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IN FOCUS: NANOMEDICINE - ARTICLE

Deciphering an Underlying Mechanism of Differential Cellular Effects of Nanoparticles: An Example of Bach-1 Dependent Induction of HO-1 Expression by Gold Nanorod

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Abstract Gold nanoparticles are extensively investigated for their potential biomedical applications. Therefore, it is pertinent to thoroughly evaluate their biological effects at different levels and their underlying molecular mechanism. Frequently, there are discrepancies about the biological effects of various gold nanoparticles among the reports dealing with different models. Most of the studies focused on the different biological effects of various nano-properties of the nanomaterials. We hypothesize that the biological models with different metabolic processes would be taken into account to explain the observed discrepancies of biological effects of nanomaterials. Herein, by using mouse embryo fibroblast cell line (MEF-1) and human embryonal lung fibroblast cell line (MRC-5) as in vitro models, we studied the cellular effects of gold nanorods (AuNRs) coated with poly (diallyldimethyl ammonium chloride) (PDDAC), polyethylene glycol and polystyrene sulfonae (PSS). We found that all three AuNRs had no effects on cellular viability at the concentration of 1 nM; however, AuNRs that coated with PDDAC and PSS induced significant up-regulation of heme oxygenase-1 (HO-1) which was believed to be involved in cellular defense activities in MEF-1 but not in MRC-5 cells. Further study showed that the low fundamental expression of transcription factor Bach-1, the major regulator of HO-1 expression, in MEF-1 was responsible for the up-regulation of HO-1 induced by the AuNRs. Our results indicate that although AuNRs we used are non-cytotoxic, they cell-specifically induce change of gene expression, such as HO-1. Our current study provides a good example to explain the molecular mechanisms of differential biological effects of nanomaterials in different cellular models. This finding raises a concern on evaluation of cellular effects of nanoparticles where the cell models should be critically considered.

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1 Introduction

Gold nanorods (AuNRs), a typical type of gold nanomaterials with attractive optical properties and easy bio-functionality, have attracted enormous interest among biomedical researchers. Potential applications of AuNRs have been demonstrated in areas of cellular imaging, diagnostics and therapy for various diseases, especially cancer [1, 2]. To considerably enhance the potential applications of AuNRs in nanomedicine, a large number of well-controlled synthesis ways were developed to improve the properties of AuNRs. For example, the surface coating of AuNRs with PEG provides better biosafety and biocompatibility [3]. Growing use of various modified AuNRs has thus aroused the need to establish a paradigm for accurately predicting their cytotoxicity in biological system.

Physicochemical properties of nanoparticles are dominant factors determining their toxicity and further biological applications [4, 5]. Several cell viability assay-based studies suggest that some surface modified AuNRs, such as PEG-, PDDAC- and PSS-coated AuNRs, exhibit little or no cytotoxicity [3, 6]. However, evidence has accumulated showing that analysis of changes in expression of genes

involved in cell apoptosis, senescence and inflammation can yield a more complete picture about effects of nanoparticles on cells [6–9]. In view of growing application of AuNRs, it is of paramount importance to determine their effects on various cellular events including reactive oxygen species (ROS) production and acute response protein induction, which are involved in many basic biological processes and various human disorders and dysfunctions [10–12].

Harmful effects of ROS occur when there is overproduction of free radicals to the extent that antioxidative enzymes are unable to counteract to maintain the cellular redox balance [12]. Heme oxygenases (HOs) are the commonly known antioxidant defense enzymes, which exert antioxidative, anti-inflammatory and anti-proliferative effects by eliminating free heme and generating iron and biliverdin [6, 13]. Heme oxygenase-1 (HO-1), the inducible form of HOs, can be upregulated by a variety of harmful stimuli in most cell types [14, 15]. Many studies suggest that up-regulation of HO-1 conferred protection to cells and organs against the harmful stimuli and subsequent injury [16, 17].

To thoroughly examine the biological effects and/or potential toxicity of some AuNRs, here we investigated the effects of three different surface coating AuNRs, namely poly (diallyldimethyl ammonium chloride) (PDDAC)-, polyethylene glycol (PEG)- and polystyrene sulfonae (PSS)-coated AuNRs, on cellular viability, ROS production and HO-1 expression as well as the molecular mechanism underlying their effects using both human (MRC-5) and mouse embryo fibroblasts (MEF-1). Our data demonstrate that the cell proliferation and the levels of ROS in the two cell lines did not affected by stimulus of any type of AuNR, but HO-1 expression was up-regulated in MEF-1 when exposed to PDDAC- and PSS-coated AuNRs. Further investigation revealed that the low fundamental expression of transcription factor Bach-1, the major regulator of HO-1 expression, were associated with up-regulation of HO-1 expression response to exposure of AuNRs in MEF-1 cells. The study suggests that cell model is critical for evaluating the cellular effects of nanoparticles.

2 Materials and Methods Experimental

2.1 Reagent

The AuNRs (PDDAC-, PEG- and PSS-coated AuNRs) were synthesized according to the Ref. [8]. HO-1 antibody was purchased from stressgen (Assay Designs Inc. USA). Bach-1 and **β**-actin antibodies were from Santa Cruz (Santa Cruz, CA, USA). The cell count kit-8 (CCK-8) was from Dojindo Laboratories (Beijing, China). 5-(and-6)-Chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) was from Invitrogen (Molecular Probes, Invitrogen, USA).

2.2 Cell Culture

Mouse embryo fibroblasts transformed with sv40 cell line (MEF-1), obtained from ATCC, were grown in Dulbecco's Modified Eagle's Medium (DMEM, HyClone) supplemented with 10% (vol/vol) fetal bovine serum (Gibco), 2 mM L-glutamine, 20 mM HEPES, 100 U/mL penicillin and 1 mg/mL streptomycin. Human embryo lung fibroblast cell line (MRC-5), obtained from ATCC, was grown in minimum essential medium (MEM, HyClone) supplemented with 10% (vol/vol) fetal bovine serum (Gibco). All cells were incubated at 37°C in a 5% CO_2 humidified atmosphere.

2.3 Experiment Procedures

2.3.1 Characterization of AuNRs

AuNRs with a mean length of 62.3 ± 7.7 nm and width of 15.5 ± 1.8 nm were prepared and coated with PDDAC, PEG and PSS, respectively, according to previous reported method [8, 18]. The major physicochemical properties of the AuNRs are characterized and listed in Table 1. Transmission electron microscopy (TEM) images of AuNRs (Fig. 1) were taken at a FEI Tecnai T20 transmission scanning electron microscope (FEI Company, USA) using an accelerating voltage of 200 kV. The size distribution analysis was performed with ImageJ software (1.41v, US National Institutes of Health, USA) by manually measuring the length and width and calculating the AR of each rod of at least 200 Au NRs for each sample.

2.3.2 Cell Viability Assay

The cell viability was determined by a cell count kit-8 (CCK-8) (Dojindo Laboratories, Japan) assay. CCK-8 contains [2-(2-methoxyl-4-nitrophenyl)-3-(4 -nitrophenyl)-5-(2, 4-disulfonicacid benzene)-2H-tetrazalium sodium] (WST-8) which can be reduced to a yellow water-soluble

Table 1 Physicochemical properties of the AuNRs

AuNRs	Length (nm)	width (nm)	Surface modification	Surface charge
AuNR-PDDAC	62.3 ± 7.7	15.5 ± 1.8	PDDAC	+56 ± 2 mV
AuNR-PEG	62.3 ± 7.7	15.5 ± 1.8	PEG	−10 ± 1 mV
AuNR-PSS	62.3 ± 7.7	15.5 ± 1.8	PSS	−34 ± 1 mV

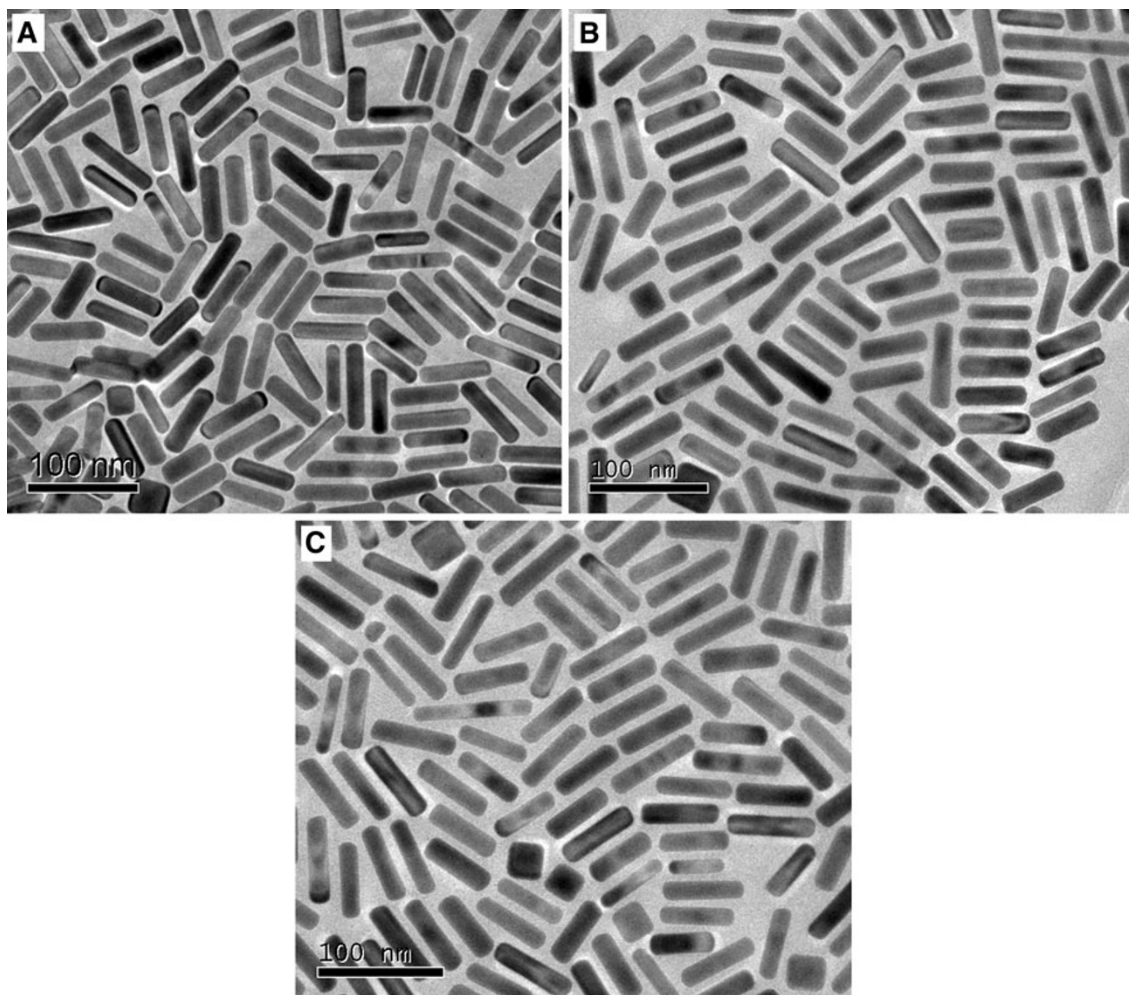

Fig. 1 TEM images of AuNRs which surfaces coated with PDDAC (**a**), PEG (**b**) and PSS (**c**), respectively

formazan dye by dehydrogenase. The cells were seeded in 96-well plates at a density of 5×10^4 cells/ml in the presence of 1.0 nM AuNRs at 37°C. After incubation for 24 h, medium was removed and 100 mL complete medium containing CCK-8 (10%) was added to each well. After incubation at 37°C for 2 h, the absorbance at 450 nm with a subtraction of reference absorbance at 650 nm was measured using a microtiter plate reader (TECAN Infinite M20, Austria) in each well. Measurement for each treatment was repeated in triplicate.

2.3.3 Measurement of Intracellular ROS Levels

5-(and-6)-Chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) purchased from Invitrogen (Molecular Probes, Invitrogen, USA), was used to evaluate the intracellular reactive oxygen species (ROS) levels following the manufacture's protocol. MEF-1 and MRC-5 cells were treated with AuNRs for 24 h. The sample was then washed twice with PBS and incubated with 5 μM CM-H_2DCFDA at 37°C for 1 h, washed twice with PBS and CM-H_2DCFDA fluorescence is measured using a flow cytometer (BECKMAN COULTER Cell lab Quanta SC, USA) with excitation and emission wavelengths of 485 and 520 nm, respectively. For positive controls (PC), cells were treated with H_2O_2 at a concentration of 100 μM for 0.5 h.

2.3.4 Western Blotting

Cells were seeded in 100 mm plates at a density of 1×10^{6} cells/mL in the presence 1 nM AuNRs at 37°C for 24 h. They were then washed and resuspended in lysis buffer containing 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1% (vol/vol) Triton-X 100 and protease inhibitor cocktail (Roche). After incubation on ice for 30 min, the total cell extracts were centrifuged at 12,000*g* for 20 min at 4°C. The protein content of the supernatant was estimated using a BCA kit (Applygen). Each sample (50 μg of protein) was subjected to SDS-PAGE and then transferred to a nitrocellulose membrane. Blots were blocked in a blocking buffer containing 5%

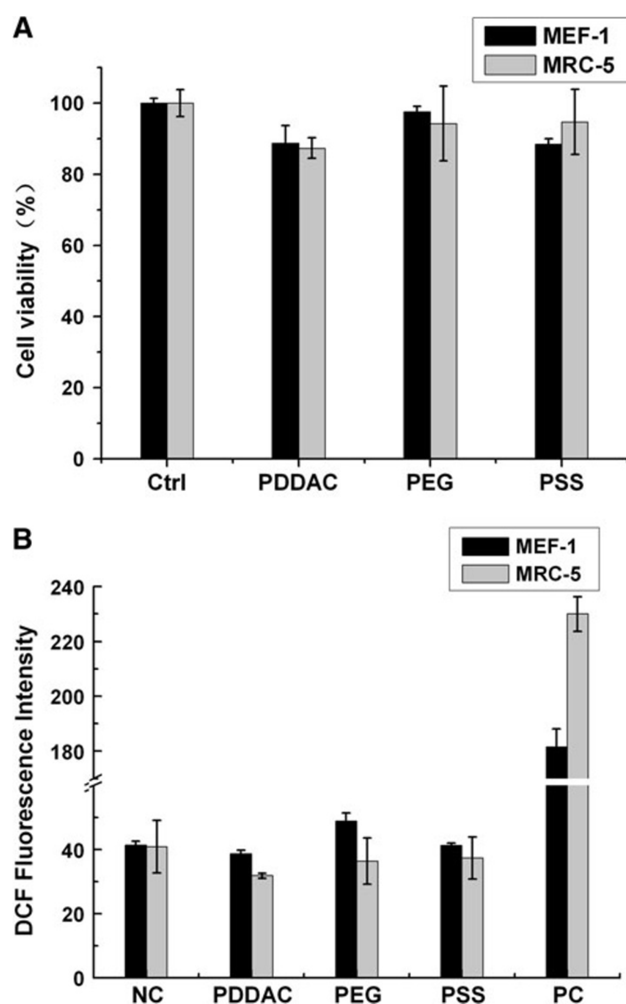

Fig. 2 Cell viability (**a**) and intracellular ROS levels (**b**) of the Au NRs in MEF-1 and MRC-5 cells. 1 nM of Au NRs was used for each type of AuNRs to treat the cells. Negative control (NC), positive control (PC); PDDAC, PEG and PSS represent AuNRs that coated with PDDAC, PEG and PSS, respectively

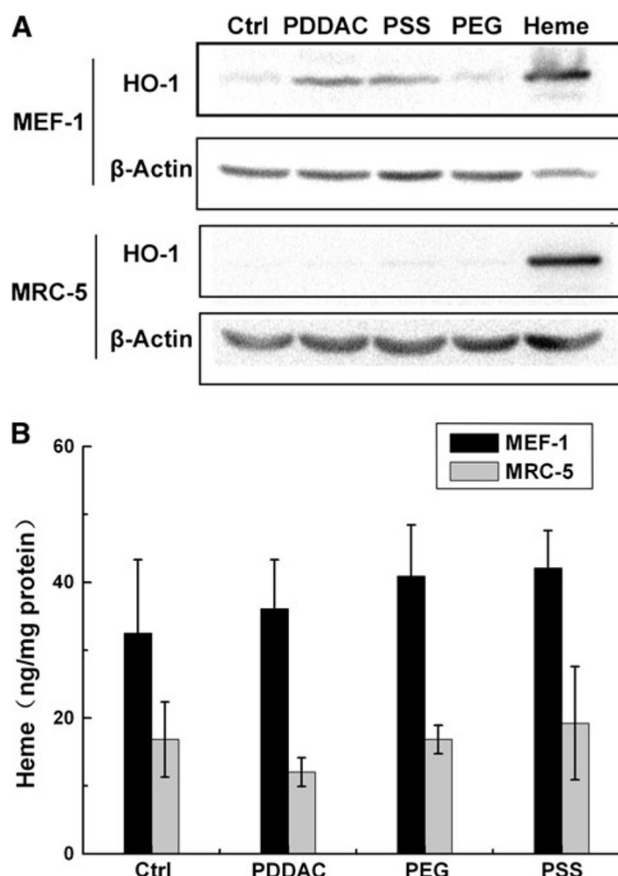

Fig. 3 The expression of HO-1 (**a**) and Heme levels (**b**) in MEF-1 and MRC-5 cells treated by AuNRs with different surface coating. Cells were treated with 1 nM AuNRs for 24 h before analysis, and heme (5 nM) treatment cells as a positive control in western blotting experiments

(wt/vol) non-fat milk, 0.1% (vol/vol) Tween 20 in 0.01 M TBS, and incubated with antibodies overnight at 4°C. The membrane was then incubated with an appropriate secondary antibody (ZSGB-BIO) for 1 h at room temperature with constant agitation, washed and reacted with supersignal chemiluminescent substrate (Pierce), scanned on a Typhoon Trio Variable Mode Imager and analyzed with Typhoon Scanner Control v5.0 (GE Healthcare).

2.3.5 TPL Imaging

Two-photon luminescence (TPL) images of AuNRs within cells were obtained using a 40× water immersion lens (N.A. = 1.2, Olympus) on a confocal microscope system (FluoView1000, Olympus, Japan) equipped with a femtosecond Ti: Sapphire laser (Mai Tai, Spectra-Physics, USA). The AuNRs were excited using an 810 nm NIR laser.

2.3.6 Heme Assay

The protein prepared for western blotting was also used for heme assay. Each protein sample (10 µg) was added to 0.5 mL of 2.0 M oxalic acid followed by heating at 100°C for 30 min to remove the iron from the heme. The autofluorescence of protoporphyrin in each sample was quantitatively measured with a microtiter plate reader (TECAN Infinite M200, Austria) at an excitation wavelength of 400 nm and an emission wavelength of 620 nm. Samples without heating were used to correct for background autofluorescence of endogenous protoporphyrin [19].

3 Results and Discussion

3.1 Cell Proliferation and ROS Assay

CCK assays were performed to determine the cell viability 24 h after exposure to 1 nM AuNRs of both MEF-1 and

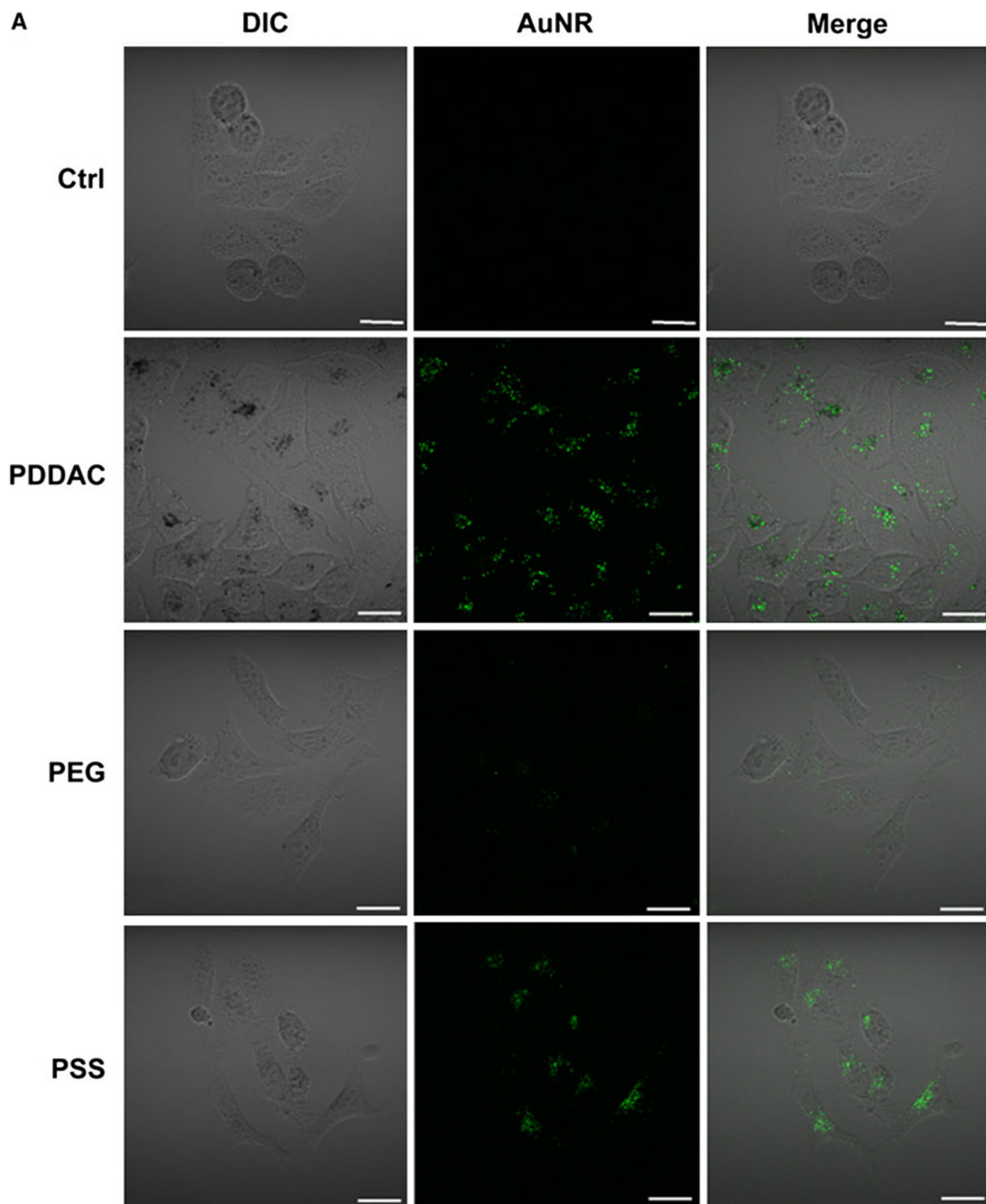

Fig. 4 Two-photon luminescence (TPL) images of AuNRs with different surface coatings in MRC-5 (**a**) and MEF-1 (**b**) cells. MEF-1 and MRC-5 cells were both treated with 1 nM AuNRs for 24 h before observation and all of the images were acquired at an accordant condition

MRC-5. 24 h exposure to AuNR-PDDAC, AuNR-PEG or AuNR-PSS did not lead to apparent difference on cell viability of the two cell lines, compared to the control cells (Fig. 2a), indicating that AuNRs showed low or non toxicity at a rod concentration of 1 nM in both MEF-1 and MRC-5. We next determined whether AuNRs in this concentration is capable of inducing endogenous ROS overproduction. CM-H_2DCFDA was used as a probe to quantify the cellular ROS levels as previously reported [20]. As shown in Fig. 2b, no elevated intensity of intracellular fluorescence was found in cells treated with AuNRs, indicating that there was no significant increase in

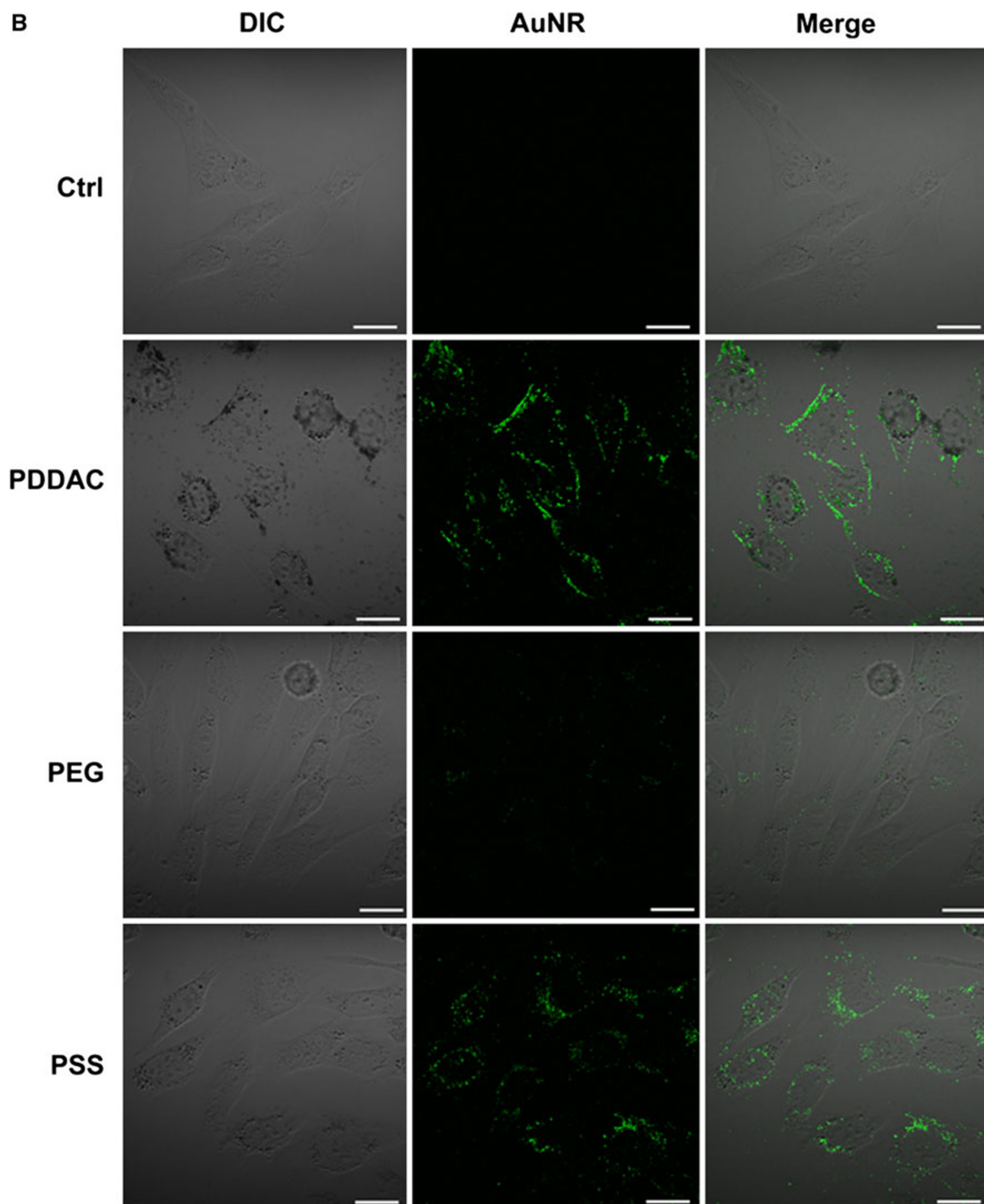

Fig. 4 continued

ROS production in both cell lines response to the three AuNRs exposure, while as a positive control, hydrogen peroxide led to dramatic increase in fluorescence intensity. These results were consistent with the previous studies [2, 3] that showed proper surface modification was one of the methods to improve the biosafety and biocompatibility of AuNRs.

3.2 HO-1 is Up-Regulated in MEF-1 Cells by PDDAC and PSS Coated AuNRs Treatment

HO-1 has been widely used as an early index to evaluate the cellular oxidative stress and stress response. The expression of HO-1 in MEF-1 and MRC-1 cells after treatment with 1 nM AuNRs with different surface coating

was detected by western blotting as shown in Fig. 3a. The protein levels of HO-1 in MEF-1 cells were up-regulated by both PDDAC and PSS coated AuNRs. Interestingly, there was no significant changes in MRC-5 cells. HO-1 serves as a protective protein by virtue of its anti-inflammatory, anti-apoptotic and anti-proliferative actions, widely manifested in endothelial, epithelial, smooth muscle and other cell types [21]. The induction of HO-1 in MEF-1 cells provided evidence that AuNRs that show little cytotoxicity, could indeed generate acute stress to MEF-1 cells. As is well known that HO-1 is the rate-controlling enzyme of heme catabolism and the increase of heme can result in an up-regulation of HO-1. We then detected the intracellular heme level in both cell lines to determine if the induction of HO-1 by AuNRs in MEF-1 cells was the result of increased intracellular heme levels (Fig. 3b). The iron in heme can be removed by heating in a strong oxalic acid solution and the resultant protoporphyrin is measured by fluorospectrometer. We calculated heme content based on a standard heme curve over the range of 0–1 nM heme per sample and the results showed that there was no obvious change of heme levels in AuNRs exposed cells. The results demonstrated that the induction of HO-1 in MEF-1 cells was not caused by the change of heme level but the effect of AuNRs. However, we observed that the AuNRs showed differential effects on the same types of cells (embryonic fibroblasts) with different species origin. Such similar conflicting results were also presented in previous studies [3, 8, 9] but little was done to explain the underlying molecular mechanism.

3.3 Cellular Uptake of the AuNRs in MEF-1 and MRC-5 Cells

In order to determine whether the differential induction of HO-1 expression by AuNRs exposure in MEF-1 and MRC-5 cells was the result of different cellular uptake between these two cell lines, two-photon laser scanning confocal microscope was conducted to estimate the amounts of AuNRs internalized by cells. The AuNRs have been shown to be capable of emitting luminescence via a two-photon excitation process that permits the direct imaging without labeling [22]. The differential cellular uptake of AuNps was observed in TPL intensity in the cell culture (Fig. 4). The PEG-coated AuNRs were the least favorable to be internalized by cells. For PDDAC-coated and PSS-coated AuNRs, the amounts of intracellular AuNRs were much more than PEG-coated AuNRs and showed no remarkable difference. This effect is consistent with previous report [23]. Interestingly, we consistently observed that the cellular uptake of these AuNRs were similar in MEF-1 and MRC-5 cells and showed the same patter (Fig. 4). Therefore, we speculated that the cellular uptake ability is not the major factor to determine the differential HO-1 induction in cell lines with different species origin.

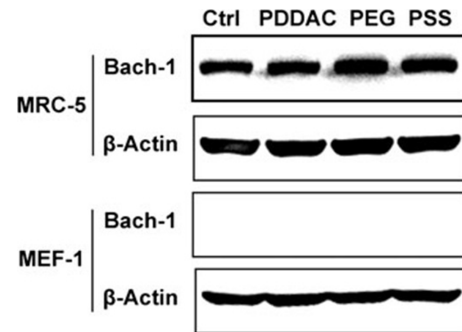

Fig. 5 The protein levels of transcription factor Bach-1 in MRC-5 and MEF-1 cells treated with the AuNRs with different surface modifications. Both cell lines were treated with AuNRs for 24 h before collected and lysed to run western blotting experiments

3.4 The Basal Level of Transcription Factor Bach-1 is the Major Influential Factor for HO-1 Expression in Different Cell Lines

To further scrutinize the underlying molecular mechanism of the differential HO-1 expression pattern in different cell lines, we focused on regulatory mechanism of HO-1 expression in the cell lines used to assess the cellular effects of AuNRs. Previous study demonstrated that regulation of the HO-1 gene involves a direct sensing of heme levels by Bach1, a member of leucine b-Zip protein family, generating a simple feedback loop whereby the substrate affects repressor-activator antagonism [24, 25]. Therefore, we detected the protein level of Bach-1 in MEF-1 and MRC-5. As shown in Fig. 5, the basal levels of Bach-1 in MEF-1 are too low to detect by western blotting; in contrary, Bach-1 showed strong signal and consistently high expression in all MRC-5 cells treated with or without AuNRs. Our results suggest that the high level of Bach-1 in MRC-5 inhibits the basal expression of HO-1 and this effect could probably further suppress the pre-existing effects of AuNPs on HO-1 induction. The basal low Bach-1 level in MEF-1 will not cover the differential expression of HO-1 caused by AuNPs.

4 Conclusions

In this study, two cell lines similarly to each other of different species have been employed to evaluate the influence of AuNRs with different surface modification on their biological effects, especially on expression of HO-1 and their underlying molecular mechanism. Although all the AuNRs used showed little cytotoxicity and the same AuNRs showed accordant cellular uptake ability in two cell

lines, there was a significant difference in HO-1 expression response to AuNRs exposure. Importantly, for the first time, we showed that the basal expression of Bach-1, a negative regulator of HO-1 expression takes into account for the differential HO-1 gene induction of AuNRs in cells with different species origin. In previous studies, mainly the materials' properties of AuNRs were considered to be the major factors to determine their cellular effects and the differences of the biological models are largely ignored. Some inconsistent results are frequently reported when evaluating the cellular effects of AuNRs, especially when proteomics and genomics methods were used. Our current study observation is not only helpful for understanding the conflict results of previous studies about the effects of AuNRs on apoptosis, senescence, inflammation, among others, but also raises a concern on evaluation of cellular effects of nanoparticles where the cell models should be critically considered.

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