Gold nanoparticles/ZnO nanorods hybrids for enhanced reactive oxygen species generation and photodynamic therapy

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**Gold nanoparticles/ZnO nanorods hybrids for enhanced reactive oxygen species generation and photodynamic therapy**

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This work demonstrated the enhanced reactive oxygen species generation of Au nanoparticle /ZnO nanorod hybrids under UV illumination, and further confirmed the feasibility of adopting such hybrids for photodynamic therapy treatment.

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Yue Zhang, nano.ustb.edu.cn/
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ABSTRACT
Gold nanoparticles (Au NPs)@ZnO nanorods (NRs) hybrids, with various molar ratios of ZnO: Au, were developed to enhance generation of reactive oxygen species (ROS) for further application in photodynamic therapy (PDT). By introducing the metal/semiconductor heterostructure interface between Au NPs and ZnO NRs, the electron transfer was modulated under UV irradiation, which dramatically suppressed the electron-hole recombination in ZnO, and simultaneously increased the amount of excited electrons with high energy at Au NPs’ surface. Hence, the ROS yield of the nanohybrid was considerably improved compared with pristine Au NPs or pristine ZnO NRs, demonstrating the 1 + 1 > 2 effect. Moreover, such enhancement was strengthened along with the increase of Au proportion in the hybrid. It was exhibited that the Au@ZnO (ZnO: Au=20:1) acquired the highest ROS yield due to the largest interface area between Au and ZnO, which in turn led to the lowest cell viability for Hela and C2C12 cells during PDT. Besides, both ROS generation and cell destruction have positive correlation with nanohybrid dosage. The Au@ZnO hybrid (20:1, 100 µg/ml) resulted in the Hela cell viability as low as 28% after UV exposure for 2 min, indicating a promising potential for therapeutic efficacy improvement in PDT.

KEYWORDS
gold nanoparticles; ZnO nanorods; reactive oxygen species; photodynamic therapy

1 Introduction
Photodynamic therapy (PDT) has been well developed in past decades as an alternative method to the traditional treatment in different oncologic field because it promises a better selectivity for tumor tissue that is accessible to light and low systemic toxicity compared to the chemotherapy and radiation therapy [1-3]. In the past decades, PDT has been applied for the treatment of early lung cancer
[4], head and neck cancers [5], bladder cancer [6], and skin cancer [7]. PDT is based on the photochemical reactions of photosensitizing agent (PS) who is capable of generating cytotoxic reactive oxygen species (ROS) to kill tumor cell when exposed to light of appropriate wave-length [8-11]. ROS include free radicals such as superoxide anion (O2·−), hydroxyl radical (·OH), as well as nonradical molecules like hydrogen peroxide (H2O2), singlet oxygen (O2), which formed upon incomplete reduction of oxygen [12]. When the level of ROS exceeds the defense mechanisms, they can pose a threat to cells by causing peroxidation of lipids, oxidation of proteins, damage to nucleic acid, and ultimately lead to various diseases and cell apoptosis and necrosis [13]. Therefore, enhanced ROS generation is crucial for PDT of cancer.

Various PSs have been approved for clinical PDT, such as porphyrins like porfimer sodium and protoporphyrin IX, chlorins like verteporfin and temoporfin, as well as phthalocyanines like sulphonated aluminium phthalocyanine mixture [14]. Recently, semiconductor nanomaterials are believed to have potentially promising future in PDT due to their unique phototoxic effect with the irradiation. Nano-scale ZnO, with direct wide band gap energy of 3.37ev at room temperature, can generate ROS in aqueous media under ultraviolet (UV) illumination. Since the UV light can be applied locally during PDT treatment, it is possible to reach an approach for conveying selective damage to targeted cancer cells, while sparing neighboring untargeted cells. It has been demonstrated that ZnO nanostructures were employed to efficiently kill cancer cells through the release of ROS [15-20]. And the antibacterial activity of ZnO nanoparticles originated from ROS induced membrane lipid peroxidation was verified [21]. On the other hand, Au nanostructures, such as nanoparticles (NPs) [22-27], nanorods [28-34], nanocages [35, 36], and nanoshells [37, 38], were developed as effective therapeutic tools in applications of photothermal therapy (PTT) [25, 28-35, 38] or PDT [22-24, 27, 28, 33-35] to induce cellular damage either via extensive temperature rise or considerable ROS generation. Specifically for Au NPs in PDT, ROS generation of Au NPs with diameters of 5-250 nm in water was investigated, indicating an inverse proportion of ROS quantity to the Au NPs’ diameter, and thus suggests that larger surface area of Au NPS is more favorable for ROS yield [22]. In addition, the size-dependent enhancement of ROS formation enabled by protoporphyrin IX conjugated with Au NPs in human breast cancer cells (MDA-MB-231) was also confirmed [23]. With the aid of antibody-coated Au NPs, the selective damage was conveyed to targeted cancer cells based on ROS accumulation within cells after femtosecond pulse irradiation [24]. More recently, Au NPs were deposited on ZnO NPs for effective promoting photocatalytic activity and antibacterial activity through ROS generation during photoexcitation [39]. To date, even though many problems regarding PDT have been solved, issue like significant enhancement of ROS generation still needs further optimization.

In this article, we proposed a novel strategy of combining ZnO nanorods (NRs) with Au NPs to enhance ROS generation under UV irradiation. A facile solution method was utilized to decorate Au NPs on ZnO NRs with tunable molar ratio of ZnO:Au. Unlike most of previous reports, in which nanostructures usually worked as carriers for PSs or light transducers [3, 35, 36, 40-42], the so-synthesized nanohybrid was also responsible for the role of PS to induce ROS release. By introducing the semiconductor/metal heterostructure between ZnO and Au, the electron transfer at the interface was modulated when excited by UV, resulting in a considerable enhancement of ROS generation compared with pristine ZnO NRs or pristine Au NPs. Moreover, the samples with molar ratio (ZnO:Au) of 20:1 yielded the highest ROS generation because of the largest interface area, which in turn led to the lowest cell viability for Hela and C2C12 Cells after PDT treatment. In this case, the therapeutic efficacy on Hela cells was demonstrated to be remarkably improved due to the enhanced ROS generation by Au@ZnO nanohybrids.
2 Results and discussion

Fig. 1a shows a typical TEM image of as-synthesized Au@ZnO nanohybrids, in which the high contrast difference between ZnO and Au is distinguishable because of the higher electron density of metal Au. The mean size of the nanorods is about 20nm in diameter (Fig. 1b) and 100nm in length (Fig. 1c), and the average diameter of the Au nanoparticles is about 5 nm. Since there are many single ionized oxygen vacancies (Vo⁺) on the surface of ZnO nanorods, the surface energy of “active center” is higher than that of the nonpolar planes and is energetically favored to Au deposition [43]. It has been demonstrated that Au nanoparticles were not absorbed onto the ZnO nanorods physically, but nucleated and grew on the ZnO nanorods [44]. By adjusting the molar ratio of ZnO to HAuCl₄, varying amounts of Au NPs in the samples were acquired (Table 1). Specifically, for the nanohybrid with the molar ratio (ZnO:Au) of 100:1, it was calculated that there should be approximately 3.5 Au NPs per single ZnO NR.

<table>
<thead>
<tr>
<th>ZnO: Au molar ratio</th>
<th>Au NPs@ZnO NRs</th>
<th>ZnO NRs (mg/ml)</th>
<th>HAuCl₄ (µmol)</th>
<th>Au NPs/ single ZnO NRs</th>
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<td>20:1</td>
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<td>50:1</td>
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Table 1 Characteristics of samples with various ZnO: Au molar ratio

In order to characterize the chemical valence state of Au in NPs, XPS was performed (Fig. 1d). The spectra were calibrated with respect to the C1s line of...
advocatory carbon at 284.8 ev. The Au 4f 7/2 peak is centered at binding energy of 83.8 eV, with a slight shift of ~0.2 eV from the binding energy of bulk metallic gold (84.0 eV) [45]. This shift toward lower value can be attributed to the small size and negative charging of Au NPs. Hence, the deposited Au NPs are confirmed to be metallic elementary substance. The obtained X-ray diffraction patterns are in good agreement with the standard hexagonal wurtzite structure of ZnO (JCPDS 75-0576) and the diffraction peaks of (111) and (002) planes of the face-center cubic (fcc) gold (Fig. 1e). In addition, no other impurity phase and no remarkable shift of all diffraction peaks were detected.

In an aim to confirm the enhanced ROS generation induced by Au@ZnO nanohybrids, quantitative measurement of ROS formation should be carried out. However, the direct quantification of ROS yield is extremely challenging due to the short lifetime of ROS, so various ROS probes were developed for indirect measurement [46]. Here, nonfluorescent dihydrododamine 123 (DHR123) was adopted as ROS tracking agent, which was oxidized to fluorescent Rhodamine 123 (R123) in the presence of ROS [47]. It is known that neither Au NPs nor ZnO NRs is fluorescent at the wavelength of 535 nm. Even though the surface-enhanced effect of Au NPs might have a little influence on fluorescence measurement, it could hardly cause any impact on the overall trend of the results due to the extremely low concentration of Au NPs in the complex solution [23]. Under this circumstance, the measured fluorescence intensity at 535 nm was basically proportional to the conversion of R123 from DHR123, that is, to the generated ROS concentration. As shown in Fig. 2a, enhanced ROS generation was confirmed through the fluorescence intensity detection. Clearly, for the Au@ZnO nanohybrids, the ROS yield was notably improved along with the variation of ZnO:Au molar ratio from 100:1 to 20:1 at a fixed concentration of 100 µg/ml. To better elucidate the enhancement effect, pristine Au NPs and pristine ZnO NRs with corresponding concentrations as that in the nanohybrids were introduced as comparisons. We found the ROS yields of the hybrids are much higher than that of the pristine ZnO NRs or the pristine Au NPs, and even higher than that of the sum of both, demonstrating the “1 + 1 > 2 effect” for the ROS generation enhancement. This phenomenon was demonstrated to be most significant in the 20:1 nanohybrids and a positive correlation between enhancement degree and the proportion of Au in the hybrid was confirmed. This indicated that such enhancement could be manipulated by the Au/ZnO interface area which was decided by the molar ratio of ZnO:Au in the nanohybrid. On the basis of fluorescence
intensity measurement, the time-resolved alteration of ROS yield in the presence of Au@ZnO nanohybrids (100 µg/ml) was also recorded in Fig. 2b. The ROS yield increased approximately linearly as the increase of the irradiation time. The 20:1 group obtained the highest slope, suggesting the fastest increase rate of the nanohybrid with the largest heterostructure interface area. Aside from these, the ROS yield against various nanohybrid concentrations ranging from 6.25 to 100 µg/ml was depicted in Fig. 3, suggesting an obvious dose-effect.

Figure 3 ROS yield after 2 min UV irradiation against various Au@ZnO nanohybrid concentration (mean ± SD, n = 3). The molar ratio of ZnO:Au was kept at 20:1.

To understand the phenomenon of enhanced ROS yield, the mechanism of ROS generation induced by photodynamic Au@ZnO nanohybrid is schematically displayed in Fig. 4. ZnO, with direct band gap of 3.36 eV, produce electron/hole pairs after exposure to UV irradiation. The photon-generated electrons in the conduction band and holes in the valence band would continuously induce a serious of photochemical reactions to generate ROS at ZnO NR surface in the aqueous solution [15]. Generally, electrons reduce O₂ to produce superoxide anion (O₂⁻) while holes extract electrons from water and hydroxyl ions to produce hydroxyl radicals (·OH). On the other hand, at the surface of pristine Au NPs, UV irradiation results in producing photoelectrons and Augar electrons, which subsequently participate into the ROS generating reactions [22]. However, ZnO NRs and Au NPs do not only separately play their own roles contributing to the ROS generation during photochemical reactions, and the simple stacking of each effect from Au and ZnO is not sufficient to explain such great enhancement of ROS yield. Therefore, a possible explanation regarding interfacial coupling between metal Au and semiconductor ZnO in the heterostructure is proposed (Fig. 5). Since the work function of gold (5.1 eV) is lower than that of ZnO (5.2-5.3 eV) [48-50], the Fermi level of gold is higher than that of ZnO. Consequently, electrons transfer from Au to ZnO in order to equilibrate the Fermi energy level during the formation of Au@ZnO nanohybrids. The transferred electrons subsequently accumulate at the interface between ZnO and Au, causing a downward band bending at ZnO side. When UV irradiation is applied, electrons in ZnO are activated from valence band to conduction band. Due to the downward bend bending at the interface, the excited electrons are more inclined to flow to the Au side. For Au NPs, UV irradiation inspires photoelectrons and Augar electrons to take part in ROS generation. At meanwhile, the visible-light photons released by electron transition process from the defect energy levels in ZnO result in the surface plasmon resonance (SPR) at Au surface. The majority of excited electrons further contribute to generate ROS, while the minority excited electrons are injected back.

Figure 4 Schematic illustration of the proposed model for enhanced ROS generation induced by Au NPs@ZnO NRs hybrid under UV irradiation.
Figure 5 The energy level diagram of ZnO NRs and Au NPs before contact, after contact and under UV radiation.

to the conduction band of ZnO. A portion of the minority electrons jump back to the valence band for recombination and the rest of them further flow into Au along the downward bending band. In general, the band bending near the interface at ZnO side significantly facilitates electron transfer to Au side, thus considerably suppressing the electron-hole recombination amount in ZnO, and simultaneously increasing the amount of excited electrons with high energy at Au surface, which finally improves the efficiency of ROS generation.

To sum up, ZnO NRs and Au NPs contribute synergistically to form metal/semiconductor heterostructure to modulate electron transfer at interface, which in turn lead to the great enhancement of ROS generation. Here, the heterostructure interface is the key point. So it is reasonable to explain that the enhancement effect could be controlled by tuning the heterostructure interface area, that is, tuning the molar ratio of ZnO: Au in the hybrid. Consequently, it is acceptable for the 1 + 1 > 2 effect on the enhancement of ROS generation.

Prior to the application in PDT, we studied the cytotoxicity of so-synthesized nanohybrids toward Hela and C2C12 cell lines through thiazolyl blue tetrazolium bromide (MTT) assays. The results based on two cell lines in Fig. 6 are consistent, indicating that Au@ZnO nanohybrids were almost nontoxic even at a relatively high concentration of 100 µg/ml with the variation of ZnO: Au molar ratio from 100:1 to 20:1. However, the negligible impact on cell viability still existed, which could be attributed to the cytotoxicity mainly coming from ZnO NRs [15, 44].

Figure 6 Toxicity test of Au@ZnO nanohybrids (100 µg/ml) based on Hela cells and C2C12 cells (mean ± SD, n = 3).

It has been reported that the light with a specific wavelength would excite the photosensitizing agent to generate ROS, which is believed to be responsible for selective tumor cell destruction [7]. And the therapeutic effect in PDT treatment closely correlated with the intracellular ROS level [23, 51]. Moreover, the cellular uptake of nanomaterials, such as gold, ZnO or even the composite in the size the same as so-synthesized nanohybrids have been widely demonstrated [15, 44, 52, 53]. As for Au@ZnO nanohybrids, the PDT efficacy against different molar ratio of ZnO: Au and different hybrid concentration on Hela (Fig. 7) and C2C12 cells (Fig.
S1) have been investigated. With the exposure to UV, 79% cell viability was acquired in the pristine ZnO group and around 56% cell viability was obtained for the 20:1 nanohybrids (25 µg/ml) (Fig. 7a). Noticeably, with the increase of the proportion of Au in nanohybrids, the PDT efficacy on Hela cells was obviously strengthened, and the cell destruction ratio was 2.01:1.31:1 with various ZnO:Au molar ratio of 20:1, 50:1 and 100:1. The result is basically consistent with ROS generation enhancement ratio of 2.22:1.25:1 characterized by ROS tracking agent (Fig. 3a). However, even though the hybrid of 100:1 yielded much more ROS than pristine ZnO, they did not perform significant difference in the cell viability. The reason might be the elaborate ROS defense mechanism of cells at a relatively low ROS concentration, so there is a threshold value of ROS concentration for cell damage. Moreover, the dose dependence of PDT efficacy was confirmed with the variation of nanohybrid concentration at a fixed molar ratio of 20:1 (Fig. 7b). The trend of cell destruction against nanohybrid concentration is also in agreement with the quantitative analysis of ROS generation in Fig 4. It is worth pointing out that the nanohybrid of 100 µg/ml yielded the Hela cell viability as low as 28% after the exposure to UV for 2 min. In Fig. 7c and 7d, it can be seen that the number of living Hela cells dramatically decreased and the cell shape obviously changed, suggesting the remarkable PDT efficacy of Au@ZnO nanohybrids. This is coherent with the MTT assay results of PDT experiments.

3 Conclusions
To summarize, we have explored the feasibility of
combining ZnO NRs and Au NPs for enhanced ROS generation and PDT. The introduction of Au/ZnO metal/semiconductor heterostructure considerably enhanced ROS generation under UV irradiation, and then a possible explanation regarding the modulation of electron transfer at the interface was proposed. Moreover, such enhancement was strengthened along with the increase of Au/ZnO interface area, that is, the proportion of Au in the hybrid. We have also shown that the cell viabilities of Hela and C2C12 cells were significantly influenced during the PDT in the presence of so-synthesized nanohybrid under UV irradiation, confirming the potential of adopting Au@ZnO hybrids in PDT application for comprehensive cancer treatment.

4 Methods

Gold (III) chloride trihydrate (HAuCl₃.3H₂O), dihydrohodamine-123 (DHR123) were purchased from sigma-Aldrich (St.Louis, MO, USA). Zn(Ac)₂.2H₂O, NaOH, ethanol, trisodium citrate and polyethylene glycol 400 (PEG-400) were purchased from Beijing Chemical Works. They were used without further purification.

For Synthesis of Au NPs@ZnO NRs Hybrid, 4 mmol Zn(AC)·2H₂O and 32 mmol NaOH were separately dissolved into 90 ml and 50 ml absolute ethanol, after mixing them together under magnetic stirring, 32 ml PEG-400 was introduced into above solution. Then the reaction was kept at 140 °C for 16 h in a Teflon-lined stainless autoclave tank. The obtained precipitations were washed by absolute ethanol and deionized water for several times, and then dried in vacuum at 60 °C. Au NPs@ZnO NRs hybrid nanocrystals with varying amounts of Au nanoparticles have been synthesized by adjusting the molar ratio of ZnO to HAuCl₃ through a general and simple aqueous-based method [44, 50]. Taking 100:1 molar ratio of ZnO: Au for example, as-prepared ZnO were suspended in 50 ml diluted trisodium citrate solution (0.648 mg/ml) by ultrasonic treatment, and then was precipitated by dropwise adding 2 ml dilute HAuCl₃ (0.002 mol/L). The mixed solution was stirred at room temperature for about 12 h, followed by a gradually color-change from pale yellow to lilac. These nanohybrid products were collected by centrifugation and washed with distilled water and ethanol several times for further characterization.

The morphology and size distribution of the product were determined by transmission electron microscopy (TEM, JEOL, JEM-100CX II, Japan). The high-resolution TEM images were acquired using acceleration voltage of 300 kV. TEM samples were prepared by placing a drop of diluted suspended product solution onto a carbon-coated copper grid and allowing it to dry in air. The crystal structure and phase identification of the nanohybrid was determined by X-ray diffraction patterns recorded on an X-ray diffractometer (Rigaku DMAX-RB, Japan, CuKα). Chemical states of surface element of Au NPs were investigated by X-ray photoelectron spectroscopy (XPS, Kratos AXIS ULTRA DLD, SHIMADZU, Japan), using Al Kα as an exciting X-ray source.

To explore the kinetics of ROS formation, Au NPs@ZnO NRs hybrid solutions (50 µL) of various concentrations and various ZnO:Au molar ratio, pristine Au NPs (50 µL) or pristine ZnO NRs (50 µL) with corresponding concentrations were mixed with 50 µL of 10 µM DHR123 under dark condition. Here, DHR123 (nonfluorescent) was oxidized by ROS to form fluorescent Rhodamine 123 (R123), thus working as ROS tracking agent. Total volume of 100 µL samples (n = 3) in 96-well plates were irradiated by UV irradiation (λ = 254 nm, 0.1 mW/cm²) for different time durations. The fluorescence measurements were done after each 1 min irradiation using a multilabel plate reader (VICTOR™ X2, PerkinElmer, USA) at an excitation wave length of 485 nm and an emission wavelength of 535 nm.

For cell culture, human uterine carcinoma cell line (HeLa) and mouse myoblast cell line (C2C12) were purchased from ATCC (Manassas, VA). The Dulbecco’s Modified Eagle Medium-high glucose (HyClone, MA), added 10% fetal bovine serum (HyClone, MA) and Penicillin/Streptomycin (100 U/mL and 100 µg/mL, respectively, PAA Laboratories GmbH, Austria) was used as the culture medium. Remove cell from liquid nitrogen and immediately
place in 37 °C water bath and quickly shake until thawed. Quickly pipette cell into a 25 cm² flask, add 5 mL medium and place back in incubator (37 °C, 5% CO₂). Change culture media to remove non-adherent cells after 16-24 h. Cells were seeded into a 96-well plate at a density of 2 × 10⁴ cells per well and then cultured for 48 h prior to the further treatment.

To determine the cell cytotoxicity, cells in 96-well plates, were exposed to pristine ZnO nanorods and nanohybrid solution at varying ZnO:Au molar ratio (100:1, 50:1, 20:1). After 24 h incubation at 37 °C and 5% CO₂, the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT, sigma) was added into each well. After another 4 h, nonreacted solution was removed and dimethyl sulfoxide (DMSO, sigma) was added to extract the formazan crystals followed by the 15 min shaking. Then the optical absorbance of the extract was measured at 490 nm (Sunrise, TECAN, Switzerland). Controls were cultivated under the same conditions without addition of any nanohybrid product. The experiment was repeated at least three times. Cell viability was expressed as follows: cell viability (%) = [A]test/[A]control*100%, where [A]test and [A]control represent the optical density at 490 nm for the test and control experiments, respectively.

For PDT experiments, the procedure of cell culture and treatment of nanohybrid was similar with the above mentioned. After incubation for 12 h with the medium containing nanohybrid products, UV irradiation (2.4 µW/cm²) was on the top of the cells for 2 min. Then the cells were incubated for another 12 h prior to cell viability assessment through MTT assay.

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