings will play a vital role in the optimization of GNP-based therapeutics in cancer treatment.

The ultimate or fundamental goal of nanoparticle (NP) based platforms is the successful targeted delivery and monitoring of therapeutics to tumors, while causing minimal damage to normal tissue and side effects to the patient. Among other NPs, colloidal gold NPs (GNPs) are being explored as a model NP-system for cancer research due to their ability to act as both a radiosensitizer and drug carrier in cancer therapy [1–3]. Previous studies have shown that the size of the NP matters [4–6]. For example, GNPs of size 50 nm showed the highest radiation dose enhancement among NPs of sizes between 14 and 74 nm [4]. Most of these studies were performed with properly oxygenated (normoxic) cells. However, if we were to use these NPs effectively for cancer therapeutic applications, it is essential to understand their uptake behavior in a tumor-like environment, such as hypoxia.

It has been shown that low levels of oxygenation (hypoxia), commonly present in solid tumors, protect cells from death by irradiation [7]. For example, damage to DNA is created via direct ionization from radiation, or is induced by the interaction with free radicals (e.g. hydroxyl radical) formed by the ionization of water surrounding the DNA. If oxygen is available, it can react with the broken ends of DNA, thereby creating stable organic peroxides. This type of DNA damage cannot be easily repaired. However, the damage is more readily repairable in the absence of molecular oxygen which would lead to less damage following radiation or chemotherapy [8,9]. One of the primary reasons for cancer recurrence is that these hypoxic cells can survive the treatment. GNPs are being explored to overcome the resistance by these hypoxic cells since they can be used in combined therapeutics of radiation therapy and chemotherapy [2]. However, it is not known how the GNP-based therapeutic response would modulate in a real tumor where hypoxia is present. If we were to use GNPs for improved cancer therapeutics, it is necessary to understand their behavior under hypoxia.

A majority of solid tumors contain regions with O_2 pressure values of less than 0.7% O_2 in the gas phase, while the partial pressure of oxygen of normal tissues is about 4–7% O_2 in the gas phase [10–12]. We conducted our experiments under 0.2% O_2 level. The effect of the size of colloidal GNPs on their cellular uptake is known under normoxic conditions [4]. For example, colloidal GNPs of size 50 nm have the highest cell uptake among the size range 14–74 nm. However, most of the cancer cells in the solid tumor are hypoxic. It is not known yet how the size of colloidal GNPs affects their cellular uptake in a real tumor-like environment (hypoxic). Hence, the goal of this study is to investigate how the size of colloidal GNPs affects their cellular uptake in a

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Diameter of GNPs (nm)	As made gold		GNPs in tissue culture media (incubated in normoxic chamber for 24 hours)		GNPs in tissue culture media (incubated in hypoxic chamber for 24 hours)	
	Hydrodynamic diameter (nm)	UV visible wavelength (nm)	Hydrodynamic diameter (nm)	UV visible wavelength (nm)	Hydrodynamic diameter (nm)	UV visible wavelength (nm)
15	18 .3 ± 4.3	519	23.2 ± 3.1	523	24.1 ± 4.6	524
50	55.2 ± 5.5	535	61.8 ± 6.1	540	61.2 ± 5.6	539
70	76.4 ± 7.2	550	80.7 ± 6.8	554	81.2 ± 7.3	555

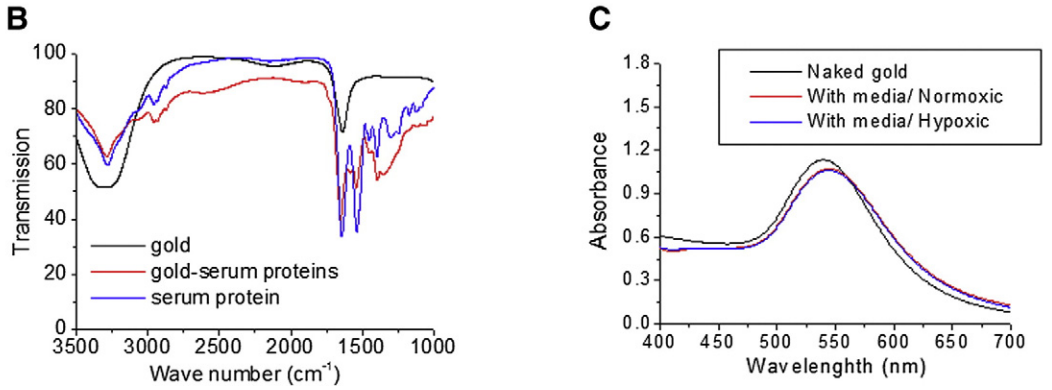

Fig. 1. Characterization of colloidal GNPs. A) Hydrodynamic diameter and UV visible peak wavelength of as-made GNPs and GNPs incubated with FBS supplemented media for duration of 24 h under normoxic and hypoxic (0.2% O_2) conditions, respectively. B–C) FTIR spectra and UV visible spectra of naked 50 nm GNPs and GNPs incubated with FBS supplemented media for duration of 24 h in normoxic and hypoxic (0.2% O_2) conditions, respectively.

hypoxic tumor environment to improve the bio-nano interface. If there is an optimum NP size, we can use that particular size for an improved outcome in therapeutic applications. For example, colloidal GNPs are being explored as radiation sensitizers in radiation therapy and drug carriers in chemotherapy [13]. Hence, it is important to evaluate the size-dependent uptake of colloidal GNPs in a real tumor-like environment, if we were to use them for such therapeutic applications. This article demonstrates how colloidal GNPs can be used to optimize the bio-nano interface in a real tumor-like environment (hypoxic), since their size and surface properties can be tailored easily.

GNPs of diameter 15, 50, and 70 nm were synthesized using the citrate reduction method. Colloidal GNPs were characterized by UV–vis spectroscopy, Dynamic Light Scattering (DLS), and Transmission Electron Microscopy (TEM) imaging. To study the effect of the hypoxic environment on the stability of NPs, they were kept in a hypoxia chamber for 24 h. UV–vis spectroscopy and DLS measurements were performed to investigate the changing characteristics of the NPs. GNPs were also incubated in the tissue culture media supplemented with FBS (Fetal Bovine Serum) under hypoxic and normoxic conditions for 24 h. The medium can have a profound influence on particle uptake

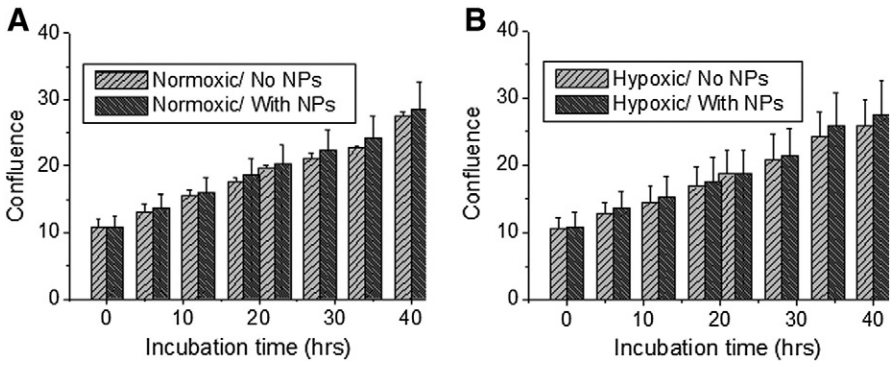

Fig. 2. Evaluation of the toxicity of GNPs. A–B) The toxicity induced by NPs was measured by monitoring cell proliferation for MCF-7 cells in normoxic and hypoxic conditions, respectively. The concentration of NPs used was 0.6 nmol. All the results are the mean of three independent experiments ± SE.

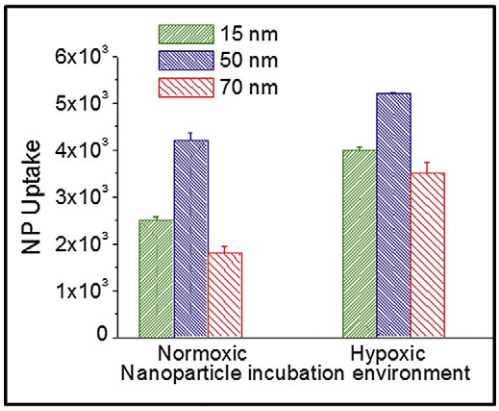

Fig. 3. Cellular uptake of GNPs in normoxic and hypoxic cells. The NP uptake in MCF-7 cells which were exposed to hypoxic conditions for 18 h prior to NP addition. The cells exposed to hypoxia had higher NP uptake compared to normoxic cells. NPs of diameter 50 nm had the highest uptake under normoxic and hypoxic conditions. All results are the mean of three independent experiments ± SE.

[14]. Hence, we measured the change in the size of the NPs in the tissue culture media under normoxic and hypoxic conditions for 24 h. The hydrodynamic diameter of the GNPs increased and the peak wavelength of the UV visible spectrum was red shifted for all three sizes (Fig. 1A). This increase in size was due to the attachment of serum proteins in the media onto the surface of GNPs [4,15]. However, there was no significant difference in the size of the GNPs incubated under hypoxic and normoxic conditions. This was further confirmed by UV visible spectroscopy and Fourier Transform Infrared (FTIR) spectroscopy. The data is shown for GNPs of size 50 nm (Fig. 1B, C). Based on these results, we can conclude that NPs are stable under normoxic and hypoxic conditions.

The toxicity introduced by NPs of different sizes was measured by comparing the cell proliferation rate with and without NPs under both normoxic and hypoxic (0.2% O_2) conditions for over 42 h. HeLa and MCF-7 cells were seeded with 0.02×10^6 cell density in 24-well dishes. Once the cells adhered to the bottom of the dishes, a group of dishes and a suspension of GNPs were transferred to the hypoxia chamber. After four hours, GNPs were added to one set of dishes in the normoxic and hypoxic chambers. One set of dishes without any added GNPs was used as a form of control in each chamber. The concentration of GNPs used was 0.6 nmol since the same concentration was used for our cell uptake studies. The cell proliferation was monitored with IncuCyte™ Kinetic Live Cell Imaging System with a 2-hour time interval for 42 h. The results are presented in Fig. 2 for the MCF-7 cell line under normoxia and hypoxia. There was no significant reduction in cell proliferation observed for both cell lines treated with GNPs under hypoxia and normoxia, as compared to the control where cells were not treated with GNPs. Hence, there was no significant induced toxicity due to the presence of GNPs within the cells.

Previous studies have shown that the cellular uptake of NPs is size dependent [4,16]. NPs of diameter 50 nm have the highest cell uptake among NP sizes between 14 and 74 nm. However, this is for properly oxygenated (normoxic) cells. The purpose of this study is to investigate how the size of NPs affects their cellular uptake in a tumor-like (hypoxic) environment. For quantification of NP uptake, cells were incubated in the hypoxia and normoxic chambers for 18 h prior to NP addition. The cells were then incubated with NPs for 24 h. Following incubation, the cells were trypsinized, counted, and processed for quantification purposes as described in our previous studies [17]. One of the major

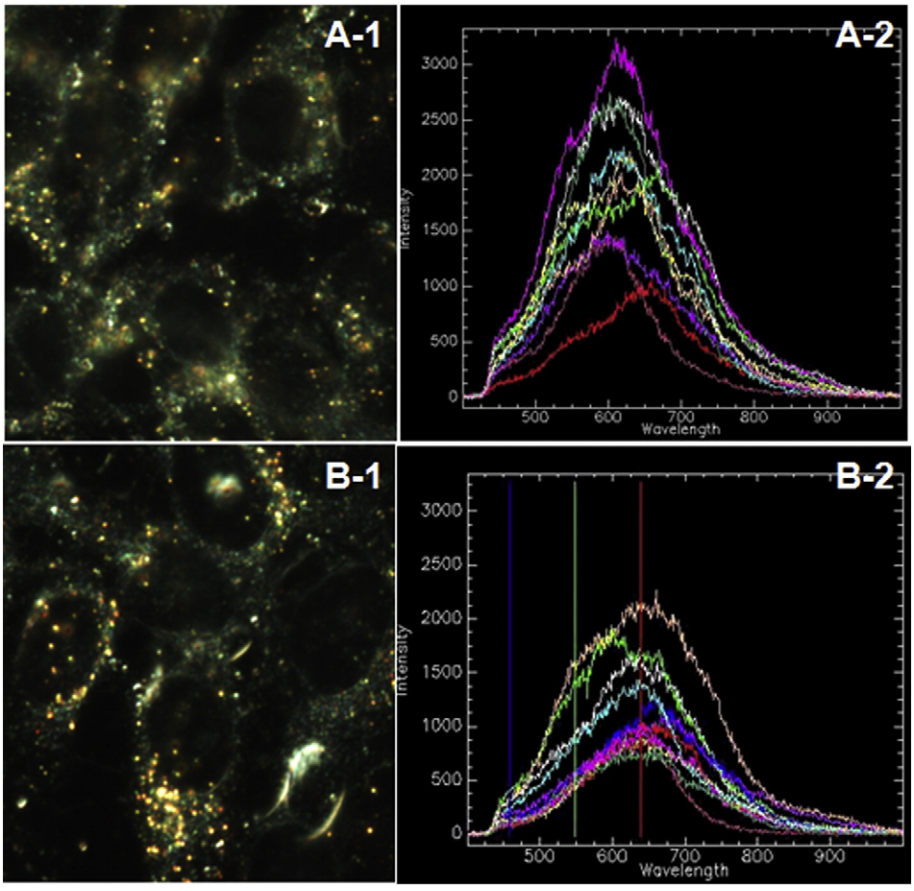

Fig. 4. Hyperspectral imaging of GNPs in normoxic and hypoxic cells. A-1, B-1) Dark field images of GNPs localized in normoxic and hypoxic cells after 24 h of incubation with NPs. The bright yellow dots were GNP clusters within the cells. A-2, B-2) Reflectance spectra from ten GNP clusters localized in cells shown in images A-1, B-1, respectively.

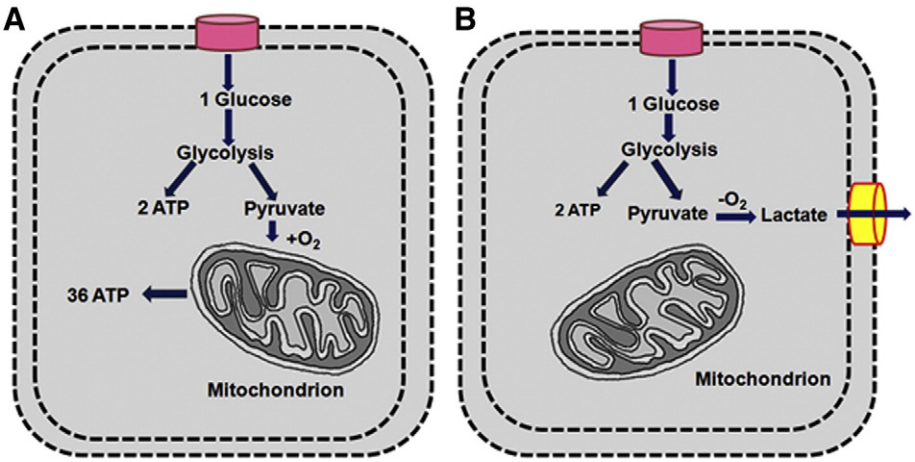

Fig. 5. Metabolic difference between normoxic and hypoxic cells. A) A normoxic cell primarily metabolizes glucose to pyruvate followed by complete oxidation of pyruvate to CO_2 in the mitochondria generating 36 ATPs per glucose. B) A hypoxic cell has limited O_2 and pyruvate is metabolized to lactate generating 2 ATPs per glucose.

objectives of this study was to compare the NP uptake under normoxia and hypoxia. Our cell uptake experiments were carried out at a concentration of 0.6 nmol, and the NP uptake per cell was quantified using the Atomic Absorption Spectroscopy (AAS) technique. As illustrated in Fig. 3, the cells pre-exposed to hypoxia for 18 h showed higher NP uptake as compared to normoxic cells following incubation with NPs for 24 h ($p < 0.05$). In addition, NPs with diameter 50 nm showed the highest uptake.

For qualitative analysis of NP uptake, we used the hyperspectral imaging technique. The dark field images in Fig. 4 (left panel) display the differences in cellular uptake of GNPs following a 24 hour incubation time period in normoxic cells (top image) and in cells pre-exposed to hypoxia (bottom image) for 18 hours, respectively. With the integrated CytoViva hyperspectral imaging capability, reflectance spectra from GNP clusters within the cell were captured as shown in Fig. 4 (right panel). The images showed an increase in NP uptake in hypoxic cells in contrast to normoxic cells. The images correspond to cells internalized with 50 nm GNPs.

The cells develop adaptive responses to survive and proliferate under hypoxic conditions. Under hypoxia, the ATP generation shifts from the phosphorylation pathway in the mitochondrion to the oxygen-independent pathway of glycolysis [18,19]. Although ATP can be generated faster with a glycolysis pathway when compared to oxidative phosphorylation, it is less efficient when comparing amounts of ATP produced [18,20]. As illustrated in Fig. 5, normoxic cells primarily metabolize glucose to pyruvate followed by the complete oxidation of pyruvate to CO_2. During this process, 36 ATPs are generated in mitochondrion per glucose molecule. In hypoxic cells, the lack of O_2 results in the activation of HIF-1 (hypoxia induced factor) pathway for the regulation of glucose metabolism [21,22]. In hypoxic cells, glucose metabolized to 2 ATP and pyruvate leaves the cell in a form of lactate. For example, a previous study has shown that under hypoxia, alveolar epithelial cells maintained their energy status near that of normoxic cells by increasing anaerobic glycolysis [22].

In this study, we investigated the effect of NP size on their uptake in cells exposed to prolonged hypoxia (18 h in the hypoxia chamber). In addition, we presented the stability and toxicity of NPs in a hypoxic environment. A recent study has shown that smaller NPs (1.9 nm) had a lower cell uptake following incubation under hypoxic conditions for 4 h [23]. In this case, the cells were exposed to hypoxic conditions for a short period of time. Our interest was to investigate NP uptake in cells exposed to hypoxia for a prolonged time since most of the tumor cells at the core are exposed to similar conditions. These differences in NP uptake can be explained by considering the extent of certain cellular processes, such as endocytosis, exocytosis, and autophagy. It is known that NP cell uptake and removal takes place via an energy dependent endo–lyso path [4,5,17]. Most of these NPs are taken up by the endocytosis process [4]. Once the NPs have entered the cells, they are trapped in the endosomes before being fused with lysosomes for processing. Once processed, these NPs are excreted from the cell via the exocytosis process.

Prolonged exposure of cells to hypoxic conditions could lead to reduced nutrients and energy supply. The process called “autophagy” is stimulated as a result of reduced nutrient availability, allowing cells to recycle cytoplasmic components. Exocytosis is also further reduced to conserve cellular constituents and energy [24,25]. We believe that the exocytosis process in cells pre-exposed to prolonged hypoxia (18 h) can be very slow, resulting in the accumulation of more NPs within the cells over time. Our future goal is to investigate the exocytosis and autophagy process in hypoxic cells to explain the outcome of our experiment in detail.

Our study proved that NP uptake was higher in cells that were under hypoxic conditions for a lengthy period of time. The increase in NPs within the hypoxic cells could be used to deliver a higher therapeutic load to overcome the drug and radiation resistance. In addition, a more aggressive combined approach can be applied since GNPs can be used as a drug carrier and radiation dose enhancer [2]. This study provides information as to how NP stability, toxicity, and uptake vary in a real tumor-like hypoxic environment. Hence, these findings will play a critical role in the use of NPs in future cancer therapeutics. Our future goal is to investigate the efficacy of such GNP-based treatment in a hypoxic environment. Proper understanding of NP behavior and the therapeutic response in a tumor-like environment (hypoxic) can be used to improve the outcome of future cancer care [2]. The biocompatibility of GNPs would accelerate the application of such innovations to existing therapeutic protocols in the near future.

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References

[1] B.D. Chithrani, Optimization of bio-nano interface using gold nanostructures as a model nanoparticle system, Insci. J. 1 (2011) 136–156.
[2] S. Jelveh, B.D. Chithrani, Gold nanostructures as a platform for combinational therapy in future cancer therapeutics, Cancer 3 (2011) 1081–1110.
[3] S. Jain, J.A. Coulter, A.R. Hounsell, K.T. Butterworth, S.J. McMahon, W.B. Hyland, M.F. Muir, G.R. Dickson, K.M. Prise, F.J. Currell, J.M. O'Sullivan, D.G. Hirst, Cell-specific

radiosensitization by gold nanoparticles at megavoltage radiation energies, Int. J. Radiat. Oncol. Biol. Phys. 79 (2011) 531–539.
[4] B.D. Chithrani, A.A. Ghazani, W.C.W. Chan, Determining the size and shape dependence of gold nanoparticle uptake into mammalian cells, Nano Lett. 6 (2006) 662–668.
[5] H. Gao, W. Shi, L.B. Freund, Mechanics of receptor-mediated endocytosis, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 9469–9474.
[6] S. Zhang, J. Li, G. Lykotrafitis, G. Bao, S. Suresh, Size-dependent endocytosis of nanoparticles, Adv. Mater. 21 (2009) 419–424.
[7] B.J. Moeller, R.A. Rachel, A. Richardson, M.W. Dewhirst, Hypoxia and radiotherapy: opportunities for improved outcomes in cancer treatment, Cancer Metastasis Rev. 26 (2007) 241–248.
[8] S. Dische, P.J. Anderson, R. Sealy, E.R. Watson, Carcinoma of the cervix-anaemia, radiotherapy and hyperbaric oxygen, Br. J. Radiol. 56 (1983) 251–255.
[9] R. Sullivan, G.C. Paré, L.J. Frederiksen, G.L. Semenza, C.H. Graham, Hypoxia-induced resistance to anticancer drugs is associated with decreased senescence and requires hypoxia-inducible factor-1 activity, Mol. Cancer Ther. 7 (2008) 1961–1973.
[10] N. Chan, C.J. Koch, R.G. Bristow, Tumor hypoxia as a modifier of DNA strand break and cross-link repair, Curr. Mol. Med. 9 (2009) 401–410.
[11] R.P. Hill, R.G. Bristow, The scientific basis of radiotherapy, in: I.F. Tannock, R.P. Hill, R. G. Bristow, L. Harrington (Eds.), The Basic Science of Oncology, McGraw-Hill Ltd., New York, 2005, pp. 289–321.
[12] K.R. Luoto, R. Kumareswaran, R.G. Bristow, Tumor hypoxia as a driving force in genetic instability, Genome Integr. 4 (2013) 1–15.
[13] B.D. Chithrani, Nanoparticles for improved therapeutics and imaging in cancer therapy, Recent Pat. Nanotechnol. 4 (2010) 171–180.
[14] D. Hühn, K. Kantner, C. Geidel, S. Brandholt, I. De Cock, S.J. Soenen, P.R. Gil, J.M.M. Martos, K. Braeckmans, K. Müllen, G.U. Nienhaus, M. Klapper, W.J. Parak, Polymer-coated nanoparticles interacting with proteins and cells: focusing on the sign of the net charge, ACS Nano 7 (2013) 3253–3263.
[15] P.R. Gil, D.J. de Aberasturi, V. Wulf, B. Pelaz, P. del Pino, Y. Zhao, J. de la Fuente, I.R. de Larramendi, T. Rojo, X.-J. Liang, W.J. Parak, The challenge to relate the physicochemical properties of colloidal nanoparticles to their cytotoxicity, Acc. Chem. Res. 46 (2013) 743–749.
[16] B.D. Chithrani, Intracellular uptake, transport, and processing of gold nanostructures, Mol. Membr. Biol. 27 (2010) 299–311.
[17] B.D. Chithrani, W.C.W. Chan, Elucidating the mechanism of cellular uptake and removal of protein-coated gold nanoparticles of different sizes and shapes, Nano Lett. 7 (2007) 1542–1550.
[18] R.A. Cairns, I.S. Harris, T.W. Mak, Regulation of cancer cell metabolism, Nat. Rev. Cancer 11 (2011) 85–95.
[19] T.N. Seagroves, H.E. Ryan, H. Lu, B.G. Wouters, M. Knapp, P. Thibault, K. Laderoute, R. S. Johnson, Transcription factor HIF-1 is a necessary mediator of the Pasteur effect in mammalian cells, Mol. Cell. Biol. 21 (2001) 3436–3444.
[20] R.J. Gillies, I. Robey, R.A. Gatenby, Causes and consequences of increased glucose metabolism of cancers, J. Nucl. Med. 49 (2008) 24S–42S.
[21] H. Takagi, G.L. King, L.P. Aiello, Hypoxia upregulates glucose transport activity through an adenosine-mediated increase of GLUT1 expression in retinal capillary endothelial cells, Diabetes 47 (1998) 1480–1488.
[22] A. Ouiddir, C. Planès, I. Fernandes, A. VanHesse, C. Clerici, Hypoxia upregulates activity and expression of the glucose transporter GLUT1 in alveolar epithelial cells, Am. J. Respir. Cell Mol. Biol. 21 (1999) 710–718.
[23] S. Jain, J.A. Coulter, K.T. Butterworth, A.R. Hounsell, S.J. McMahon, W.B. Hyland, M.F. Muir, G.R. Dickson, K.M. Prise, F.J. Currell, D.J. Hirst, J.M. O'Sullivan, Gold nanoparticle cellular uptake, toxicity and radiosensitisation in hypoxic conditions, Radiother. Oncol. 110 (2014) 342–347.
[24] H. Shorer, N. Amar, A. Meerson, Z. Elazar, Modulation of N-ethylmaleimide-sensitive factor activity upon amino acid deprivation, J. Biol. Chem. 280 (2005) 16219–16226.
[25] X. Ma, Y. Wu, S. Jin, Y. Tian, X. Zhang, Y. Zhao, L. Yu, X.-J. Liang, Gold nanoparticles induce autophagosome accumulation through size-dependent nanoparticle uptake and lysosome impairment, ACS Nano 5 (2011) 8629–8639.