Iranian Journal of Medical Physics

ijmp.mums.ac.ir

An In-Vitro Study on Photochemical Internalization of Methylene Blue with Gold Nanoparticles Coated By Thio-Glucose

Maryam Shabanzadeh[1], Alireza Shirazi[2], Armin Imanparast [3,4], Zahra Mohammadi [3,4*], Ameneh Sazgarnia[3,4]

1. Department of Medical Radiation, Faculty of Engineering, Islamic Azad University, Science and Research Branch, Tehran, Iran.
2. Department of Medical Physics and Biomedical Engineering, Faculty of Medicine, Tehran University of Medical Sciences, Tehran, Iran.
3. Medical Physics Research Center, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran
4. Department of Medical Physics, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

ARTICLE INFO

Article type:
Original Paper

Article history:
Received: Mar 25, 2020
Accepted: Oct 16, 2020

Keywords:
Breast Cancer
Photodynamic Therapy (PDT)
Gold Nanoparticles (GNPs)
Methylene Blue (MB)

ABSTRACT

Introduction: Photochemical internalization is a novel PDT-based technology for the intracellular delivery of hydrophilic macromolecular therapeutic agents and other drugs limited in penetration into cellular membranes with intracellular targets. In this regard, one of the approaches is to use nanoparticles along with photosensitizing agents. In this study, the presence of thioglucose-coated gold nanoparticles in the efficiency of the photodynamic effect of methylene blue (MB) caused by the photochemical internalization phenomenon was investigated.

Material and Methods: First, Glu-GNPs was synthesized, and then the toxicity of Glu-GNPs and MB were determined to achieve their optimal concentrations. Afterward, the photodynamic effects of Glu-GNPs combined with MB by Luma-Care source light were evaluated at different doses using MTT assay and colony assay (12 days after treatment).

Results: According to the MTT assay, the photodynamic effect in the Glu-GNPs group revealed no significant efficacy, whereas the colony-formation capability in all groups with an optical dose of 15.6 J / cm^2 decreased, compared to the similar group without light exposure ($P < 0.05$).

Conclusion: The photodynamic efficiency of MB with the Glu-GNPs group was reduced at 15.6 J/cm^2, compared to the free MB group. The decreased efficiency can have various reasons such as the photochemical bleaching of the free MB because of ROS and $^1O_2$ produced by the plasmonic photodynamic phenomenon of Glu-GNPs or changing the optophysical properties of surface plasmon resonance of final product (MB+ Glu-GNPs) due to the possible electrostatic bonding of the drug with the nanoparticles.

► *Please cite this article as:*
Shabanzadeh M, Shirazi A, Imanparast A, Mohammadi Z, Sazgarnia A. An In-Vitro Study on Photochemical Internalization of Methylene Blue with Gold Nanoparticles Coated By Thio-Glucose. Iran J Med Phys 2021; 18: 361-368. 10.22038/IJMP.2020.39283.1518.

Introduction

Photodynamic therapy (PDT) is a minimally invasive treatment eliminating tumor cells. PDT is based on three essential non-toxic components, which, if used simultaneously, lead to the induction of photochemical cytotoxicity. The components include photosensitizer macromolecules (PS), molecular oxygen level, and the overlap of the PS absorption peak with an emission spectrum of the light source [1]. The PDT products include the reactive oxygen species (ROS) and singlet oxygen ($^1O_2$), which can generate remarkable phototoxicity, leading to cell death by apoptosis or necrosis within the target tumor cells [2]. The "photosensitizing drug macromolecules" have become one of the attractive agents for PDT in cancer and other diseases. Recent advances in photobiology and biphotonic processes have made it possible to improve cellular targeting and design novel photo cytotoxic nanocomplex and other photosensitizer agents for clinical use [3]. In many cases, one of the main prerequisites for achieving the optimal biological effect and the expected effects of optical toxicity from these macromolecules is to internalize these agents in the cytosol [4]. Macromolecules are usually hindered to be entered the cell by membranes because of endocytosis as such they are destroyed by hydrolytic enzymes in lysosomes with no active intervention [5]. Accordingly, the membrane of endocytic vesicles at an intracellular level is the most basic obstruction for the cytosolic localization of macromolecules [6]. Photochemical internalization (PCI) is a novel branch of the photodynamic process for hydrophilic drug delivery and other types of drugs or macromolecular agents with limited ability to penetrate into cellular membranes [7]. In this approach, endolysosomal membranes are disrupted, and their content is escaped from vesicle to cytosol by being exposed to light [8].

*Corresponding Author: Tel: ????; Email: mohammadilz931@mums.ac.ir

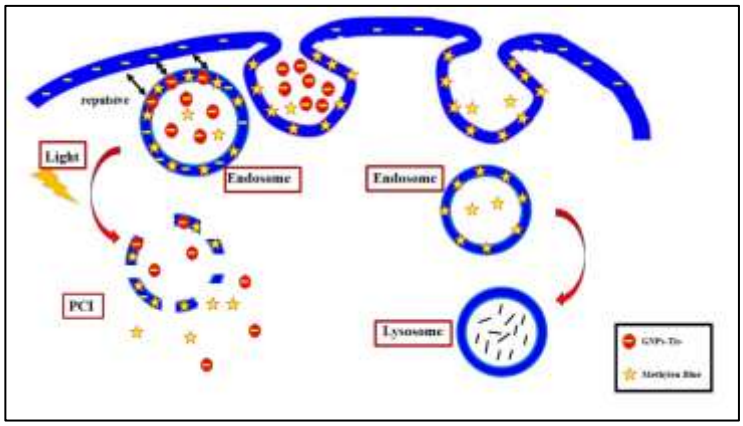

Figure 1. Photochemical internalization process along with the natural mechanism of photosensitizer cell uptake and finally its destruction inside the vesicle by lysosomal enzymes.

Methylene blue (MB) is hydrophobic drug photosensitizers with an absorption peak at 660 nm, which can destroy cancerous cells [9]. The effective uptake of MB in cells is low, and it should be introduced into the cells by endocytosis. Nanostructures are one of the appropriate methods increasing the endocytosis likelihood [10, 11]. There are two main solutions in this regard: The first one is to conjugate the drug to the nanostructure, and another one is the simultaneous use of the drug with the nanostructure without conjugating it. The anionic gold nanostructures are one of the most suitable options in plasmonic photodynamic/photothermal therapies due to their unique optophysical properties [12]. In this study, gold nanoparticles coated with thioglucose and methylene blue (without conjugation) were used for the following reasons: (i) preventing the decrease in the endocytosis likelihood by increasing the size of gold nanostructures, (ii) increasing the endocytosis likelihood by interacting with the positive side of the proteins in the membrane following the surface modification of gold nanoparticles with thioglucose, and (iii) capturing the anionic gold nanoparticles remarkably by cells due to their repulsive interactions with the negatively-charged cell surface after endocytosis. Moreover, thioglucose (like polyethylene glycol) reduces the agglomeration of nanostructures [13, 14].

This study aimed to evaluate the efficacy of photodynamic therapy of methylene blue (based on photochemical internalization) due to the presence of gold nanoparticles coated with thioglucose (Figure 1).

Materials and Methods

Synthesis of Glu-GNPs

Glu-GNPs was synthesized according to Rostami's et al. study [15, 16]. Gold (III) chloride trihydrate ($HAuCl_4 3H_2O$), 1-thio-β-D-glucose (Glu, T6375) (Sigma-Aldrich, USA), and sodium borohydride (NaBH4, 16940-66-2, Sharla, Spain) were used to synthesize the coated Thio-glucose on the gold nanoparticles (Glu-GNPs). In this regard, three sub-steps were as follows:

First, 3.2 ml of deionized water and 0.03 g of $HAuCl_4$ were shaken, and the solution was then added to 60 ml of 25 deionized water in an ice bath under moderate stirring.

Second, 0.004 g of $NaBH_4$ (as a reducing agent) was added to 4 ml of deionized water, and it was then added to the solution prepared in the previous step to obtain GNPs.

Finally, 0.05 g of 1-thio-β-D-glucose was added to 12 ml of deionized water to functionalize GNP, When the solution was uniform, it was added to the solution containing gold nanoparticles. After 30 minutes, the resulting solution contained thioglucose-coated gold nanoparticles bonded together by covalent bonding. After completing the synthesis process, Glu-GNPs were centrifuged to remove free thioglucose.

Glu-GNPs Characterization

The size distribution of the synthesized particles and their zeta potential were determined using a particle size analyzer (Malvern Instruments, Southborough, MA). The absorption spectrum of the gold nanoparticles was recorded by a UV-Visible spectrophotometer (Shimadzu Model UV-1700, Japan). Moreover, transmission electron microscopy (TEM Leo 912-ab, Zeiss Germany) was used to detect the morphology of GNPs.

Cell line and culture conditions

This study was performed on the MCF-7 cell line derived from the adenocarcinoma tumor of the human breast. The cells were provided by the Pasture Institute of Iran.

The MCF7 cells were grown in culture flasks of 75 cm^2 in the culture medium of RPMI-1640 supplemented with 10% fetal calf serum (FCS) and 1% antibiotics (penicillin 50 µg/ml and streptomycin 50 mg/ml) in an incubator at 37 °C in the presence of 5% CO_2. When the cells reached the exponential growth phase and coated the flask floor as a monolayer, they were detached from the flask using trypsin- EDTA solution and transferred to new flasks or for the designed experiments. The cell

culture media was changed every other 2-3 days. Trypan-blue was used for staining and counting cells. Before the treatments, the cell survival >98% was ensured.

Cytotoxicity of the treatment agents

In this phase, the cytotoxicities of MB and Glu-GNPs were determined separately. First, a cell suspension with the cell density of 10^4 cells per ml was prepared in the culture medium, and 200 µL of this suspension was added to each well in the 96-well plates and incubated for 24 h. Then the culture medium of the cells was extracted, and the cell incubation process continued in the presence of the different concentrations of MB (6, 12,16,18,24 µg/mL) or Glu-GNPs (20, 40, 60, 80, 100 µg/mL) for 2 h. Afterward, the MTT assay was used to determine the percentage of cellular survival and evaluate cytotoxicity. In the next step, 18µg/mL concentration of MB and with different Glu-GNPs concentrations was considered in separate experimental groups to determine the combined toxicity of MB and Glu-GNPs (Glu-GNPs combined with MB). After 24 h, the MTT assay was used to determine the percentage of cellular survival and evaluate the cytotoxicity of Glu-GNPs combined with MB.

MTT assay

To perform the MTT test, first, the contents of the 96-well plate wells were drained, and then 10 µl of MTT solution and 100 µl of fresh medium were added to each well and incubated for 4 h. Following the incubation phase, the culture medium in the wells was replaced with 200 µl of DMSO (dimethyl sulfoxide, Sigma, USA) and put in a shaker for 10 min in order for the contents of each well to be mixed thoroughly and the content color to be stabilized. Finally, the optical density of the wells was measured at 545 nm vs. 630 nm (Stat Fax 2100, USA) using the ELISA reader (Awareness company, USA) and the cell viability percentage was compared with that of the control group.

Colony assay

To perform the colony assay, 10^4 cells were cultured for the survival analysis in 6-well plate wells. Twelve days later, relatively acceptable colonies were formed. The colonies attached to the bottom of the plate were fixed with pure methanol and stained with Giemsa (Sigma Aldrich, USA). Then the colonies were counted to determine the cell survival fraction. Colonies with >50 cells were scored as the markers of surviving cells.

Experimental groups

After determining the optimal concentrations of Glu-GNPs combined with MB, the MCF-7 cells were divided into different groups to evaluate the effect of PDT on cell survival. The concentrations of Glu-GNPs and MB were set as 80 and 18 µg/ml, respectively. For this purpose, the cells were incubated with the agents (namely MB, Glu-GNPs, and Glu-GNPs combined with MB) for 2 h, separately.

After washing the cells, a fresh complete culture medium was added to each well, and PDT was conducted. The separate samples were illuminated using a Luma-Care light source (LumaCare, USA) equipped with the fiber optic probe at a wavelength of 670 nm, bandwidth of 30 nm, and a power density of 65 mW/cm^2 at three different exposures. Then the cells were incubated, and MTT was performed after 24 h to determine the cell survival of different control and treatment groups. To confirm the results, each test was reiterated at least three times.

Judgment criteria and data analysis

In this study, the following indexes were utilized to interpret the collected data:

IC_{10} and IC_{50} are the required concentration of the agent (MB or Glu-GNPs) to induce 10% and 50% cell death, respectively. The exposure dose was required for 50% cell death at a specific sensitizer concentration (ED_{50}).

The findings were analyzed by SPSS software version 24. First, the Kolmogorov-Smirnov test was used to evaluate the normality of data. When the normal distribution of data was confirmed, one-way ANOVA and Tukey's tests were run to compare the data. The previous processes are repeated at least 3 times in all groups.

Results

Glu-GNPs Characterization

According to the results, the size and zeta potential of Glu-GNPs are 54 nm and -24 mV, respectively (Appendix 1). Figure 2 shows the UV-Vis spectrum of Glu-GNPs, and Figure 3 presents the transmission electron microscopy (TEM) image of GNPs [15]. The UV-Vis spectrum was also recorded from Glu-GNPs to determine the surface plasmon resonance spectrum of Glu-GNPs. As presented in Figure 2, the absorption peak was estimated at 553 nm.

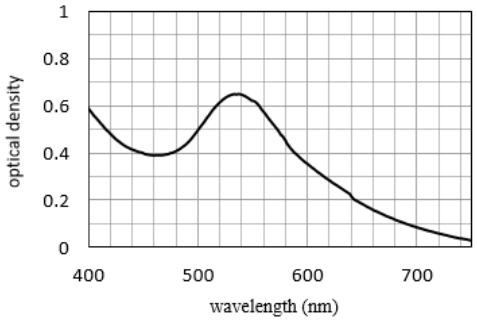

Figure 2. UV-Vis spectrum of the Glu-GNPs

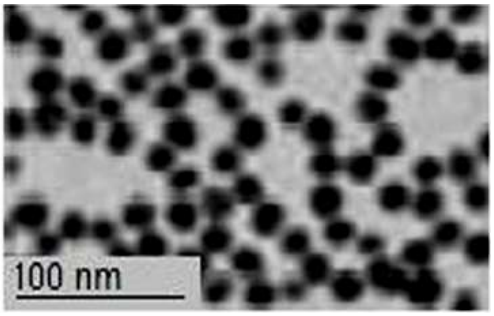

Figure 3. The TEM image of Glu-GNPs

Cytotoxicity of the agents

Figures 4 and 5 present the cell survival results at different concentrations of MB and Glu-GNPs, respectively. According to these figures, there was no significant variation in cell survival, and these two agents had no cytotoxicity at the different concentrations. Moreover, the cell survival decreased by 93.1% in the presence of MB (18µg/ml) with Glu-GNPs (80µg/ml) (Figure 6).

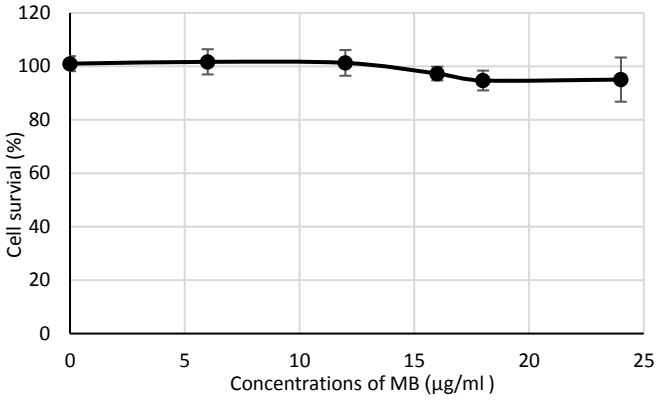

Figure 4. Percentage of the cell survival in the presence of various concentrations of Methylene Blue. The selected cell incubation period was 1h. The data represent mean ± standard deviation obtained from three experiments.

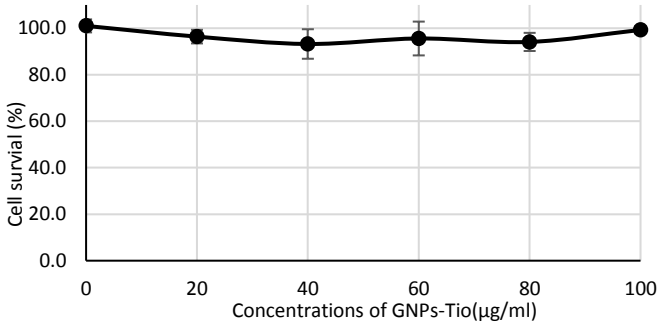

Figure 5. Percentage of the cell survival in the presence of various concentrations of Glu-GNPs. The selected cell incubation period was 2h. The data represent mean ± standard deviation obtained from three experiments.

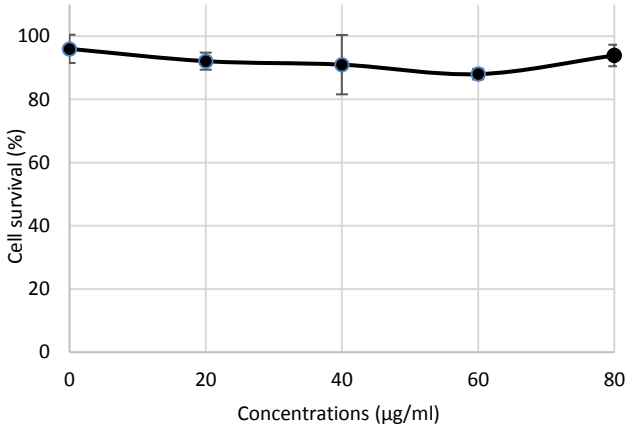

Figure 6. Percentage of the cell survival in the presence of various concentrations of Glu-GNPs combined with MB (with 18-µl/ml concentration of Methylene Blue). The selected cell incubation period was 2h. The data represent mean ± standard error of the mean obtained from three experiments.

PDT findings

To perform PDT, the cells were incubated by the treatment agents at different concentrations for 2h, and the treatment was then performed in three exposure doses. As shown in Figure 7, the MTT assay revealed that light alone did not affect the cell. The presence of MB with an exposure dose of 15.6 J/cm^2 resulted in ~80% cell death, and Glu-GNPs combined with exposure doses of 15.6 J/cm^2 had no effect on the cells. The simultaneous presence of Glu-GNPs and MB with an optical dose of 15.6 J/cm^2 induced ~34% cell survival. Furthermore, a significant difference was observed between some groups and the control group.

ED_{50} (the optical dose required to induce 50% cell death) was determined based on the findings of photodynamic treatments with MB in the presence or absence of Glu-GNPs. Figure 8 and Table 1 indicate that ED_{50} increases in the presence of Glu-GNPs.

Report plating efficiency of control group

After counting clones, plating efficiency (PE) and cell survival fraction (SF) were calculated based on the following equations:

$$\text{Plating efficiency (PE)} = \frac{Number\ of\ colonies\ formed}{number\ of\ cell\ seeded} \times 100 \quad (1)$$

$$\text{Survival fraction (SF)} = \frac{Number\ of\ colonies\ formed\ after\ irradiation}{Number\ of\ cell-seeded\ \times PE} \times 100 \quad (2)$$

According to the Clonogenic observations in this study, the PE value under all conditions was 0.4. Regarding the colony assay in Figure 9, the optical dose of 15.6 J/cm^2 alone decreased cell survival by about 70%, and 15.6 J/cm^2 of irradiation with MB induced the highest cell death rate without Glu-GNPs.

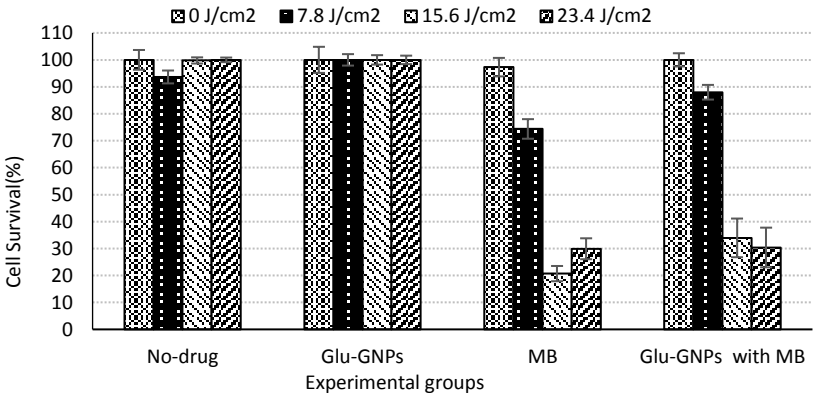

Figure 7. The percentage of cell survival for MCF-7 in terms of three exposure doses of 7.8, 15.6 and 23.4 J/cm^2 at a wavelength of 670 nm concentrations of Glu-GNPs and MB are 80 and 18 µg/mL, respectively (according MTT assay).

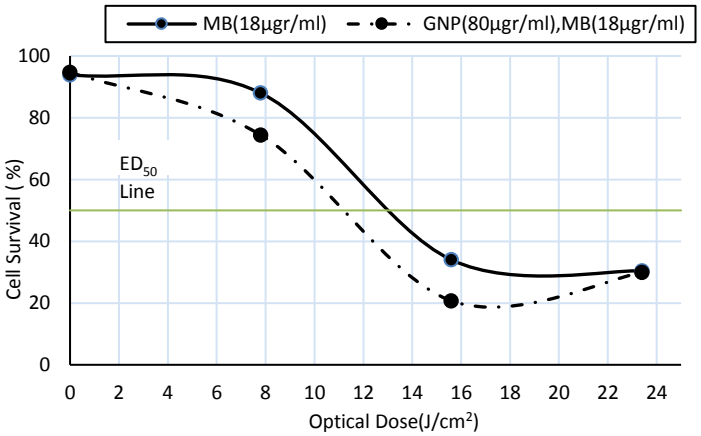

Figure 8. ED_{50} determination graph for MB in the presence and absence of Glu-GNPs.

Table 1. ED_{50} values in the presence of (80 µg/ml) Glu-GNPs and (18 µg/ml) MB.

	MB	Glu-GNPs + MB
ED_{50}(J/cm^2)	11.2	12.9

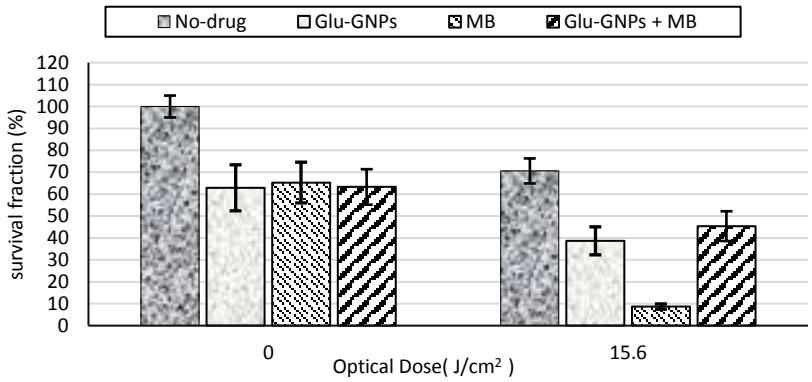

Figure 9. The survival fraction for MCF-7 in terms of exposure dose 15.6 J/cm2 at a wavelength of 670 nm concentrations of Glu-GNPs and MB are 80 and 18 µg/mL, respectively (according colony assay).

Discussion

MB is one of the photosensitizers, which has been of great interest to researchers because of features such as proper physico-chemical stability, optimal loading capability in pharmaceutical nanocarriers, and minimal toxicity [17]. MB, as a potent drug for PDT, has been approved for topical periodontal treatment due to its lower toxicity and the generation of singlet oxygen [18]. However, it has some drawbacks limiting its clinical application as a photosensitizer. When MB is injected intravenously or into a brain tumor, its efficacy is reduced due to its low accumulation in the tumor. Moreover, the optical absorption coefficient of MB decreases in the biological and cellular environment, and this can be compensated by loading MB in a nanostructure and conjugating with a targeting group such as antibodies or specific tumor peptides [19]. GNP has several advantages as a drug carrier, including acceptable solubility, high loading of medications, and limited effects for the multi-drug resistance of cancerous cells [20].

Previous studies have documented that the thio-glucose coated on GNPs results in increased cellular uptake by the endocytosis process [21]. In this study, we evaluated the effects of photodynamic treatment of MB alone and in combination with Glu-GNPs on the MCF-7 cells.

The toxicity of GNPs- Tio is escalated with an increase in concentrations, thereby decreasing the percentage of cell survival. A majority of studies (e.g., Rostami et al.) on the cytotoxicity of Glu-GNPs have confirmed that toxicity depends on the concentration and size of nanoparticles.

In the combinational experiments, the optimal concentration (lower than IC_{10}) of Glu-GNPs based on its cytotoxicity data was set to be 80 µg/ml. Moreover, the similar concentration of MB was 18µg/ml.

The treatment efficacy was determined by using cell survival determination (MTT), as a metabolic-based test, and colony assay, as a test for determining cells ability colony formation). The MTT assay has confirmed the best responses at the doses of 15.6 and 23.4 J/cm^2 in the presence of MB and MB-Glu-GNPs. At the light dose of 15.6 J / cm^2 in the colony assay, the findings were slightly different from MTT; however, in the presence of MB, a similar decrease in the cell survival was observed based on the cell colony formation ability and the cell metabolic activity. However, Glu-GNPs did not significantly affect cell colony formation ability after light exposure without altering the metabolic activity (P= 0.070). Further, the highest efficacy of photodynamic therapy was documented in the presence of MB and based on the cellular metabolic activity and the colony formation ability. According to the MTT and colony assay findings, the simultaneous presence of two agents did not positively improve photodynamic treatment efficiency.

The 'Enhanced Therapeutic Index' (ETI) is defined to describe the effect of GNPs on the PDT efficacy as the ratio of "cell death after PDT in the presence of GNPs" to the "cell death after PDT without GNPs." Accordingly, the MB-PDT ETI was estimated by using the MTT and colony assay findings to be 0.83 and 0.32, respectively. It is worth noting that Glu-GNPs not only provided no additive effect on MB- PDT also acted as a restrictor. This finding is in agreement with the precipitated ED_{50} for MB with and without GNPs. ED_{50} was estimated to be higher in the presence of GNPs than in their absence.

The decrease in photodynamic efficiency of MB with Glu-GNPs, compared to the free MB, can be due to the following phenomena, which necessitates further studies to delve into the exact mechanisms:

1) When vesicles containing MB and Glu-GNPs are exposed to light, any ROS produced by the plasmonic photodynamic phenomenon of Glu-GNPs causes MB bleaching because they are trapped in an endolysosomal vesicle (a short distance from each other).
2) Due to the difference in surface charge of anionic nanostructures and cationic drugs, these components form an unwanted electrostatic bond during the endocytosis process, and their optophysical properties (e.g., adsorption peak displacement) change, thereby

decreasing ROS production efficiency and optical toxicity.

Narband's et al. report may justify this finding as they showed that dye molecules belonging to the Thiazines family are capable of adsorption onto GNPs. MB is a dye from the thiazines family, and its adsorption onto GNPs is up to 0.5 μM . However, agglomeration occurs at a concentration of ≥0.79 μM [22]. Similarly, the present findings could be caused by the adsorption of MB on Glu-GNPs, which decreased the MB extinction coefficient. In other words, increasing the cellular uptake of MB and decreasing the extinction coefficient were two factors that neutralized each other and inhibited the increase in the efficiency of photodynamic treatment in the simultaneous presence of MB and Glu-GNPs.

It should be noted that only two studies on the microbial microorganisms have reported the use of MB and its conjugate with GNPs; however, none of these studies have focused on cancer cells. In one of the studies, Darabpour et al. (2016) examined the photodynamic effect of MB-conjugated GNPs on the biofilm of a Methicillin-Resistant Staphylococcus Aureus (MRSA) using 650 nm laser light and reported that the PDT efficiency was higher in the presence of conjugated MB than MB alone [23].

In the second study, Maliszewska et al. (2014) also showed an increased photodynamic effect in the presence of MB-conjugated GNPs on Staphylococcus epidermidis, compared to MB alone, during xenon lamp light (550–780nm) and He-Ne laser (632 nm) with 5 and 10 min irradiations [24].

Conclusion

PCI is a branch of photodynamic therapy for delivering macromolecular (or photosensitizer) into the cytosol by the endocytosis process. When the cells are exposed to a low dose of specific wavelength light in PCI, the endosomal/lysosomal membranes are disrupted, and their content is released in the intracellular space. PCI seems to facilitate the uptake of PS or macromolecules not readily transmitting from the plasma membrane. This study aimed to evaluate the efficacy of the photodynamic effects of methylene blue due to the presence of gold nanoparticles coated with thioglucose regarding photochemical internalization. The photodynamic efficiency of MB with the Glu-GNPs group was reduced at 15.6 J / cm^2, compared to the free MB group, which might have various reasons such as the photochemical bleaching of the free MB because of ROS and $^1O_2$ produced by the plasmonic photodynamic phenomenon of Glu-GNPs or changing the optophysical properties of surface plasmon resonance of final product (MB+ Glu-GNPs) due to the possible electrostatic bonding of the drug with the nanoparticles. Further studies are recommended to delve into the exact mechanism involved in this process.

Acknowledgment

The authors would like to thank the Research Deputy of the Mashhad University of Medical Sciences, Mashhad, Iran, for their financial support. The findings described in this paper are part of a master's thesis in Nuclear Engineering.

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