

CONSTRUCTION OF GOLD NANORODS CARRYING IFN γ AND METHIONINE TO TARGET MCF-7 CELLS

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Development of gold nanoparticles (GNPs) conjugated with interferon gamma (IFN γ) and methionine along with the use of near-infrared (NIR) laser beams for treatment of cancer cells was the main aim of this study. MCF-7 cells were cultured and incubated with conjugated GNPs with IFN γ and methionine at different concentration followed by irradiation with NIR laser beam. Samples were then evaluated for their viability by MTT assay which showed that the GNPs complex was toxic on tumor cells ($P < 0.05$) at 0.64 μ g/ml concentration. Exposure to hyperthermia decreased the viability of cells significantly at 0.3 μ g/ml concentration of gold nanorods complex.
(11/28/2017)

(Received December 19, 2017; Accepted February 9, 2018)

*Keywords:*Hyperthermia, gold nanorods, MCF-7, NIR laser, interferon -gamma.

1. Introduction

Cancer is the second leading cause of death in the world [1] and in recent years there have been many studies to carry out new methods for prevention, diagnosis, and treatment of tumor cells and to decrease the side effects of treatment [2]. In many of these methods, a trace of nanotechnology can be found using a different type of nanoparticles. In general, nanoparticles have dimensions between 1-100 nm². Metal nanoparticles are at the center of attention because of their unique physical and chemical properties [2]. Due to the remarkable optical, electrical and conductive properties of gold nanostructures, they have been extensively studied in a variety of applications [3]. However, the use of these materials has recently expanded beyond traditional applications to include exciting functions in the biomedical, diagnostic and catalytic areas. Spherical gold nanoparticles have been one of the most studied materials in researches leading to photo thermal cancer therapies during the last 20 years. However, due to their limited peak absorptions which are usually ~580nm for 100nm diameter, their effects on biological entities such as connective tissues, skin, and hemoglobin have not been optimized. Gold nanorods behave similarly to the gold nanoparticles but are elongated to optimize their peak absorption and scattering characteristics (Figure 1). This allows the ability to tune the nanorods absorption from 550nm to 1400nm through different manufacturing processes as shown on the right in Figure 1. This tuning results in the ability of gold nanorods to scatter at wavelengths across the visible and NIR regions [4].

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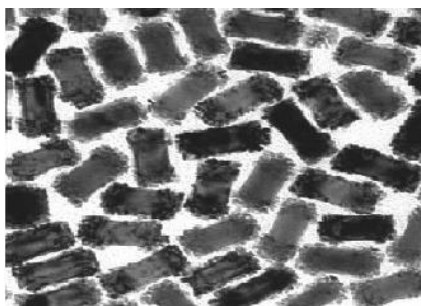


Fig. 1. Gold nanorods characteristics. A) The TEM image of typical gold nanorods (10nm x 40nm) (Aldrich Prod No. 716820).

Due to the existence of an amine or carboxyl terminal on nanorods, one of the most important applications of these molecules is conjugation with a variety of biomolecules including proteins, antibodies, and even DNA. Nanorods can be used for multi channel lateral flow assays in the visible region [5] and in the NIR region; they can also aggregate in tumors after being injected intravenously. When nanorods are concentrated around the diseased cells, they can be subjected to a continuous wave NIR laser in order to generate heat for photo thermal cancer therapy [6]. Gold nanostructures can also be used as drug delivery vectors where they can concurrently assist to destroy cancer cells (therapy) using pulsed lasers [7, 8].

In the last forty years, the use of hyperthermia has been developed as an adjuvant in cancer treatment. Depending on the vascular system, tumors and normal tissues show different behavior against the heat applied on these tissues. A temperature range of 41- 42°C seems to be lethal to tumor cells, whereas normal cells are not affected. In fact, the behavior of the various tumors and tumor response to hyperthermia mainly depends on their vasculature the differences against normal tissues [9]. It has previously been shown that the combination of laser beams and application of gold nanoparticles in imaging and non-invasive treatment of cancer with gold nanospheres could deform cylindrical nanotubes and remove the tumor cells [10]. On the other hand, IFN- γ and interleukins secreted from T cells showed destructive effects on tumor cells. However, in normal conditions, a cancer cell cannot be identified as an enemy by immune cells and therefore no cytokine is secreted. Therefore, a combination of thermotherapy with the delivery of cytokines to the core of cancer using gold nanorods and nanotechnology seem to be useful for the treatment of tumor cells. In this study, we will apply this combination on MCF-7 cells because are useful for in vitro breast cancer studies and the cell line has retained several ideal characteristics particular to the mammary epithelium.

2. Materials and Methods

The proliferation of cell line and culture conditions

MCF-7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FBS, 100 U/ml penicillin, and 100 μ g/ml streptomycin in a 25 t-flask at 37°C with 5% CO₂, 95% air and complete humidity. Once the cells reached approximately 90% confluency, they were detached using 0.05% trypsin/EDTA and counted using trypan blue on a hemocytometer slide under an inverted microscope (10).

Conjugate Production

Conjugation of Gold nanorods with Interferon-gamma

Diethylene Triamine Pentaacetic Acid (DTPA) was used as a linker to conjugate gold nanorods and IFN- γ . DTPA (0.1 mg) was first dissolved in 0.5 ml sterile PBS and the content was added to 0.5 ml PBS containing 50 μ g IFN- γ and vortexed for 10 minutes at room temperature. The mixture was then incubated at 4°C for 24 hours. On the next day, 3 mg 1_ethyl_3(3_dimethyl amino propyl) carbodiimide (EDC) was mixed with 1.5 mg methionine in 0.2 ml PBS. The content

was then added to 0.5 ml PBS containing 10 μg nanorods and mixed using a 2 ml syringe. The complex of DTPA and IFN- γ from the previous day was then added to the mixture of nanorods, methionine, and EDC and vortexed for 20 minutes at room temperature. Finally, a dialysis bag (Sigma, Canada) with 1000 Dalton cut off was used to purify the final product. The color of the final complex was clear and kept at 4°C until further use. The structure of the conjugate was then confirmed by UV spectroscopy (Figure 2. A, B, and C). The size and charge of gold nanorods were measured before and after conjugation by a zetasizer machine (Zetasizer Nano S90, Malvern Instruments Ltd., U.K). It is expected that after the conjugation process the size and charge of the complex increased.

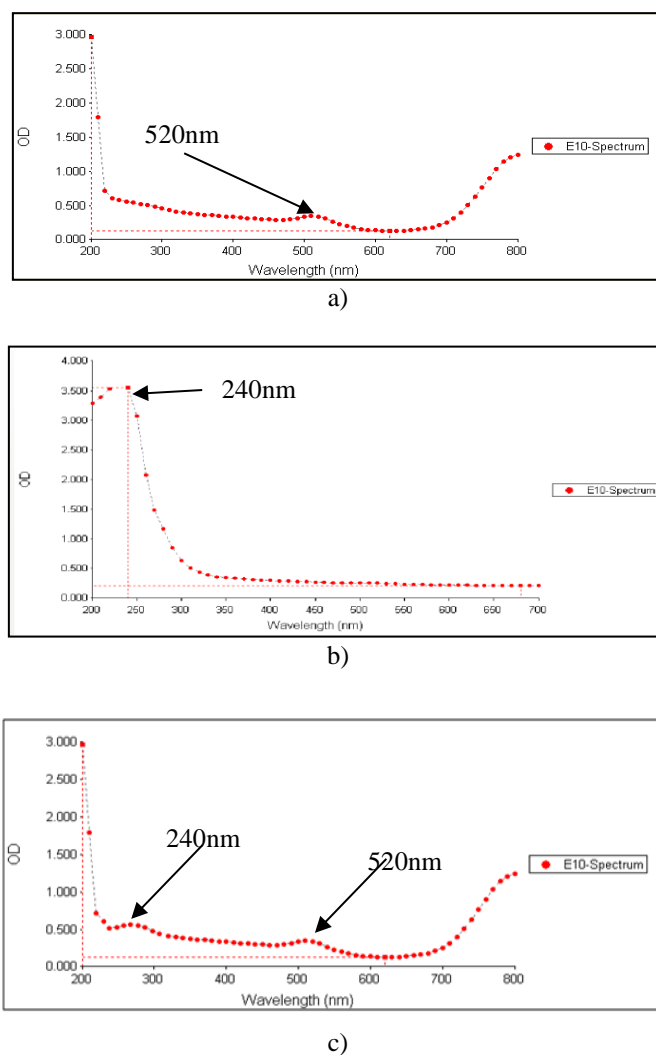


Fig. 2. UV spectrophotometry of the conjugated and unconjugated gold nanorods.

a) A pick absorbance of unconjugated gold nanorods at 520 nm wavelength.

b) IFN- γ at 240 nm wavelength .

c) The Complex at 240 nm and 520 nm wavelength.

Cells treated with various concentrations of unconjugated or conjugated gold nanorods and IFN- γ (table1).

Table 1: Used concentration and their effects

Materials for test	Cell type	Concentrations ($\mu\text{g/ml}$)						
		Toxic concentration	9.8	4.9	2.5			
Gold nanorods	Normal cell	nontoxic				1.2	0.6	0.3
		Toxic concentration	9.8	4.9	2.5	1.2	0.6	
	Cancer cell	nontoxic						0.3
		Toxic concentration	9.8	4.9	2.5			
Complex	Normal cell	nontoxic		1.2	0.6	0.3	0.16	0.08
		Toxic concentration	2.5					
	Cancer cell	nontoxic				0.3	0.16	0.08
		Toxic concentration	2.5	1.2	0.6			
IFN- γ	Normal cell	nontoxic	100	50	25	12.5		
		Toxic concentration	100					
	Cancer cell	nontoxic		50	25	12.5		
		Toxic concentration	100					

Test groups

The effects of conjugated gold nanorods with interferon and methionine on MCF-7 cell line was evaluated by incubating cells at a concentration of 2×10^4 cell/well on 96 well plates [11]. Cells were divided into 3 groups on each plate and repeated 6 times per group.

Group 1 contained six different concentrations of conjugated complex including 0.08 $\mu\text{g/ml}$, 0.16 $\mu\text{g/ml}$, 0.3 $\mu\text{g/ml}$, 0.6 $\mu\text{g/ml}$, 1.2 $\mu\text{g/ml}$ and 2.5 $\mu\text{g/ml}$ gold nanorods. Group 2 contained six different concentrations including 0.3 $\mu\text{g/ml}$, 0.6 $\mu\text{g/ml}$, 1.2 $\mu\text{g/ml}$, 2.5 $\mu\text{g/ml}$, 5.0 $\mu\text{g/ml}$, and 10 $\mu\text{g/ml}$ of unconjugated gold nanorods. Group 3 contained four different concentrations of IFN- γ at 12.5 $\mu\text{g/ml}$, 25 $\mu\text{g/ml}$, 50 $\mu\text{g/ml}$, and 100 $\mu\text{g/ml}$.

Briefly, the culture media was removed and 50 μl of each concentration was added to each well and cell incubated for 24 hours followed by MTT assay to measure the viability of cells.

Hyperthermia Test

The medium of all plates including the treated and untreated cells was replaced with fresh medium after twenty-four hours treatment with conjugated complex or gold nanorods alone, and the cells were exposed to NIR laser light at a wavelength of 980 nm using a (980 nm Infrared Laser Diode Module, China) with a power of 37 W/cm^2 for a period of 1 to 6 minutes. Each test was repeated three times.

MTT assay for evaluating cell viability

Cell survival rate and cell proliferation were evaluated based on the ability of mitochondria to convert MTT to formazan crystals with a pale blue color. Briefly, 10 μl of MTT plus 100 μl of RPMI1640 were added to each well, except the cell-free blank wells (the concentration of MTT is 12mM). Cells were incubated for 4 hours at 37°C with 5% CO_2 , 95% air and complete humidity. After 4 hours incubation, the MTT solution was removed and replaced with 50 μl of DMSO and the plates were further incubated for 20 min at 37°C, and the optical density (OD) of the wells was determined using a Bio-Rad plate reader machine at a test wavelength of 570 nm and a reference wavelength of 630 nm [12, 13].

All tests in addition to cancer cells over normal cells were also performed and the results were examined.

Statistical Analysis

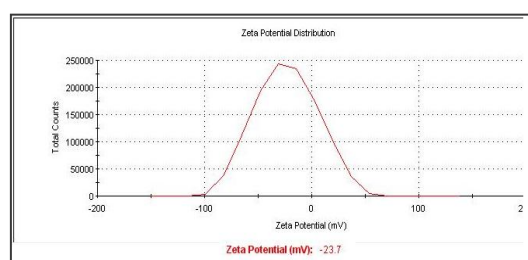
To determine the effect of treatment in different groups based on cell viability, all data were collected and analyzed using a T-test of SPSS 17 software and the charts were drawn in Excel 2007.

3. Results

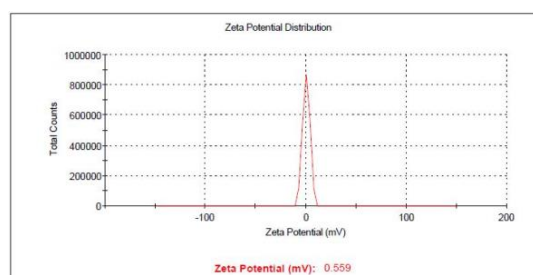
Confirmation of the complex structure

UV spectrophotometry

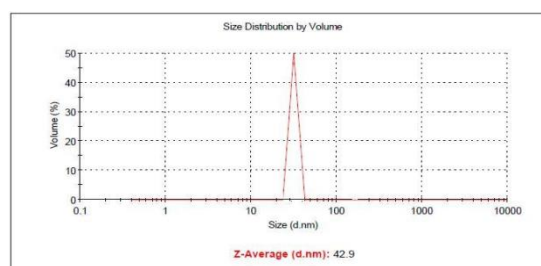
To confirm the structure of conjugated and unconjugated gold nanorods a UV spectrophotometer was used which indicated a pick absorbance at the 520nm wavelength corresponding to gold nanorods alone (figure 2A). IFN- γ , however, created pick absorption at 240 nm wavelength (figure 2B). After conjugation of gold nanorods with IFN- γ , two different picks appeared that confirming the attachment of IFN- γ to gold nanoparticles (figure 2C).



a)



b)



c)

Fig. 3. Measurement of the complex's size and charge by Zetasizer machine.

- a) Negatively charged IFN- γ due to the presence of its carboxylic groups.*
- b) Positively charged IFN- γ after conjugation with gold nanorods (Gold nanorods have positive charge and it affects on charge of IFN- γ).*
- c) The increased size of the complex due to the attachment of IFN- γ to 10 nm gold nanorods.*

Measurement of the complex's size and charge by a zetasizer machine

A Zetasizer machine was used to identify the negative charges of IFN- γ due to its carboxylic group (Figure 3A). Due to the conjugation of IFN- γ with gold nanorods, the zetasizer machine detected an exchange of electrons with the positively charged gold (figure 3B). In the mean time, the size of the complex including gold nanorods and IFN- γ was increased to 42.9 nm whereas the size of gold nanorods alone is 10 nm confirming the attachment of the protein to gold

nanorods (figure 3C). As it was expected, the size and charge of the complex were increased after conjugation.

MTT assay on BSR cell

Test data was obtained from MTT assay at a wavelength of 570nm with a reference wavelength of 630nm and evaluated using SPSS software. The MTT was performed to evaluate the toxicity effect of pure gold nanorods on normal BSR cells, cells were first treated with different concentrations of gold nanorods for 24 hours. MTT assay results revealed that gold nanorods were significantly toxic to the cells at the concentration of 2.5 $\mu\text{g/ml}$ and higher (Figure 4A). Therefore, a non-toxic concentration of 1.2 $\mu\text{g/ml}$ gold nanorod was used for the rest of experiments. IFN- γ alone showed no toxicity effect on BSR cells even at its highest concentration of 100 $\mu\text{g/ml}$ (Figure 4B). The complex was prepared based on different concentrations of gold nanorods conjugated with 50 $\mu\text{g/ml}$ IFN- γ . Normal BSR cells exposed to the complex with various concentrations of gold nanorods showed no significant toxicity effects except at the concentration of 2.5 $\mu\text{g/ml}$ as we observed previously in figure 4B which is mainly due to the gold nanorods toxicity effects at this concentration (Figure 4C).

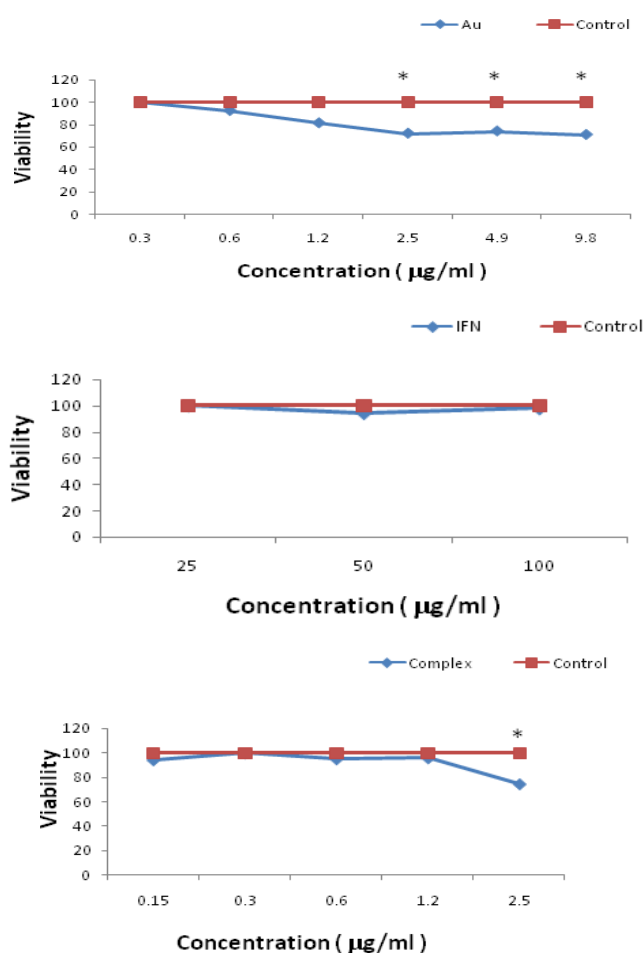


Fig. 4. MTT assay evaluations on viability BSR cell line. A) The toxicity effect of pure gold nanorods on normal BSR cells. B) The toxicity effect of IFN- γ on normal BSR cells. C) The toxicity effect of the complex on normal BSR cells. All data were statistically analyzed using SPSS software. The significance considered as $p < 0.05$.

Effects of conjugated and unconjugated gold nanorods on MCF-7 cancer cells

As compared to normal BSR cells, unconjugated gold nanorods demonstrated similar effects with the same pattern on MCF-7 cells as was shown in Figure 4A on 9.8 $\mu\text{g/ml}$, 4.9 $\mu\text{g/ml}$,

2.5 $\mu\text{g/ml}$ concentration and two lower concentrations (1.2 $\mu\text{g/ml}$ & 0.6 $\mu\text{g/ml}$) had toxicity effect on MCF-7 cells and only 0.3 $\mu\text{g/ml}$ concentration which is its lowest had no toxicity effect on MCF-7 cells. (Figure 5A). IFN- γ alone, as well, showed no significant toxicity effects on MCF-7 cells except at the highest concentration of 100 $\mu\text{g/ml}$ (Figure 5B) which allowed us to use the none toxic concentration of 50 $\mu\text{g/ml}$ for treatment of cancer cells.

Exposed MCF-7 cells with the complex of gold nanorods, methionine, and IFN- γ , revealed an interesting result showing the increase of cell death at a lower concentration of gold nanorods conjugated with IFN- γ (Figure 5C).

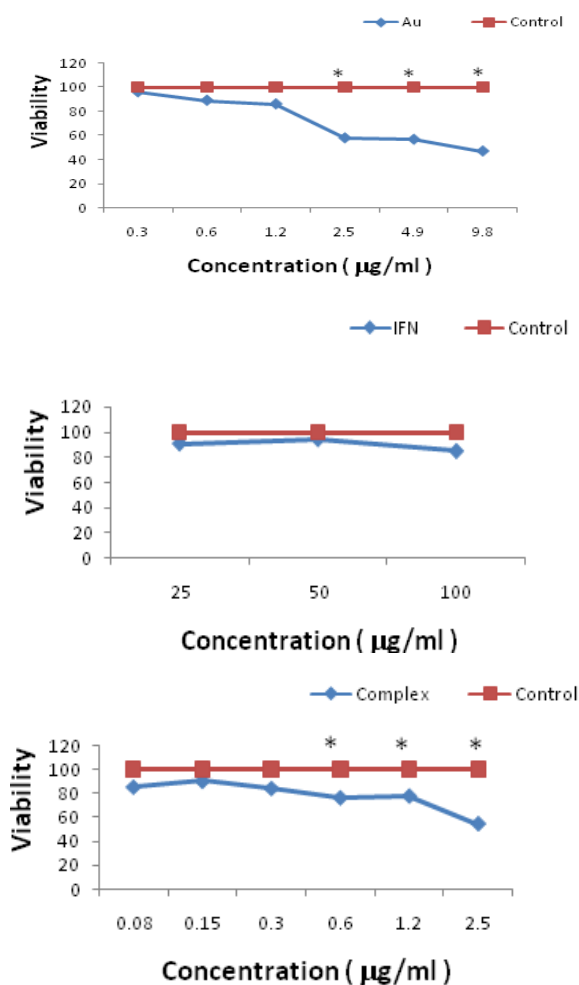


Fig. 5. MTT assay evaluations on the viability of MCF-7 cell line. A) The toxicity effect of pure gold nanorods on MCF-7 cancer cells. B) The toxicity effect of IFN- γ on MCF-7 cancer cells. C) The toxicity effect of the complex on MCF-7 cancer cells. All data were statistically analyzed using SPSS software. The significance considered as $p < 0.05$.

Laser radiation on cells in the absence of any material

Single cells were exposed to a laser beam for 1 to 6 minutes with a viability rate of more than 90% for all cells (Figure 6A).

Laser irradiation on cells in the presence of gold nanorods at different time periods

Exposure of cells to laser irradiation in the presence of the toxic and nontoxic concentration of gold nanorods had no effect on cell viability on MCF-7 cells, indicating that the particle remains unattached to the cells because the gold nanorods cannot bind to cells specifically (Data not shown).

Laser irradiation on cells in the presence of the complex at different time periods

Irradiation of MCF-7 cells in the presence of the complex at the 0.3 $\mu\text{g/ml}$ and higher concentrations increased the cell death after 6 minutes of exposure. This phenomenon could be due to the presence of methionine attached to the complex that is in favor of MCF-7 cells and causes the attachment of the complex to the cells. Uptake of methionine by cancerous cells is more than normal cells, therefore, the cells absorbed more complex and laser irradiation is more affected on them. As a result, the heat generated by gold nanorods in the course of laser irradiation could kill the cells (Figure 6B).

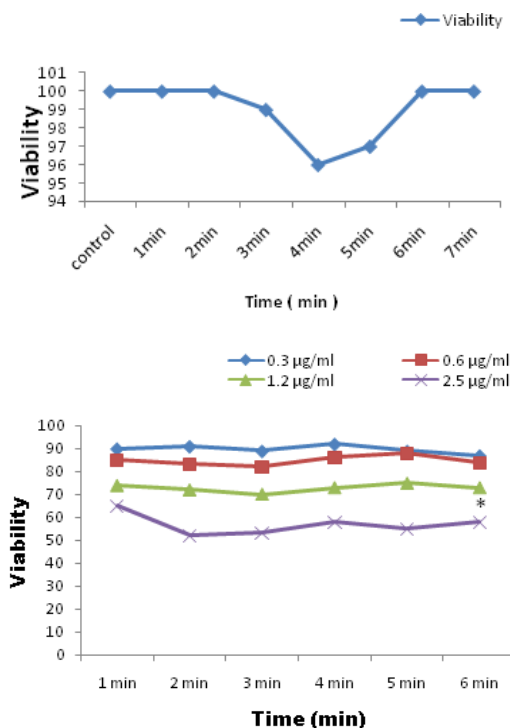


Fig. 6. Evaluation of the effect of laser irradiation on MCF-7 cells viability. A) Effects of laser irradiation on MCF-7 cancer cells. B) Effects of laser irradiation on MCF-7 cancer cells in the presence of the complex. All data were statistically analyzed using SPSS software. The significance considered as $p < 0.05$.

4. Discussions

Interferon gamma ($\text{IFN}\gamma$) is a key moderator of cell-mediated immunity with diverse, mainly pro-inflammatory actions target tissues. Moreover, methionine (Met) is a key factor and one of the essential amino acids cell metabolisms [14]. Met is necessary for growth and differentiation of normal and the cancer cells. Therefore, tumor cells have a much greater requirement for Met than normal cells [15]. Numerous lines of cancer cells such as MCF7 show Met-dependent phenotypes that are unable to survive and grow when the amino acid, Met, is replaced with homocysteine precursors in the medium [15].

Gold nanorods (GNs) are known as carriers with high loading capacity and large functionalizable surface area for target-directed delivery. In this study, we constructed a gold nanorods complex conjugated with both methionine and $\text{IFN}\gamma$ and the results showed that the gold nanorods were successfully synthesized (Fig 2. A, B, C).

MTT assay was used to evaluate the cytotoxicity on BSR cell line as control (Fig. 4). As we expected, there was no significant differences between the GNs complex and the free GNs. The cytotoxicity of gold nanorods conjugated with $\text{IFN}\gamma$ and methionine was then compared with free gold nanorods at equivalent concentrations on MCF-7 cells as shown in Figure 5. At lower

concentration of 0.6 $\mu\text{g/ml}$, free GNs showed no significant effect on cell viability as compared to the control (Fig. 4A). This concentration was nominated for synthesis of the complex. All concentrations of IFN γ also showed no significant differences with the control and therefore, the highest concentration of 100 $\mu\text{g/ml}$ was selected for construction of the complex (Fig. 4B). The cytotoxicity effect of the complex was then examined on BSR cells that revealed no significant cytotoxicity at the concentration of 1.2 $\mu\text{g/ml}$ (Fig.4C). The effects of nanoparticles to induce lethal Hyperthermia after laser irradiation, as well as the synergistic effect of gold nanorods with methionine and laser hyperthermia in the complex were also evaluated. Treatment of MCF-7 cell with gold nanorods complex significantly reduced tumor cells whereas, more than 90% of MCF-7 cells survived in the presence of free gold nanorods indicating the size and concentration of nanoparticles do not induce cytotoxicity in MCF-7 cell line as it was reported by Phadnis and colleagues [15] and Leggio et al. [16]. In another similar study, gold nanoparticles with a size of 10 nm were incubated with MCF-7 cells and the results indicated none toxic effects of these nanoparticles to the cells at low concentrations due to their specific shape that absorbs more heat and destroys tumor cells and it was described as a safe, none invasive treatment [17]. It has also been reported that the deformation of the spheres to rod-shaped gold nanoparticles can be replaced by the visible light spectrum that would be used for gold nanospheres using near-infrared spectroscopy [10]. Moreover, the nanoparticles with more elongated dimensions show specific optical properties [18]. It is known that laser irradiation induces hyperthermia and increases cellular death in the presence of nanoparticles. In the mean time, the immune system plays an important role in eliminating and destroying cancer cells by releasing the cytokines [19]. IFN- γ as one the most important cytokines induces apoptosis and has anti proliferative effects on a large number of tumor cells with IFN- γ receptor [20]. The results of this study show that IFN- γ in the concentrations used was not toxic to cells. To accept or reject any of the hypotheses and to determine the mechanism of the cell death in the presence of these factors will be the subject of future studies.

Gold nanorods attached to methionine can effectively bind to cancer cells. The amino groups of methionine and its nitrogenous material is often being used by cancer cells, therefore, it induces a better binding of nanoparticles to cancer cells. Gold nanorods can absorb photons from the laser beam and convert them into heat energy and thus raises the temperature and cause the destruction of the cells.

The laser used in this study was in the form of IR and at the wavelength of 980 nm with a power of 37 W/cm^2 and exposure time of 1 to 5 min did not kill any cells, whereas, exposing cells to the laser for 6 minutes at a concentration of 0.3 micrograms per milliliter of the complex destroyed them. Because of the annexing methionine to the nanorods, it facilitates the attachment of the complex to the cells which in turn produces heat after exposure to laser beams and brings about a marked cell death. Perhaps, by increasing the duration of laser beam exposure a larger population of tumor cells will be destroyed. Researchers in other studies have demonstrated the cell death after using laser beam at a wavelength of 820nm [21, 22].

5. Conclusions

Rod-shaped gold nanoparticles allow us to use optical spectra with a lower frequency than visible light spectrum. In other words, instead of using light spectrum that is normally used for gold nanospheres, we can use infrared rays. Moreover, the elongated shape of gold nanorods can possibly absorb more energy and convert it to heat to destroy malignant cells in deeper tissues. Conjugation of Gold nanorods with IFN- γ and methionine can show a new way for treatment of cancer cell in the future. Further studies are required to evaluate these results in vivo and to confirm the effects of conjugated nanoparticles on tumor cell in live animals. The results of this study can be promising for the development of novel anti cancer treatment in future studies.

Acknowledgments

The authors declare no conflict of interest in this study. This study was supported by a grant from Iran National Science Foundation (Code number: 91002993) to Dr. Masoud Ghorbani.

References

- [1] M. Akram, M. Iqbal, M. Daniyal, A. U. Khan. *Biol Res.* **50**(1), 33 (2017).
- [2] W. Tao, J Zhang, X Zeng, D. Liu, G. Liu, X. Zhu. *Adv Healthc Mater.* **4**(8), 1260 (2015).
- [3] J. N. Fryer, K. D. Burns, M. Ghorbani, D. Z. Levine. *Kidney Int.* **60**(5), 1792 (2001).
- [4] N. Rizk, N. Christoforou, S Lee. *Nanotechnology* **27**(18), 185704 (2016).
- [5] W. H. Walker II, J. C. Borniger, A. Surbhi, A. A. Zalenski, S. L. Muscarella, J. A. Fitzgerald, N. Zhang, M. M. Gaudier-Diaz, A. C. DeVries. *Sci Rep.* **15**(7), 8152 (2017).
- [6] E. Fourkal, I. Velchev, A. Taffo, V. Khazak, N. Skobeleva. *IFMBE Proceedings* **25**, 761 (2009).
- [7] N. Mohseni, F. Salehi Sarvestani, M. Shafiee Ardestani, F. Kazemi-Lomedasht, M. Ghorbani. *Asian Pac J Trop Biomed.* **6**(2), 173 (2016).
- [8] S. M. Bagheri, A. A. Asl, A. Shams, S. A. Mirghanizadeh-Bafghi, Z. Hafizibarjin. *Indian J Med Paediatr Oncol.* **38**(2), 116 (2017)
- [9] W. Li, P. Rong, K. Yang, P. Huang, K. Sun, X. Chen. *Biomaterials* **45**, 18 (2015).
- [10] H. Sharma, P. K. Mishra, S. Talegaonkar, B. Vaidya. *Drug Discov Today* **20**, 1143 (2015).
- [11] N. H. Aliabad, B. Bakhshi, M.R. Pourshefah, A. Sharifnia, M. Ghorbani. Molecular diversity of CTX prophage in *Vibrio cholera*. *Letters in Applied Microbiology* **55**, 1, 27 (2012).
- [12] N. Cihoric, A. Tsikkinis, G. van Rhooon, H. Crezee, D. M. Aebersold, S. Bodis. *Int J Hyperthermia* **31**, 609 (2015).
- [13] G. H. Edrissian, M. Mohammadi, A. Kanani, A. Afshar, R. Hafezi, M. Ghorbani, A. R. Gharagozloo. Bacterial infections in suspected cutaneous leishmaniasis lesions (Article). *Bulletin of the World Health Organization* **68**, 4, 473 (1990).
- [14] M. A. Benavides, D. K. Oelschlager, H. G. Zhang. *Am J Surg.* **193**(2), 274 (2007).
- [15] A. Phadnis, S. Kumar, A. Srivastava. *J Therm Biol.* **61**, 16 (2016).
- [16] L. Leggio, O. de Varona, E. Dadrasnia. *Prog Electromagn Res.* **150**, 73 (2015).
- [17] Y. J. Wang, S. m. Zhou, G. Xu, Y. Q. Gao. *Molecules* **20**(5), 8060 (2015).
- [18] M. A. Gobin, M. H. Lee, M. J. Halas, W. D. James, R. A. Drezek, J. L. West. *Nano Letter* **7** (7), 1929 (2007).
- [19] E. Gomaa, S. A. A. Gaber, S. Bhatt, T. Liehr, Glei, T. A. El-Tayeb. *J Nanopart Res.* **17**, 102 (2017).
- [20] W. K. Cho, S. Kang, H. Choi, C. R. Rho. *Cornea* **34**(4), 456 (2015).
- [21] Hao, Z. Yan, Y. Shuang, J. Sun, S. Tao, W. Fu. *Int J Clin Exp Pathol.* **8**(6), 7409 (2015).
- [22] P. P. Adisheshaiah, S. T. Stern. *Methods Mol Biol.* **1682**, 241 (2018).