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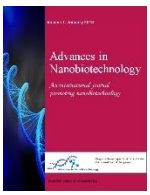

Design and Synthesis of an Efficient Nanophotosensitizer used in Photodynamic Therapy

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ABSTRACT

Gold nanoparticle (GNP) was used for the delivery of a hydrophobic protoporphyrin IX (PpIX) as an efficient photosensitizer for photodynamic therapy. This conjugated GNPs were made by a single coupling reaction. Their optical properties indicate that PpIX-GNP conjugates could be used as a photosensitizer in photodynamic therapy (PDT) through the formulation of singlet oxygen. The GNP conjugates have an average diameter of 7 nm. Also, the 'dark' toxicity test of each of these individual components of these nanoparticles were carried on Hela cells.

1. Introduction

Todays, nanotechnology has attained a profound impact within medical science and nanoparticles can accumulate at the tumor site due to enhance endocytotic activity and leaky vasculature in the tumors [1]. Drugs on the NPs could be shielded from being uptaken by the reticuloendothelial system (RES). Therefore, the circulation time of NPs in the blood can be extended [2].

At present, the predominantly used photosensitizers are represented by porphyrins and their analogs [3]. Porphyrins are a class of tetrapyrroles that have attracted the attention of researchers owing to their extensive application as photosensitizing drugs in medicine. Protoporphyrin IX (PpIX) is a naturally occurring porphyrin constituent of hemoglobin, cytochrome *c* and other biologically relevant molecules. PpIX is produced in the body by the conversion of 5-aminolevoulinic acid through the heme biosynthetic pathway [4]. It has been shown that PpIX is photodegraded by a photooxidation process and its photoproducts have a characteristic absorption band around 630 nm [5]. PpIX is used as a drug in PDT but its direct application is limited due to its aggregation and low solubility in a physiological medium.

In a recent study about the systems as photosensitizer delivery carriers, it was speculated that the surface bound photosensitizer on nanoparticles may present an advantage over an encapsulated photosensitizer because of facility diffusing the generated singlet oxygen [6]. Gold nanoparticles, because of their unique properties and ease of functionalization, traditionally have biological applications such as biosensors for assays, and targeted delivery of drugs into cells [7, 8].

In this paper, GNPs which have been widely investigated and officially approved as drug carriers in clinical applications were conjugated with PpIX via thiol linkage. To attach the PpIX molecule to the gold surface, GNPs were functionalized with 6-mercapto-1-hexanol. The thiol moieties of the substrate provided a direct linkage to the gold nanoparticle surface via self-assembly using a gold salt, 6-mercapto-1-hexanol and sodium borohydride. Then the photosensitizer was bound to the gold nanoparticle surface by a single

coupling reaction. Also, the 'dark' toxicity test of each of these individual components of these nanoparticles were carried. This study has been focused on the synthesis of PpIX conjugated gold nanoparticles that can be used in photodynamic therapy.

2. Experimental Section

2.1. Chemicals

Protoporphyrin IX, hydrogen tetrachloroaurate (III) trihydrate ($HAuCl_4.3H_2O$, 99.5% purity), sodium borohydride (NaBH4, 99.99% purity), and Thiazolyl blue tetrazolium bromide (MTT) were purchased from the Sigma–Aldrich Company. Fetal bovine serum and RPMI 1640 were provided by the Hyclone Laboratories, Inc. (HyClone, Logan, UT, USA). All other reagents used in this research were purchased from Merck, Germany and Fluka, Switzerland.

2.2. Instrumentations

UV-visible (UV-vis) absorption spectroscopic measurements were recorded on a UV-vis spectrometer, Shimadzu UV1700, using quartz cells of 1 cm path length and methanol as the reference solvent at room temperature. Transmission Electron Microscopic (TEM) images of the nanoparticles were taken with a LEO 912AB instrument operating at an accelerating voltage of 120 kV with line resolution of 0.3 nm at room temperature. The samples for TEM measurements were prepared by placing a droplet of the colloidal solution onto a carbon-coated copper grid and allowing it to dry in air naturally. Based on the TEM images we determined the size distribution of the final product by counting at least 300 particles. We determined the gold percentage in the conjugated gold nanoparticles by Shimadzu model AA-670 atomic absorption spectrophotometer.

Preparation of protoporphyrin-conjugated with GNPs

For preparation of protoporphyrin-conjugated with GNPs on the basis of our previous work [9], first, a solution of $HAuCl_4.3H_2O$ (13 mg, 0.03 mmol) in CH_3OH (2 mL) was added to a stirred solution of 6-mercapto-1-hexanol (20 mg, 0.03 mmol) in CH_3OH (1.5 mL). After 30 min, a freshly prepared solution of sodium borohydride (19 mg, 0.5 mmol) in CH_3OH (1.5 mL) was added to the vigorously stirred reaction mixture. The reaction mixture turned deep brown indicating the formation of GNPs. The stirring was continued for 2 hrs. Finally, the conjugates of GNPs with 6-mercapto-1-hexanol were separated from methanol by precipitation under centrifuge at 10,000 rpm and purified by successive washing with CH_3OH.

Second, a 0.49 μmol quantity of 58% of above synthesized nanoparticles was treated with 53 μmol (11 mg) of N, N′-dicyclohexylcarbodiimide (DCC) and 53 μmol (6.5 mg) of dimethylaminopyridine (DMAP) in 5 ml of THF. Following a brief activation period (10 min), 43 μmol (13.5 mg) of protoporphyrin (PpIX) in THF (1 ml) was added to the reaction mixture, and the solution was stirred at room temperature for 24 hrs. The solvent was removed under reduced pressure, and the residue was washed with hexane (20 ml) and acetonitrile (15 ml) successively to give the desired conjugated gold nanoparticles.

Cell Culture Condition

HeLa cells (derived from a human cervical cancer) were cultured in 75 cm^3 tissue culture flasks in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 1% penicillin–streptomycin (P–S) and 1% *L*-glutamine (all purchased from Invitrogen Life Sciences). The cells were grown at 37 ^oC in a humidified 5% CO_2/95% air atmosphere.

Dark toxicity test

HeLa cells (2×10^4 cells per well) were seeded in 96-well tissue culture plates. One day after the cultivation, a 200 μl stock solution of PpIX-conjugated GNPs (2.5- 30 μg/ml) was added to a HeLa cell plate. 200 μl stock solutions of GNPs and PpIX were added to another HeLa cell plate for a control experiment. A forth HeLa cell plate without any treatment was used as another control. HeLa cells were incubated with these three compounds in 5% CO_2 atmosphere at 37 ^oC for 3 hrs. Afterward, compounds containing media were removed, and the cells were washed with sterile phosphate buffered saline (PBS) and complete culture medium was added.

The viability of Hela cells was assessed 16 hrs after treatment. To determine cell viability, the colorimetric MTT metabolic activity assay developed by Mossman [10] and subsequently modified by Prasad and co-workers, was used [11]. Cell viability was assessed using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. MTT was dissolved in PBS (5 $mgmL^{-1}$), and 10 μL of it was added to each well following the 16 h incubation. The cells were then incubated for a further 4 h. The resultant formazan crystals were dissolved in dimethyl sulfoxide (200 μL) and the absorbance intensity was measured at 570 nm.

Data analysis

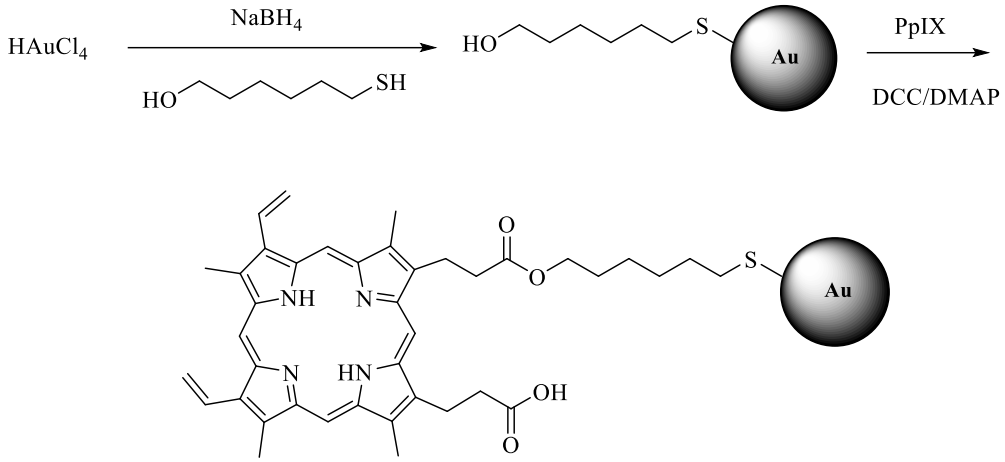
Each experiment was repeated four times as triplicate. First, Kolmogrov-Smirnov test was applied in order to assure their normal distribution. Then data analysis was performed by one-way ANOVA and Tukey test with SPSS software version 16. All data are presented as means ± SD. A value of $P < 0.05$ was considered as significant.

3. Results and discussion

3.1. Identification of protoporphyrin IX-conjugated GNPs

We designed, synthesized and characterized conjugated with GNPs, for using in photodynamic therapy. For this purpose, as shown in Scheme 1, we synthesized GNPs

conjugated with mercaptohexanol based on standard wet chemical methods using sodium borohydride as a reducing agent. Then the PpIX was bound on the functionalized gold nanoparticles' surface by a single coupling reaction to produce PpIX-conjugated GNPs.



Scheme. 1. Protoporphyrin IX-conjugated gold nanoparticle synthesis.

For in vivo applications of PDT, preferred photosensitizers are those efficiently absorbing red light (λ > 600 nm). Such wavelengths exhibit a relatively deep penetration power into most human tissues. The extinction spectra and emission spectra of PpIX conjugated gold nanoparticles in water are shown in Fig. 1.

The absorption peak of the PpIX after conjugation was 630 nm, which had red-shifted 10 nm in comparison to the absorption peak of the PpIX before conjugation; this change may be attributed to the surrounding media. Accordingly, the appearance of a red-shifted absorption band due to coupling of the individual surface plasmon of nanoparticles in the aggregated structures will be observed if the interlinked nanostructures are formed.

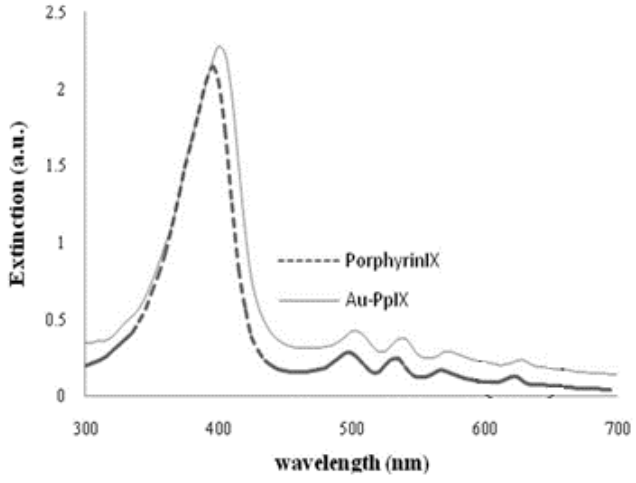


Fig. 1. Extinction spectra of PpIX before and after conjugation with gold nanoparticles.

Figure 2 shows the TEM image of the PpIX-conjugated gold nanoparticles. These nanoparticles were all spherical in shape, and had an average diameter of 7 nm. Cellular uptake and intracellular fate of AuNPs depends on surface functionality, charge, size and hydrophobicity. Furthermore, tumoral cells need PpIX for their growth, DNA nucleotide synthesis and cell division. On the other hand, the sign of surface charge on AuNPs significantly affects the constant affinity between the particles and cell membranes. So, due to both or either of the surface charge interaction or functional interaction with receptor mediated endocytosis, the uptake of nanoparticles conjugated with PpIX would be efficient.

Moreover, GNP is a very versatile support material, structurally more stable, biocompatible, and chemically more resistant than other metals, offering ease of surface modification and biotargeting for site-specific delivery. The methodology here reported is very general and can be applied to several other drug-conjugated gold nanoparticles.

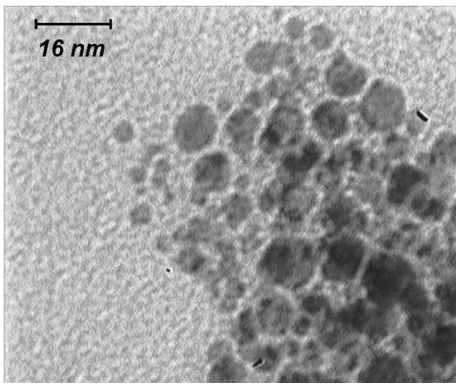


Fig. 2. TEM image of PpIX-conjugated GNPs (scale bar is 16 nm).

3.2. Dark Toxicity Results

In this study, were needed. Cell viability (as % metabolic

activity) for each parameter using the colorimetric MTT reagent was determined. In order to achieve whether a specific component of the conjugates was cytotoxic, each constituent (including varying concentrations (2.5–20 μg/ml)) was tested by measurement of cell viability. Control experiments were undertaken for each variable. The component tested included GNPs and the free PpIX both at concentrations determined to be present in nanoparticle conjugates. The results of the 'dark' toxicity test of each of these individual components are shown in Fig. 3. The results show that the HeLa cell viability increases when the concentration of the PpIX is 2.5 μg/ml (P = 0.027), however the increase in concentration up to 10 μg/ml causes significant inhibition in cell proliferation (P < 0.05). A similar pattern in the presence of similar concentrations of GNPs is observed. However, conjugated nanoparticles at all concentrations reduce cell proliferation in comparison to the control group (P < 0.032). Considering the small size of the gold nanoparticles, we can anticipate that the HeLa cells would internalize the photosensitizer nanoparticle conjugates from the extracellular environment through endocytotic processes.

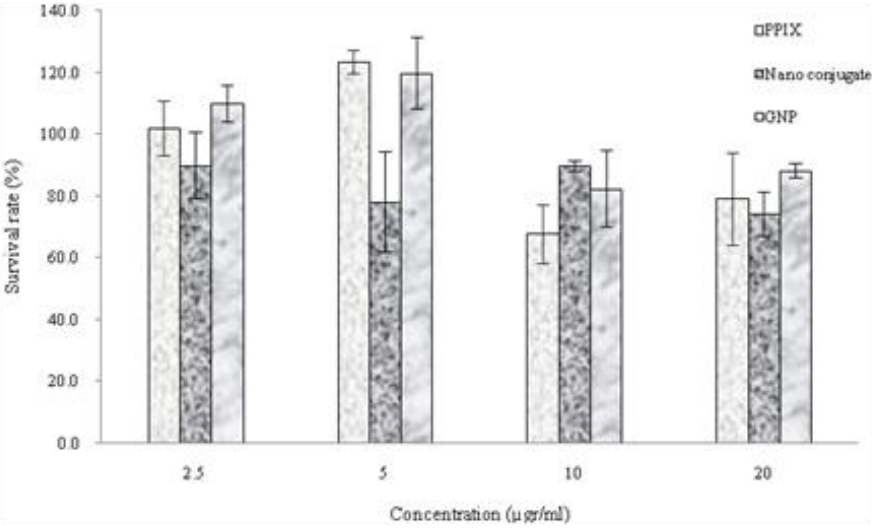


Fig. 3. The effect of 'dark' toxicity and the phototoxicity of each individual component (from 2.5 to 20 μg/ml) on HeLa cell viability was assessed by using the MTT assay.

4. Conclusions

In this paper, GNPs which have been widely investigated and officially approved as drug carriers in clinical applications were conjugated with PpIX via thiol linkage. The photosensitizer was bound to the gold nanoparticle surface by a single coupling reaction. Also, the 'dark' toxicity test of each of these individual components of these nanoparticles were carried. This study has been focused on the synthesis of PpIX conjugated gold nanoparticles that can be used in photodynamic therapy. Further specified localization of the drug within tumor sites by conjugating receptor-specific targeting moieties onto the nanoparticle surface and in vitro and in vivo experiments are currently under study.

Acknowledgements

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References

[1] A. Roby, S. Erdogan, V.P. Torchilin, Solubilization of poorly soluble PDT agent, meso-tetraphenylporphin, in plain or immunotargeted PEG-PE micelles results in dramatically improved cancer cell killing in vitro. *Eur. J. Pharm. Biopharm.* **2006**, *62*, 235-240.

[2] G.F. Paciotti, D.G.I. Kingston, L. Tamarkin, Colloidal Gold Nanoparticles: A Novel Nanoparticle Platform for Developing Multifunctional Tumor-Targeted Drug Delivery Vectors. *Drug. Deliv. Res.* **2006**, *67*, 47-54.

[3] G. Jori, W. Horspool, F. Lenci, (Eds) CRC Handbook of Organic Photochemistry and Photobiology. *CRC Press, Boca. Raton.* **2003**, 1461–1471.

[4] Z. Xiao, Y. Tamimi, K. Brown, J. Tulip, R. Moore, Interstitial photodynamic therapy in subcutaneously implanted urologic tumors in rats after intravenous administration of 5-amino-levulinic acid. *Urol. Oncol.* **2002**, *7*, 125-132.

[5] M.B. Ericson, S. Grapengiesser, F. Gudmundson, A.M. Wennberg, O. Larko, J. Moan, A. Rose´n, A spectroscopic study of the photobleaching of protoporphyrin IX in solution. Lasers Med. Sci. **2003**, *18*, 56-62.

[6] S.Z. Wang, R.M. Gao, F.M. Zhou, M. Selke, Nanomaterials and singlet oxygen photosensitizers: potential applications in photodynamic therapy. *J. Mater Chem.* **2004**, *14*, 487-493.

[7] G. Justin, Electrochemical DNA Hybridization Biosensors. *Electroanalysis*, **2002**, *14*, 1149-1156.

[8] S. Trudel, Unexpected magnetism in gold nanostructures: making gold even more attractive. Gold Bull. **2011**, *44*, 3-13.

[9] H. Eshghi, A. Sazgarnia, M. Rahimizadeh, N. Attaran, M. Bakavoli, S. Soudmand, Protoporphyrin IX—gold nanoparticle conjugates as an efficient photosensitizer in cervical cancer therapy, *Photodiagnosis and Photodynamic Therapy*, **2013**, *10*, 304-312.

[10] T. Mossman, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods*, **1983**, *65*, 55-63.

[11] I. Roy, T.Y. Ohulchanskyy, H.E. Pudavar, E.J. Bergey, A.R. Oseroff, J. Morgan, T.J. Dougherty, P.N. Prasad, Ceramic-based nanoparticles entrapping water- insoluble photosensitizing anticancer drugs: a novel drug-carrier system for photodynamic therapy. *J. Am. Chem. Soc.* **2003**, *125*, 7860-7865.