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Development of dual-fluorescence cell-based biosensors for detecting the influence of environmental factors on nanoparticle toxicity

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HIGHLIGHTS

- We created a novel dual-fluorescence biosensor system that exhibited good accuracy and high sensitivity via flow cytometric analysis.
- The toxicity of silver nanoparticles was evaluated by the biosensors and confirmed by conventional assays.
- The cell-based biosensors can potentially be applied to assess nanoparticle-toxicity risks.

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ABSTRACT

With the expanding use of engineered nanoparticles (NPs), development of a high-throughput, sensitive method for evaluating NP safety is important. In this study, we developed cell-based biosensors to efficiently and conveniently monitor NP toxicity. The biosensor cells were obtained by transiently transfecting human cells with biosensor plasmids containing a *mCherry* gene regulated by an inducible promoter [an activator protein 1 (AP-1) promoter, an interleukin 8 (IL8) promoter, or a B cell translocation gene 2 (BTG2) promoter], with an enhanced green-fluorescent protein gene driven by the cytomegalovirus promoter as the internal control. After optimizing flow cytometric analysis, these dual-fluorescence cell-based biosensors were capable of accurately and rapidly detecting NP toxicity. We found that the responses of AP-1, BTG2, and IL8 biosensors in assessing the toxicity of silver nanoparticles (Ag NPs) showed good dose-related increases after exposure to Ag NPs and were consistent with data acquired by conventional assays, such as western blot, real-time polymerase chain reaction, and immunofluorescence. Further investigation of the effects of environmental factors on Ag NP toxicity revealed that aging in water, co-exposure with fulvic acid, and irradiation with ultraviolet A light could affect Ag NP-induced biosensor responses. These results indicated that these novel dual-fluorescence biosensors can be applied to accurately and sensitively monitor NP toxicity.

1. Introduction

The rapid development of nanotechnology promotes the wide application of engineered nanoparticles (NPs) in many fields, including industry, the pharmaceutical chemistry, and consumer goods (Emerich and Thanos, 2003; Mu and Sprando, 2010; Kaloyanova et al., 2016). Increasing types of novel NPs with special characteristics are constantly being synthesized (Piccinno et al., 2012). In the interests of biosecurity, health and environmental

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risks should be assessed prior to NP application. Furthermore, engineered NPs are inevitably released into natural aquatic environments during the process of production and consumption. Environmental factors, such as ultraviolet A (UVA) irradiation, dissolved organic matter (DOM), pH, and ionic strength, can interact with nanoparticles, making the toxicity of transformed NPs in the environment a concern (El Badawy et al., 2010; Levard et al., 2012).

Some conventional analysis methods, including cell viability assays (AshaRani et al., 2009; Zhang et al., 2015, 2016a; Cheng et al., 2016), comet assays (Wang et al., 2014; Zhang et al., 2016b), and micronucleus testing (Li et al., 2012), are commonly applied to detect NP toxicity; however, these methods are elaborate and time-consuming (Karlsson et al., 2015). Additionally, NPs may interact with marker molecules or chemical agents, resulting in problems when conducting common toxicity tests (Kroll et al., 2012; Karlsson et al., 2013; Guadagnini et al., 2015). Investigation of apoptosis via AnnexinV/propidium-iodide staining has returned false-positive results in situations where NPs were able to bind propidium iodide and be taken up by intact cells (Darzynkiewicz et al., 2001; Shukla et al., 2005). NPs also interfere with enzyme-linked immunosorbent assays owing to the ability of NPs to adsorb cytokines (Monteiro-Riviere and Inman, 2006; Veranth et al., 2007).

The promoter-based biosensor system could be an alternative tool enabling the avoidance of interference and capable of performing high-throughput toxicity testing through acquisition of signals from reporter-gene expression. In these biosensors, the reporter gene is fused to a stress-responsive promoter, providing quantitative information in the form of a fluorescent signal when biosensor cells are exposed to conditions triggering a stress response. This method involving flow cytometric measurement is rapid and inexpensive as compared with conventional bioassays.

Cell-based biosensor systems have been employed to detect immunotoxicity (Oostingh et al., 2008; Stoehr et al., 2015), genotoxicity (Hever and Belkin, 2006; Geng et al., 2012; Blagus et al., 2014; Karlsson et al., 2014), and cell stress (Hofmann et al., 2014) induced by chemical compounds, with results comparable to those of conventional analysis methods [e.g., comet assays, immunocytochemistry, and real-time polymerase chain reaction (PCR)] (Oostingh et al., 2008; Hofmann et al., 2014; Karlsson et al., 2014). Some biosensor systems have been used to detect NP toxicity, based on responses triggering the activator protein 1 (AP-1) promoter (Prasad et al., 2013; Magaye, 2014), the interleukin 8 (IL8) promoter (Kohl, 2011; Stoehr et al., 2015), or the B cell translocation gene 2 (BTG2) promoter (Chen, 2012). AP-1 is a homodimer or heterodimer composed of the Jun, Fos, and activating transcription factor subgroups of transcription factors. Genes regulated by members of the AP-1 family are associated with proliferation, differentiation, cell death, and especially oncogenic transformation (Ding, 1999; Khanjani et al., 2012; Shaulian and Karin, 2001; Shaulian, 2002). IL8 is an important pro-inflammatory cytokine produced by different cell types in response to various stress stimuli. The 5′-flanking region of the IL8 gene contains a variety of potential binding sites for transcription factors, including nuclear factor kappa B, AP-1, and CAAT/enhancer-binding protein, along with the TATA box (Mukaida, 1994). BTG2 is a member of the BTG/TOB (B-cell translocation gene/transducers of ErbB2) gene family and acts as a DNA-damage marker used for detecting cytotoxicity involving DNA-strand breaks (Wada et al., 2009). The biosensor system is the basis for promising tools for toxicity assessment; however, current biosensors are normally limited by high background signals and internal-control deficiencies.

Here, to accurately and sensitively assess NP toxicity, we developed an improved dual-fluorescence biosensor system exhibiting reduced background signal and a validated internal control. Each biosensor plasmid was comprised of a *mCherry*-reporter gene regulated by an inducible promoter and an enhanced green-fluorescence protein (*EGFP*)-reporter gene driven by the cytomegalovirus (CMV) promoter as an internal control. AP-1, IL8, and BTG2 promoters were chosen as the inducible promoters. Using this dual-fluorescence biosensor system, we investigated the toxicity of silver NPs (Ag NPs) and the influence of environmental factors [aging, fulvic acid (FA), and UVA irradiation] on NP toxicity. Our results indicated that this novel cell-based biosensor system constituted an accurate and sensitive method for rapidly evaluating NP toxicity and exhibited great potential for applications involving the assessment of toxic risk.

2. Materials and methods

2.1. Cell lines and reagents

HeLa cells and 293T cells were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS). Human non-small cell lung carcinoma H1299 cells were cultured in Roswell Park Memorial Institute 1640 medium supplemented with 10% FBS. Cells were maintained in a humidified atmosphere with 5% CO_2 at 37 °C. Ag NPs (polyvinylpyrrolidone-coated; <100 nm) and zinc oxide NPs were purchased from Sigma-Aldrich (St. Louis, MO, USA). Titanium dioxide NPs were purchased from NanoAmor (Houston, TX, USA). Lip2000 was purchased from Invitrogen (Carlsbad, CA, USA). Trizol and Power SYBR Green PCR master mix were purchased from Life Technologies, Inc. (Carlsbad, CA, USA), and TransScript One-Step gDNA removal and cDNA synthesis supermix was purchased from TransGen (Beijing, China).

2.2. Biosensor plasmid construction

The biosensor plasmid pAP1-MC-EGFP was constructed in four successive cloning steps. First, the *EGFP* fragment was subcloned into the Flag-CMV2 plasmid via *Hin*dIII and *Bam*HI restriction sites, using the forward primer 5′-TCGACT***AAGCTT***ATGGTGAG-CAAGGGCGAGGAG-3′ and the reverse prime 5′-TCACTG***GGATCC***T-TACTTGTACAGCTCGTCCATGCCG-3′ to yield pCMV-EGFP. The *mCherry* fragment was then digested with *Eco*RI and *Xba*I and introduced into the corresponding sites in the pcDNA3.1(+) plasmid to construct pcDNA-MC. Next, the AP-1 promoter was digested using *Bgl*II and *Kpn*I and inserted into pcDNA-MC upstream of the *mCherry* promoter to create pAP1-MC. Finally, the sequence containing the AP-1 consensus-binding sites and the *mCherry* gene was inserted into pCMV-EGFP by adding a *Bgl*II site to generate plasmid pAP1-MC-EGFP. The *CMV-EGFP* and *AP-1-mCherry* genes were designed to transcribe in opposite directions in the pAP1-MC-EGFP plasmid. An interference-blocking element consisting of two SV40 poly (A) sites was defined as transcription blocker (TB) and located upstream of the AP-1 promoter to prevent transcriptional interference of the downstream promoter.

The plasmids (pBTG2-MC-EGFP and pIL8-MC-EGFP) containing the BTG2 promoter (−110 bp to +71 bp) and the IL8 promoter (−179 bp to +69 bp) were constructed using a similar approach. Constructs were verified by sequencing.

2.3. Transfection for biosensor cells and nanoparticle exposure

Exponentially growing H1299 cells were seeded into 60-mm diameter Petri dishes (5×10^5 cells/dish) for 24 h. Transfection with empty plasmid or biosensor plasmid was performed using Lip2000 (Invitrogen) according to manufacturer instructions. After transfection, the cells were passaged into 12-well plates at 25,000 cells/well. Following overnight culture, the cells were