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Detection of nitric oxide in macrophage cells for the assessment of the cytotoxicity of gold nanoparticles

Libo Du [a,b,1], Xiaoxiang Miao [a,b,1], Hongying Jia [b], Yanli Gao [b], Ke Liu [a,*], Xueji Zhang [c,***], Yang Liu [b,**]

[a] Ministry of Education Key Laboratory of Bio-resources and Eco-environment, School of Life Sciences, Sichuan University, Chengdu 610064, PR China
[b] State Key Laboratory for Structural Chemistry of Unstable and Stable Species, Institute of Chemistry, Chinese Academy of Science, Beijing 100190, China
[c] Research Center for Bioengineering and Sensing Technology, University of Science and Technology Beijing, Beijing 100083, China.

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ABSTRACT

In this paper, an electrochemical method using a nitric oxide sensor was employed for quantitative evaluation of NO released from AuNPs-treated macrophage cells. Our results indicate that the AuNPs initiate NO release from macrophage cells and the amount of NO released is positively correlated with concentration of AuNPs. Meanwhile, total nitrite/nitrate concentrations in the AuNPs-treated macrophage cells have been determined via the Griess reaction and we demonstrate that the variation of the nitrite/nitrate concentrations is in accordance with that measured by the electrochemical method. In contrast to the citrate-coated gold nanoparticles (CT-AuNPs), when AuNPs were protected by thiolated poly ethylene glycol (PEG), the NO-releasing in macrophage upon the addition of AuNPs was relieved, implying that the PEG-coated AuNPs having less cytotoxicity and oxidative stress potential is probably due to inhibition of NO production. In conclusion, this work has demonstrated an effective sensing platform for the evaluation of the cytotoxicity of AuNPs by detecting the extracellular NO released from macrophage cells.

1. Introduction

Gold nanoparticles (AuNPs) have become an indispensable tool in the development of clinical diagnostics and therapy. Because AuNPs can be readily functionalized with multiple targeting molecules and have so far shown excellent potential for the delivery of small molecule drugs [1,2], several AuNPs-based drugs are currently under development by CytImmune, with their lead drug, Aurimune, in clinical trials [3]. Along with the wide applications of AuNPs in biology and medicine, the cytotoxicity of AuNPs has become one of the most significant threats that should be primarily considered and evaluated, before making their application in practice [4,5].

Previous studies have demonstrated that the *in vitro* cytotoxicity of AuNPs is associated with the generation of oxidative stress [6], which is accompanied with the production of a variety of reactive oxygen species (ROS), including $^{\bullet}OH$, $O_2^{\bullet -}$ etc. More recently, a fewer studies have further indicated that the *in vivo* toxicity of AuNPs is also relevant with oxidative stress and ROS production, which were generally mediated by Kupffer cells (liver macrophages) hyperplasia [7,8]. Meanwhile, authors previously found that AuNPs might induce potential oxidative stress *via* inducing endogenous release of nitric oxide (NO) by catalyzing cleavage of S-nitroso adduct, such as S-nitrosoalbumin (AlbSNO), S-nitrosocysteine (CysNO), and S-nitrosoglutathione (GSNO) in blood serum [9]. On the other hand, overproduction of NO (up to 1 μM or more) may lead to nitrosative stress that is responsible for a variety of degenerative disease processes [10]. In addition, considering that after AuNPs administration they are mainly accumulated in the liver and cause the activation of Kupffer cells [11,12], we speculate that the cytotoxicity of AuNPs is probably not only related to the generation of ROS, but is also associated with inducible NO generation in liver macrophage cells. To test the hypothesis, we examined herein whether the AuNPs trigger the overproduction of NO in a mouse (RAW 264.7) cell line. Meanwhile, to alleviate the nitrosative and oxidative damage initiated by AuNPs, the PEGylated ligands-coated AuNPs (i.e. PEG-AuNPs) were prepared and then the extracellular NO-releasing was comparatively measured upon the addition of PEG-AuNPs.

A range of techniques have been developed for analyzing NO in a biological environment, including chemiluminescence, capillary electrophoresis chemiluminescence (CE-CL), high-performance liquid chromatography, mass spectrometry, electron paramagnetic resonance (EPR) spectroscopy, and fluorescence [13–21]. Each of these techniques has certain benefits but also suffers from low sensitivity, poor specificity, or expensive experimental apparatus, which restrict its application in practice [22,23]. However, in comparison with the

* Corresponding author. Tel.: +86 28 8541 5008; fax: +86 28 8541 5300.
** Corresponding author. Tel.: +86 10 6257 1074; fax: +86 10 6255 9373.
*** Corresponding author. Tel.: +86 10 8237 6993; fax: +86 10 8237 5840.
E-mail addresses: liuke@scu.edu.cn (K. Liu), xzhang@cas.usf.edu (X. Zhang), yliu@iccas.ac.cn (Y. Liu).
[1] Both authors contributed equally to this work.

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above methods, the electrochemical approach [24,25] is demonstrated to be advantageous in direct, rapid, and real-time measurements. There is no need to suffer from the poor stability and photobleaching of fluorescent probes, the influence of foreign substances on the chemiluminescent reagents, or detecting under rigorous environment conditions any more [26]. For example, a previous study has reported the use of amperometry at platinized carbon microelectrodes to detect and quantify ROS/RNS released by human lymphocytes or skin cells [27,28]. The release of ROS/RNS by macrophages cultured in a microfluidic chamber and stimulated by the microinjection of a calcium ionophore was measured with platinized band electrodes [29]. The obvious major advantage of an electronic sensor for NO is that it makes it easy to quantitatively measure real-time production of extracellular NO [30], and therefore the electrochemical method has been chosen to evaluate the overproduction of NO in macrophage. In addition, the AuNPs-induced total nitrite/nitrate accumulations were contrastively assessed by means of the Griess method.

2. Experiment and materials

2.1. Chemicals

L-arginine, Phorbol 12-myristate 13-acetate (PMA), D-glucose, gentamycin, Lipopolysaccharide (LPS) and S-nitroso-N-acetyl-D, L-Penicillamine (SNAP) were purchased from Sigma-Aldrich (St. Louis, USA). Hydrogen tetrachloroaureate (III) trihydrate ($HAuCl_4 \cdot 3H_2O$), trisodium citrate ($Na_3C_6H_5O_7$), sulfanilamide, naphthyl ethylenediamine dihydrochloride, phosphoric acid, and all the other chemicals were of analytical grade and were from Beijing Chemical Reagent (Beijing China). Dulbecco's modified Eagle's medium (DMEM), and fetal bovine serum were from Invitrogen. Mouse macrophage RAW 264.7 cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA).

2.2. Synthesis of CT-AuNPs and PEG-AuNPs (13 nm)

AuNPs of 13 nm in size were used in this work. CT-AuNPs of sized 13 nm were synthesized as described by Frans [31]. The gold ion solution (120 mg of $HAuCl_4$ dissolved in 250 mL of water) was prepared and boiled. Then, the 1% citric acid solution (50 mL) acting as a reducing agent was added and the solution was further boiled for 10 min. The CT-AuNPs solution was cooled down to room temperature. The average diameter of as-prepared CT-AuNPs was determined to be 13 ± 2 nm using the transmission electron microscopy. AuNPs were modified using thiol-terminated poly (ethylene) glycol (HS-PEG, of MW 5000). Then, transmission electron microscopy images of PEG-coated AuNPs were obtained using a JEM-2011 (JEOL) at an acceleration voltage of 120 KV. The hydrodynamic diameter of CT-AuNPs and PEG-coated AuNPs determined in this paper was calculated by the cumulant method provided in software of the dynamic light scattering instrument (DLS, Malvern, England).

2.3. Cell culture and treatment

The murine macrophage RAW 264.7 (American Type Culture Collection) cell line was cultured at 37 °C under a 5.5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) containing 1.0 g L^{-1} D-glucose and sodium pyruvate (Invitrogen). The medium was supplemented with 5% fetal bovine serum (Invitrogen) and 20 g mL^{-1} gentamycin (Sigma). 24 h prior to electrochemical studies, confluent monolayers of RAW 264.7 cells were harvested mechanically, and resuspended in a glass bottle, with or without the addition of L-arginine (100 μM).

2.4. Electrochemical measurements

Electrochemical measurements were performed with the Apollo 4000 instrument (WPI Europe) connected to NO selective carbon fiber electrodes (ISO NOPF, 200 μm, WPI Europe). As described above, the macrophage cells were incubated in glass bottle for 12 h at 37 °C and 5% CO_2, and then the macrophage cell were washed 3 times with degassed PBS (pH 7.4, 0.1 M). Subsequently, the electrode tip was inserted into the glass bottle containing macrophage cells, allowing the background current to stabilize. The CT-AuNPs or PEG-AuNPs (15, 30, 60 and 90 μM) was then added in solution to generate NO. The rapid decline in the NO signals generated after the addition of each concentration of AuNPs precluded the use of a cumulative protocol. Consequently, the maximum change in current (Δp_A) produced by each new addition was recorded and used to assess the generation of nitric oxide. All the experiments were carried out at room temperature.

2.5. Electrochemical determination of S-nitrothiols

In this method, RSNO in cells were decomposed in the presence of AuNPs and the NO released was measured using the NO-selective electrode. A calibration curve was obtained using a similar procedure as described in our previous study [32]. Briefly, the different volumes of the standard SNAP were added to the AuNPs solution. The final concentrations of SNAP were 0, 5, 20, 41, 51, 102, 203, 407, 509, 1017 nM. The changes of the current were plotted against SNAP concentrations that were added to the AuNPs solution. In order to demonstrate that the assay was specific for NO release from RSNOs, a variety of control (GSH, nitrite, and nitrate) were performed. The precision of additions of SNAP (S-nitroso-N-acetyl-D, L-Penicillamine; 10 and 20 nM final concentration, respectively) to AuNPs solution.

2.6. Measurement of nitrite concentration

Accumulation of nitrite in the medium was measured by a colorimetric assay based on the Griess reaction [33]. Briefly, after CT-AuNPs and PEG-AuNPs treatment with RAW 264.7 cells (1×10^6 cells/ml) for 24 h, the supernatant was collected from each well. Then, samples were reacted with 1% sulfanilamide, 0.1% naphthyl ethylenediamine dihydrochloride, and 2.5% phosphoric acid at room temperature for 10 min, and nitrite concentration was determined by absorbance at 540 nm in comparison with sodium nitrite as a standard.

3. Results and discussion

3.1. Preparation and characterization of CT-AuNPs and PEG-AuNPs

Citrate-coated gold nanoparticles (CT-AuNPs) were prepared by the reduction of aqueous chloroaurate with sodium citrate [8]. CT-AuNPs were further modified using thiol-terminated poly (ethylene) glycol (HS-PEG, of MW 5000), giving the production of PEG coated gold nanoparticles (PEG-AuNPs). The CT-AuNPs and PEG-AuNPs were characterized using a transmission electron microscopy (TEM) and dynamic light scattering (DLS). The TEM image clearly showed that the AuNPs were highly monodispersed with sizes of $13 \text{ nm} \pm 2$ nm, which were also consistent with those of DLS analysis of aqueous solutions of the AuNPs (Fig. 1b–e). In addition, the characteristic absorption bands of CT-AuNPs and PEG-AuNPs were observed at 521 nm and 525 nm, respectively, indicating the formation of AuNPs.

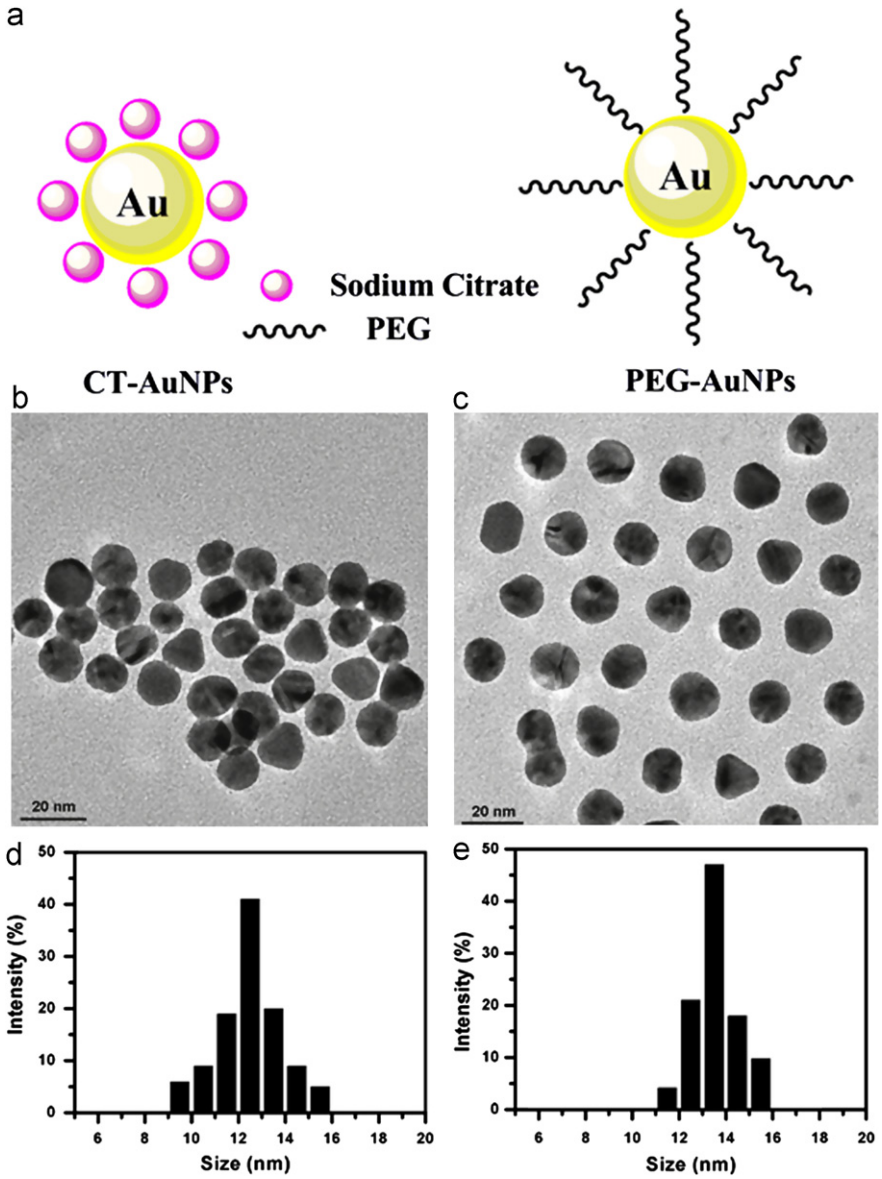

Fig. 1. Characterizations of CT-AuNPs and PEG-AuNPs. (a) Schematic illustration of the synthesized AuNPs (CT-AuNPs and PEG-AuNPs.); The TEM images of CT-AuNPs (b) and PEG-AuNPs (c); Nanoparticle size distribution measured by dynamic light scattering (DLS) in H_2O: CT-AuNPs (d), PEG-AuNPs (e).

3.2. Sensor selectivity

The interference of species such as NOx (e.g., nitrite and nitrate), thiol reducing agents (RSH; e.g., glutathione and cysteine) in the determination of NO was studied according to a similar method described in our previous study [32]. Briefly, interfering species were injected along with SNAP into a constantly stirred solution of AuNPs and their response was measured. NO was found to respond in the same way in the presence or absence of interferences with different concentrations, such as 0.1 mM GSH and 100 μM nitrate. Although the current response was detectable when 5 μM nitrite solution was added, the response was less than 3% compared with that of 20 nM SNAP. It indicated that the quantitative determination of NO would not affected by interfering species. Moreover, the NO-selective electrode coated with WPI NO selective membranes exhibits high resistance towards electrode fouling caused by macromolecular nonspecific absorption [34]. It therefore can be indicated that the method based on the gold nanoparticles decomposing RSNOs is special, it is used to detect RSNOs in biological system and obtain accurate results.

3.3. Analytical performance

The amperometric response of the AuNPs (0.9 mM) to SNAP was investigated by successively increasing the concentration of SNAP under room temperature. As expected, the microsensor clearly showed substantial increase in current upon successive additions of SNAP. A linear relationship with a correlation coefficient of 0.9921, between the current and the concentration of SNAP was observed. The linear range is from 5 to 1000 nM with a sensitivity of 18 pA/nM. The calculated detection limit was 5.1×10^{-11} M at a single-to-noise ratio of 3. The linear range is wider and the sensitivity is higher as compared with other assays [35,36], which indicated that the sensor has high sensitivity. Moreover, the intra-day precision measurement was carried out in order to investigate the reproducibility of sensor. The intra-day precision was shown to

be within a RSD at 4.91% at 10 nM and 2.83% at 20 nM, indicating that sensor displayed an acceptable reproducibility.

3.4. The cellular nitric oxide release induced by AuNPs

As described above, the overproduction of NO can readily trigger oxidative and nitrosative stress and then induces cell apoptosis or necrosis. To evaluate the possibility of nitrosative/oxidative stress induced by AuNPs, it is necessary to verify the concentration of NO release from AuNPs-treated macrophage cells. In this paper, the amount of NO-releasing from macrophage cells were monitored by the Apollo 4000 system equipped with a NO microsensor according to the electrochemical method described in our previous studies [9,32]. Standard solutions of SNAP were prepared to construct standard curves for conversion of the NO signal (output measured in current) to molar concentration. Briefly, the electrode tip was inserted into a stirring gold nanoparticles solution (10 mL), allowing the background current to stabilize. SNAP or sample was then added in solution to generate NO. The concentration of SNAP could be regarded as the concentration of NO because the excess of AuNPs solution could completely converse the SNAP to NO. Consequently, the maximum change in current produced by each new addition was recorded and used to assess the generation of nitric oxide. The molar amount of NO from the cells was then determined by using the standard curve (Fig. 2). To examine the signal response to the addition of AuNPs, various concentrations of CT-AuNPs (15, 30, 60, and 90 μM) were added into cells. It could be obviously observed that the signal of NO was simultaneously detected upon the addition of CT-AuNPs to the cells (Fig. 3).The level of NO-release significantly increases from 0.28 μM to 1.32 μM with the concentration of AuNPs increasing from 15 to 90 μM, exhibiting a dose-dependent increasing with the addition of CT-AuNPs. These results imply that a higher concentration of CT-AuNPs (above 60 μM) might cause nitrosative/oxidative stress of macrophage cells through inducing the overproduction of NO-release [37]. As expected, the electrochemical results are consistent with our previous study, in which when concentration of AuNPs was higher than 50 μM, a significant decrease of cell viability and an evident elevation of intracellular malondialdehyde (MDA) level could be observed [38].

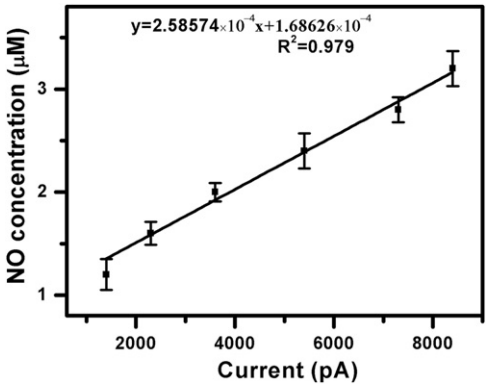

Fig. 2. Plots of current density as a function of the concentration of NO (the concentration of S-Nitroso-N-acetyl-D, L-penicillamine).

Furthermore, to explain the reason for the overproduction of NO induced by AuNPs, we propose two possibilities: (i) As is well documented, when macrophage is activated with exogenous stimulus (such as PMA, LPS), iNOS (inducible nitric oxide synthase, NOS II) generates large quantities of NO through the conversion of L-arginine to L-citrulline [39]. Recent studies have demonstrated that nanoparticles have the potential to activate the NADPH oxidase and induce the mRNA expression of pro-inflammatory cytokines (IL-1β, TNF-α, and IL-6) or other inflammatory-related enzymes (iNOS, COX-2) in peritoneal macrophage [40]. It is therefore speculated that the overproduction of NO in CT-AuNPs-treated cells might be attributed to the activation of NADPH oxidase and induction of iNOS expression; (ii) On the other hand, our previous study showed that CT-AuNPs could induce NO release from serum by catalyzing RSNOs (i.e. AlbSNO, CysNO, and GSNO) decomposition to NO [8]. In macrophage, it has been demonstrated that RSNOs could be formed under physiological conditions [41]. We therefore suggested that the release of NO from AuNPs-treated macrophage cells might derive from RSNOs decomposition catalyzed by CT-AuNPs.

Since the availability of L-arginine is a major determinant for NO synthesis in activated macrophage [42,43], it could be predicted that the amount of NO production in PMA-activated macrophage would be significantly different in the absence or presence of L-arginine. As expected, in the presence of L-arginine, PMA-activated macrophages produced a higher level of NO when comparing with cells stimulated by PMA only (Fig. 4a). Furthermore, NO production from macrophage was significantly decreased after addition of N-nitro-L-arginine (a commonly used inhibitor of NO synthesis). It could be reasonably speculated that if the hypothesis that the AuNPs induced NO release was associated with the activation of iNOS is true, the CT-AuNPs, as exogenous stimulus, will initiate an additional release of NO from macrophage in the presence of the exogenous L-Arginine. However, when the macrophage were stimulated with CT-AuNPs, no apparent differences in the level of NO production by activated macrophage were observed in the absence or presence of L-arginine, as shown in Fig. 4a and b. We therefore prefer to accept the possibility that the NO-releasing initiated by CT-AuNPs is derived from CT-AuNPs-catalyzed decomposition of RSNO in cells, rather than trust that the NO-releasing is ascribed to the contribution of iNOS.

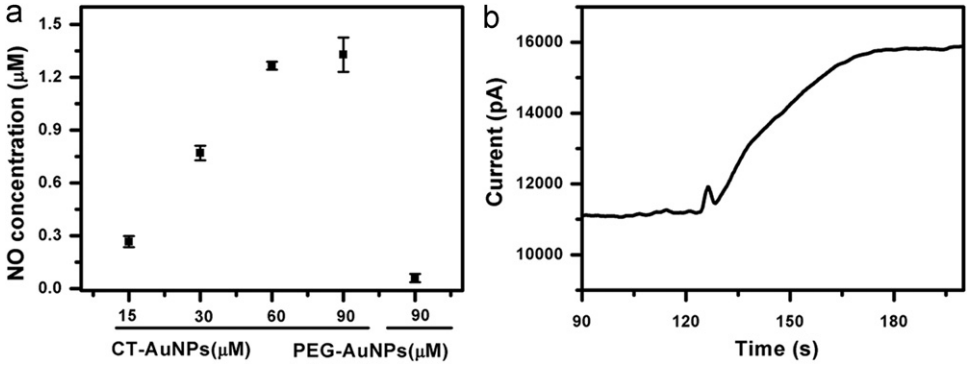

Fig. 3. (a) The amount of NO release in macrophage cells induced by CT-AuNPs (15, 30, 60, 90 μM) and PEG-AuNPs (90 μM), respectively. (b) Typical amperograms with adding CT-AuNPs (60 μM) to macrophage cells in PBS (pH 7.4).

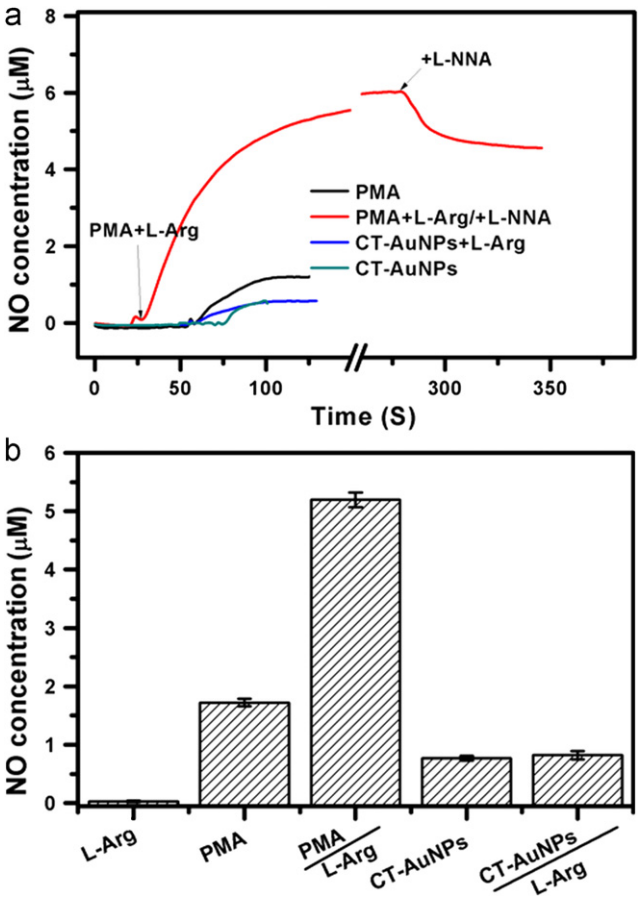

Fig. 4. (a) Current responses when PMA, PMA (preincubated with L-Arg and then added with L-NNA), CT-AuNPs and PEG-AuNPs were added to RAW 264.7 cells. PMA (100 nM), L-Arg (200 μM), L-NNA (200 μM), and CT-AuNPs (30 μM); (b) The statistical analysis of the NO concentration obtained from (a) was profiled in (b). Three replicates were taken at each time point.

3.5. Determination of the nitric oxide using the Griess method

To further confirm the results of our electrochemical analysis, we measured the level of NO in macrophage cells induced by CT-AuNPs using the Griess method that has been extensively used for the detection of NO through determining nitrite/nitrate (the stable products of NO oxidation) concentration [44]. Referring to the results in Fig. 4, a concentration of 60 μM AuNPs was used as an example for the detection of nitrite/nitrate level in macrophage cells. As expected, when macrophage cells were incubated with AuNPs, we did observe an increase of nitrite/nitrate level in macrophage cells. However, the nitrite/nitrate level in CT-AuNPs-treated macrophage cells did not show any significant difference in the absence or presence of L-Arginine (data not shown), indicating that the production of NO in macrophage cell induced by AuNPs did not relate to the existence of L-Arginine. However, when compared with the results in Fig. 5, the higher NO (nitrite/nitrate) concentrations can be detected by the Griess method. The reason might be explained as follows. The main disadvantage of the Griess method is low specificity. It is possible to be disturbed by various biological and supplementary agents, such as ascorbic acid, dithiothreitol, thiol-containing amino acids, etc; another disadvantage of this method is that it measures only the overall concentration of nitrite/nitrate without detail information about transient nitric oxide production. In comparison with the Griess method, the electrochemical sensor provides a more sensitive and specific real-time assay for the detection of the NO in macrophage cells induced by CT-AuNPs. As a result, it could be concluded that

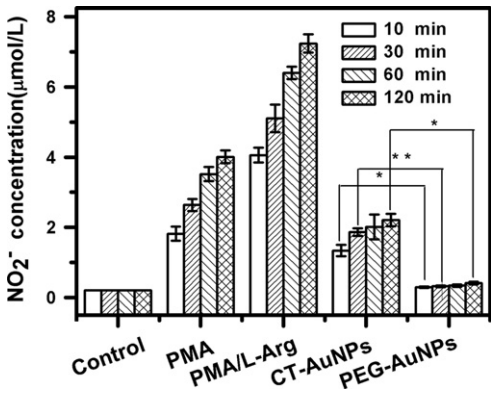

Fig. 5. Effects of PMA (100 nM), PMA (preincubated with 200 μM L-arginine) (100 nM), CT-AuNPs (60 μM), and PEG-AuNPs (60 μM) on nitrite/nitrate production using the Griess method with time increasing (10, 30, 60, 120 min) in RAW 264.7 cells. Three replicates were taken at each time point.

the concentration of NO in macrophage cells detected by Griess assay were obviously higher than using the electrochemical method.

3.6. Effect of PEG-AuNPs on the nitric oxide release in cells

To reduce the cytotoxicity of AuNPs, various strategies have been investigated previously [45,46]. Among them, one of the most attractive methods is forming a protective PEG layer around AuNPs, by which it has been proven that the cytotoxicity and oxidative damage of AuNPs could be significantly decreased *in vitro* and *in vivo*. It could therefore be speculated that, while using PEG as the ligands on AuNPs surface, the AuNPs-induced oxidative and nitrosative stress would be relieved by decreasing the amount of ROS and NO generation. To confirm the protective effect of PEG on macrophage cells, the NO-releasing induced by PEG-AuNPs was monitored using an amperometric NO sensor. As expected, the amount of NO-release in PEG-AuNPs treated macrophage (0.05 μM) was much less than that of CT-AuNPs (1.32 μM). The result of Griess assay also shows that the level of nitrite/nitrate in PEG-AuNPs treated macrophage cells are obviously lower than that of CT-AuNPs (Fig. 5), which imply that the oxidative and nitrosative stress induced by AuNPs could be suppressed when using thiolated PEG as a protective layer. Au atoms in the outer shell of AuNPs are occupied via forming covalent Au–S bond between Au and thiolated PEG, thereby inhibiting the catalytic reaction for the RSNOs decomposition and then simultaneously suppressing the NO release in macrophage. This inhibiting effect is in accordance with our previous result, in which GSH-coated AuNPs release much less NO in serum than CT-AuNPs do and probably further support the hypothesis of AuNPs-initiated RSNOs decomposition in macrophage.

4. Conclusion

In this paper, we demonstrate that the NO release from AuNPs-treated macrophage can be quantitatively evaluated by real-time electrochemical detection. Our results further show that the NO-release in CT-AuNPs-treated macrophage cells is associated with a catalytic reaction between AuNPs and endogenous RSNOs. Meanwhile, total nitrate/nitrite concentrations have been measured by Griess reagent in the CT-AuNPs-treated macrophage cells. The results from the Griess assay show a similar tendency with that from the electrochemical detection. The finding for the overproduction of NO initiated by AuNPs can probably provide a comprehensive understanding of the cytotoxicity effect of AuNPs on macrophage cells. Optimistically, the electrochemical detection method for NO might have great application potential in the

studies related to nitric oxide physiology and pathology, especially on the assessment of the cytotoxicity of AuNPs.

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