Toxicology 370 (2016) 60–69

Contents lists available at ScienceDirect

Toxicology

journal homepage: www.elsevier.com/locate/toxicol

A cell-based biosensor for nanomaterials cytotoxicity assessment in three dimensional cell culture

Monika Dubiak-Szepietowska[a,*], Aleksandra Karczmarczyk[a], Thomas Winckler[b], Karl-Heinz Feller[a]

[a] Department of Medical Engineering and Biotechnology, Ernst-Abbe-University of Applied Sciences Jena, Carl-Zeiss Promenade 2, 07745 Jena, Germany
[b] Institute of Pharmacy, Friedrich Schiller University Jena, Semmelweissstrasse 10, 07743 Jena, Germany

ARTICLE INFO

Article history:
Received 9 August 2016
Received in revised form 22 September 2016
Accepted 26 September 2016
Available online 28 September 2016

Keywords:
Cell-based biosensor
Nanoparticles
3D cell culture
NF-κB signaling

ABSTRACT

Nanoparticles (NPs) are widely used in consumer and medicinal products. The high prevalence of nanoparticles in the environment raises concerns regarding their effects on human health, but there is limited knowledge about how NPs interact with cells or tissues. Because the European Union has called for a substantial reduction of animal experiments for scientific purposes (Directive 2010/63), increased efforts are required to develop *in vitro* models to evaluate potentially hazardous agents. Here, we describe a new cell-based biosensor for the evaluation of NPs cytotoxicity. The new biosensor is based on transgenic human hepatoblastoma cells (HepG2) that express a secreted form of alkaline phosphatase (SEAP) as a reporter protein whose expression is induced upon activation of a stress response pathway controlled by the transcription regulator nuclear factor-κB (NF-κB). The NF-κB_HepG2 sensor cells were cultured in a Matrigel-based three dimensional environment to simulate the *in vivo* situation. The new biosensor cells offer the advantage of generating fast and reproducible readout at lower concentrations and shorter incubation time than conventional viability assays, avoid possible interaction between nanomaterials and assay compounds, therefore, minimize generation of false positive or negative results and indicate mechanism of toxicity through NF-κB signaling.

1. Introduction

Nanotechnology has provided a basis for innovation in a wide range of fields including biomedical, pharmaceutical, and cosmetic applications. The widespread use of manufactured nanoparticles will almost certainly lead to the release of these materials into the environment and, as a consequence, into the human body. However, there is limited knowledge about what the molecular targets of NPs might be and how their interaction with cells and tissues may influence human health. This demands the development of new methods to predict the toxicity of NPs. Although it is conceivable that toxicity testing is most reliably performed *in vivo* in animal studies, the premise to reduce experiments involving animals demands the development of *in vitro* models that most faithfully reflect the *in vivo* situation (Dusinska et al., 2015). Therefore, a whole cell-based biosensor (CBB) was developed to monitor and screen toxic substances in nano range. Cell-based biosensors typically have three interconnected modules to accomplish the task, that is, the input of the sensor (a receptor in the cell membrane or cytoplasm), intracellular gene regulatory networks and an output system (Wang and Buck, 2012). One of the first commercialized sensors was based on naturally luminescent bacteria (Microtox® test). Nearly 40 years ago, this simple sensor used the inhibition of light emission after exposure to a pollutant and was developed for monitoring aquatic samples (Bulich and Isenberg, 1981). To date, there is a wealth of published reports in which whole cells of microorganisms such as bacteria, yeasts, and algae were used to evaluate environmental hazards (Chouteau et al., 2004, 2005; Singh and Mittal, 2012; Shing et al., 2013; Vopálenská et al., 2015). In addition, mammalian cell-based sensors were introduced that can predict the safety of chemicals and their clinical potency (Xing et al., 2006; Banerjee and Bhunia, 2009; Wang et al., 2010; Hofmann et al., 2014; Liu et al., 2014). Cell-based sensors using human cells for nanomaterials toxicity screening still represent only a small percentage of developed assays e.g. (Taniguchi, 2010; Chen, 2012).

A variety of different response elements suitable in cell-based reporter assays have been described, such as binding sites for AP-1

* Corresponding author.
E-mail addresses: Monika.Dubiak-Szepietowska@eah-jena.de, dubiak.monika@gmail.com (M. Dubiak-Szepietowska).

http://dx.doi.org/10.1016/j.tox.2016.09.012
0300-483X/

(activator protein 1), STAT3 (signal transducer and activator of transcription 3), HSPs (heat shock proteins), or ISRE/IRF (IFN-stimulated response elements/interferon regulatory factor) (King et al., 2007; Hofmann et al., 2014). Another prominent transcription factor that responds to numerous external stimuli by inducing the expression of target genes is the nuclear factor-κB (NF-κB). This protein was first described as a B cell-specific transcription factor, but it was later found that this factor is inducible in virtually all cells and regulates many genes, most of which are involved in immune response, inflammation, cell proliferation, differentiation, cell survival and death (Karin and Lin, 2002; Bonizzi and Karin, 2004; Hayden and Ghosh, 2008). In most cells, NF-κB forms dimers that are retained in the cytoplasm by binding to a member of the inhibitory IκB proteins such as IκBα, IκBβ, or IκBε. In the canonical way of activating NF-κB, pathogen-associated molecular structures activate IκB kinase complex (IKK). The most common forms of IKK consist of IKKα and IKKβ catalytic subunits bound to the IKKγ regulatory subunit (also called NEMO – NF-κB essential regulator). Phosphorylation of IκB by IKK leads to its ubiqutinylation and subsequent degradation via the cells proteasome, such that the released NF-κB dimers can translocate to the nucleus and bind their cognate response elements in promoters of NF-κB target genes.

Interestingly, the NF-κB pathway is described as being strongly activated after exposure of cells to various kinds of NPs (Prasad et al., 2013; Stepkowski et al., 2014; Chen et al., 2016). Thus, the NF-κB pathway is an excellent candidate for the development of a cell-based biosensor for the assessment of NPs cytotoxicity. Whether this is a direct interaction or a secondary effect after exposure to nanoparticles is not completely understood. Some studies indicate an indirect activation of NF-κB signaling through oxidative stress (Ye et al., 2009; Nishanth et al., 2011; He et al., 2011), pro-inflammatory cytokines, while others strongly indicate an overriding role of TLR receptors (Chen et al., 2013; El-Said et al., 2014). After binding of NPs TLRs may directly activate cascade of NF-κB pathway-specific kinases, IKKα (or IKK1), IKKβ (or IKK2) and regulatory protein NEMO enabling active NF-κB to translocate into the nucleus. It is likely that there are still other unknown ways to activate NF-κB signaling (Ghosh and Karin, 2002). A variety of stimuli coalesce on NF-κB activation, which can in turn mediate varied transcriptional programs. Consequently, NF-κB-dependent transcription is not only tightly controlled by positive and negative regulatory mechanisms (Oeckinghaus and Ghosh, 2009) but also closely coordinated with other signaling pathways. Whether activation of NF-κB pathway results in stimulation or suppression other intracellular pathway depends of the type of toxic factor, its concentration, frequency but as well cell type. Liu et al. showed that silica nanoparticles provide dysfunction of endothelial cells through oxidative stress and thus activation of both JNK/P53 and NF-κB pathways (Liu and Sun, 2010). In turn, titanium dioxide nanomaterials activate pro-inflammatory response in hepatic cells via activation of MAPK and NF-κB pathway (Chen et al., 2016). Silver nanomaterials incubated with Jurkat cells activates p38 mitogen-activated protein kinase through nuclear factor-E2-related factor-2 and nuclear factor-kappaB signaling pathways, subsequently inducing DNA damage, cell cycle arrest and apoptosis (Eom and Choi, 2010). Nanoparticles may activate various other intracellular signaling pathways such as Rho GTPase, p38-Nrf-2, and SAP/JNK signaling (Sniadecki, 2010; Eom and Choi, 2010; Hyun-Jeong Eom, 2009). To date, the development of whole cell-based biosensors mostly uses well-established two dimensional (2D) cell culture. However, interpretation of results obtained with 2D cell culture is often limited by large discrepancies when compared with in vivo animal models. This is certainly due to the highly artificial environment present in the culture dish, as the majority of cells in tissues is adapted to perform their functions in a three dimensional (3D) environment with the opportunity of multiple cell contacts regulating specialized functions of the cells.

The presented research aimed to improve the prediction of nanomaterial cytotoxicity on cell and tissue- level and provide the essential screening steps prior to any *in vivo* or environmental applications. Therefore, the initial objective was the use of a genetically modified human hepatoblastoma cell line (HepG2) that joins a cellular signaling system (NF-κB pathway) with a reporter gene (human secreted alkaline phosphatase) to create a whole cell-based biosensor with an optical readout. This enable the monitoring of toxic effects of chemicals in real time and in early stage before cell death. Then, it was intended to establish an environment for sensor cells with improved physiological relevance of cell-based assays. Since cells in the body grow in a three dimensional milieu, a 3D approach as an alternative to 2D culture was to be established to reduce the gap between cell culture and living tissue. Moreover, implementation of a whole cell biosensor based on human cells with an extracellular reporter in a three dimensional milieu and its application for screening of nanoparticle toxicity has not been investigated before.

2. Materials and methods

2.1. Cell culture

The human hepatoblastoma cell line HepG2 was obtained from Cell Line Service GmbH (CLS Eppelheim, Germany). Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) with low glucose and supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, 100 μg/ml penicillin and 100 μg/ml streptomycin (referred to as complete cell culture medium). All mentioned reagents were purchased from Biowest (Nuaillé, France). The cells were kept at 37 °C under a humidified atmosphere with 5% CO_2. In all cytotoxicity assays, cells were kept under serum-deprived conditions (1% FBS) for at least 24 h before performing experiments.

For spheroid cell culture, Corning® Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix (phenol red-depleted; Amsterdam, Netherlands), was prepared at 1 mg/ml in DMEM and diluted 1:1 with complete cell culture medium before cell seeding. Cells were mixed with the above mentioned hydrogel and cultured at 37 °C.

The morphology of spheroids was evaluated using an LSM 710 microscope (Carl Zeiss) equipped with an EC Plan-Neofluar 20x/0.50 M27 objective. Staining of vital cells was performed with calcein AM (4 μM) (Sigma) and nuclei of cells were visualized using DAPI staining (AppliChem) in Hanks' Balanced Salt Solution (Sigma) for 10 min in darkness. The respective excitation and emission wavelengths were 490/540 nm for calcein AM and 358/461 nm emission for DAPI.

2.2. Assessment of nanoparticle-induced cytotoxicity (Alamar Blue assay)

For cell viability assays 15,000 cells were seeded in each well of 96-well plates in the form of monolayers or spheroids. After 8 days the cells were treated with varying concentrations of the following nanoparticles: silver nanoparticles (AgNP, 10 nm), zinc oxide nanoparticles (ZnONP, 14 nm and 25 nm), silicon dioxide nanoparticles (SiO_2NP, 10 nm and 20 nm), titanium dioxide nanoparticles (TiO_2NP 4–8 nm and 16–26 nm) (all obtained from PlasmaChem). All NPs were sonicated (Bandelin, Sonorex super RK 102H) for 15 min at room temperature directly before use to resuspend formed agglomerates. After 24 h of incubation of cells with NPs, all wells were washed 3 times with PBS and 50 μM AlamarBlue® Cell Viability Reagent (Alfa Aesar) was added.

Absorbance was recorded at 540 nm with reference at 630 nm using a Tecan Infinite® M200 plate reader. Cytotoxicity (in percent) was expressed by dividing the absorbance recorded for treated cells with the absorbance obtained for untreated control cells. The characterization of nanomaterials including their tendency to form agglomerates in medium and the influence of cell culture medium on final toxicity measurements was performed as recently described (Dubiak-Szepietowska et al., 2016). Based on these results, NPs solutions were sonicated directly before experiments and all experiments were performed with serum deprived medium (1% FBS).

2.3. Transfection of HepG2 cells

HepG2 cells were seeded in 24-well cell culture plates at a density of 4×10^4 cells per well in 0.5 ml of complete cell culture medium 24 h before transfection. Transfection was performed according to the manufacturer's manual by adding 2 μl of Lipofectamine 2000 reagent (Life Technologies) and 500 ng of plasmid pNiFty2-SEAP (InvivoGen, Toulouse, France). Complete cell culture medium was replaced with selective medium (DMEM, 10% FBS, 300 μg/ml zeocin) 24 h after transfection. After five days of cell culture, these cells were used as "transiently transfected cells" in intial experiments.

For the generation of stably transfected NF-κB_HepG2 cell lines, transfection was performed as described above, but selection in zeocin-containing medium was continued until untransfected cells had died. To enable single clone selection, remaining zeocin-resistant cells were diluted to ~50 cells per well (12-well plate) in selective medium (DMEM, 10% FBS, 300 μg/ml zeocin). Clones were isolated using paper disks (~6 mm; Whatman filter paper; sterilized by autoclaving) soaked with trypsin. Cells from 20 single colonies (scaled up to T75 flask) displaying the highest growth rate and viability were induced with 30–200 ng/ml TNF-α (Sigma) and 0.001–60 μg/ml lipopolysaccharides from *Escherichia coli* (LPS; Sigma) and screened using the NF-κB_HepG2 assay (see below) to identity the most sensitive clones as potential sensor cell lines.

2.4. NF-κB_HepG2 assay

Transiently or stably transfected NF-κB_HepG2 cells were treated with the indicated NPs for 1–24 h (kept in 37 °C, 5% CO_2 and humidified atmosphere). Cell culture supernatants were collected, centrifuged for 10 min at 13,000 rpm, and 20 μl per well were transferred to 96-well plates. Endogenous alkaline phosphatase was inactivated by heating the samples to 65 °C for 10 min. Note that the reporter enzyme in the NF-κB_HepG2 assay, secreted alkaline phosphatase (SEAP), is heat-stable. Next, samples were mixed with 46 μl of 20 mM L-homoarginine (Sigma), 1 mM $MgCl_2$, Tris-HCl pH 9.0 (Dean, 2002) and incubated for 15 min at 37 °C. Finally, 34 μl of 120 mM freshly prepared *p*-nitrophenyl phosphate (pNPP; Sigma) were added. The mixtures were incubated for 1 h at 37 °C. SEAP catalyzes the hydrolysis of pNPP producing a yellow product (*p*-nitrophenol) under alkaline conditions that was conveniently measured at 405 nm using a microplate reader. SEAP activity determined in cell culture medium is directly proportional to changes in intracellular SEAP gene expression. Results are presented as relative values of absorbance calculated according to equation:

$$RA = \frac{A - A_B}{A_C - A_B}$$

RA is the relative absorbance, *A* is the absorbance of analyzed samples (supernatant of stimulated cells), A_B is the absorbance of the blank (cell culture medium), and A_C is the absorbance of the control (supernatant of unstimulated cells).

2.5. RNA isolation and cDNA synthesis

Total RNA was extracted from pellets of 1×10^6 cells using Qiagen's RNeasy Mini Kit according to the provided protocol. 2 μg of total RNA was used for cDNA synthesis in 20 μl reaction volume using the First Strand cDNA Synthesis kit purchased from Thermo Scientific.

2.6. Quantitative reverse-transcription PCR (RT-qPCR)

Primers were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Ye et al., 2012) and purchased from Eurofins Genomics (Ebersberg, Germany). The following primers were used: GAPDH-fwd: 5′-GAAGGTGAAGGTCGGAGTC-3′, GAPDH-rev: 5′-GAAGATGGTGATGGGATTTC-3′ (226 bp); IκBα-fwd: 5′-GGCAGCAACGACACAGAAAC-3′, IκBα-rev: 5′-GCGGTGGGTAATGGAGACAT-3′ (128 bp). RT-qPCR was performed on a Rotor-Gene 6000 (Corbett) system using the GoTaq qPCR Master Mix (Promega). The PCR profile was as follows: Hot-Start activation 95 °C, 3 min; denaturation 95 °C, 15 s; annealing/extension 60 °C, 60 s; melting curve analysis/termination 65 °C to 95 °C. Data were analyzed with the Pfaffl quantification model (Pfaffl, 2001) using GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as reference gene.

2.7. Statistics

Quantitative data are expressed as arithmetic mean ± standard deviation. The Student's *t*-test was used to analyze the statistical significance of the data ($p < 0{,}05$ vs. control; STATISTICA 9). All measurements were performed at least three times.

3. Results and discussion

3.1. Generation and characterization of NF-κB_HepG2 sensor cells

Numerous of reporter proteins have been used in mammalian cells to facilitate the determination of gene expression levels, represented predominantly by chloramphenicol acetyltransferase, β-glucuronidase, β-galactosidase luciferase and fluorescent proteins (Bennett et al., 2015; Fang et al., 2015; Hofmann et al., 2013; Soboleski et al., 2005). Nonetheless, to overcome some of the limitations connected to above mentioned reporter systems, such as the presence of similar endogenous enzyme activity, requirement for cumbersome assay, separation product from the substrate, cell lysis performance, difficulties with measurements of fluorescence or luminescence signal emitted by cells inside spheroids, intracellular pathway was linked with an extracellular reporter. Thus, eliminating the need to extract the valuable spheroids and providing the opportunity for repeated measurements of the same cultures. Berger et al. (1988) described a reporter gene, the human secreted alkaline phosphatase (SEAP), which is a secreted form of the human placental alkaline phosphatase (PLAP, EC 3.1.3.1). The enzyme removes phosphate groups from many types of molecules, including simple compounds such as *p*-nitrophenyl phosphate. PLAP is initially anchored to the membrane via a C-terminal 26 amino-acid hydrophobic domain. SEAP lacks this domain allowing the protein to be efficiently secreted in an active form (Millan, 1986; Nakashima et al., 2013). SEAP activity accumulating in cell culture medium is directly proportional to changes in intracellular SEAP gene expression. Another important advantage of SEAP as a secreted reporter protein is a low background of alkaline phosphatase

activity in cell culture medium. In fact, this background of alkaline phosphatase activity can be further reduced by simply pre-incubating the samples at 65 °C (SEAP is heat-stable) or by addition of L-homoarginine in the assay (Cullen and Malim, 1992).

To generate SEAP-expressing sensor HepG2 cells, transfections were performed using the commercially available plasmid pNiFty2-SEAP. The plasmid features an engineered human E-selectin minimal promoter (ELAM) equipped with five NF-κB binding sites (5′-GGGGACTTTCC-3′). In initial experiments, transiently transfected HepG2 cells were examined to analyse whether induction of NF-κB in response to exposure of cells to nanoparticles would induce SEAP reporter gene activity in conventional 2D monolayer culture. After cells were incubated with 15 μg/ml AgNP, 250 μg/ml SiO_2NP or 80 μg/ml ZnONP for 8 h, SEAP reporter activity increased by 4- to 6-fold above background of untreated cells, depending on the NPs analyzed (Fig. 1A).

In the next steps, the validation and optimization of the sensor cell line was carried out with two well established activators of the NF-κB pathway TNF-α and LPS (Schütze et al., 1992; Bellezzo et al., 1996; Bouwmeester et al., 2004) as a proof of concept setup. Firstly, stably transfected NF-κB_HepG2 cell lines were established. Individual clones were tested for their performance in the NF-κB_HepG2 reporter assay after stimulation with TNF-α (30–200 ng/ml) or LPS (0.001–60 μg/ml). Among tested clones, clone #13 stood out with the lowest background of alkaline phosphatase activity, best linearity and smallest standard deviation in the NF-κB_HepG2 assay readout (Fig. 1B, C). Depending on the place of genomic DNA in which vector was inserted and in how many copies, transfected cells may display different expression levels of proteins coded in the vector. Which in turn, explains response with different sensitivity of analyzed clones after stimulation with LPS and TNF-α. Moreover, mentioned above linearity of the signals is an essential feature of sensors because it indicates that an output is directly proportional to input over its entire range while, standard deviation emphasize precision of developed system. Then, the stably transfected sensor cell line was compared with untransfected cells to examine whether the genetic modification had an impact on the general properties of the cells. Growth rates of transfected cells and untransfected cells were determined. Both cell populations displayed generation times of ~36 h (Fig. 2A). Then, the stability of the sensor cell response upon storage of cells was verified after 3, 25 and 35 subcultivations. NF-κB_HepG2 cells were stimulated with TNF-α (30 ng/ml) and LPS (0.02 μg/ml) for 8 h and it was found that even after one year of storage and repeated re-activation the transfected cells produced stable reporter signals (Fig. 2B). Moreover, the genetic modification did not change sensitivity of cells to well known inducers of cell death, DMSO and ethanol. After incubation of the cells in the presence of 2–16% ethanol for 24 h, both the transfected and control cells were similarly sensitive (EC_{50} = 8%; Fig. 2C). Cells were also similarly sensitive to the presence of 1–10% DMSO (EC_{50} = 4%; Fig. 2D). Obtained results clearly indicate that genetic modifications of HepG2 cells display any influence on cells viability and growth rate. Moreover, one year monitoring of sensor cells point out their stable signal.

Further, the required cell number to obtain reliable measures of SEAP reporter activity was examined. As shown in Fig. 3, SEAP activity increased with cell number with a maximal induction level determined when using 10^7 cells. The minimum cell number needed for detectable SEAP activity at 8 h post-plating was ~250. Moreover, the stability of the secreted reporter protein upon storage was examined. One week storage of samples in −80 °C did not significantly change the outcome compared to fresh samples; however, storage for more than a month resulted in considerable loss of reporter enzyme activity (data not shown). Therefore, all data presented in this study result from measurements of fresh samples.

3.2. NF-κB_HepG2 biosensor in 3D cell culture for testing nanoparticles cytotoxicity

Realizing that immortal HepG2 cell line differs from hepatocytes which can be found in native tissue (Guo et al., 2011; Wilkening et al., 2003; Guillouzo et al., 2007), adoption of three dimensional environment was a balanced solution.

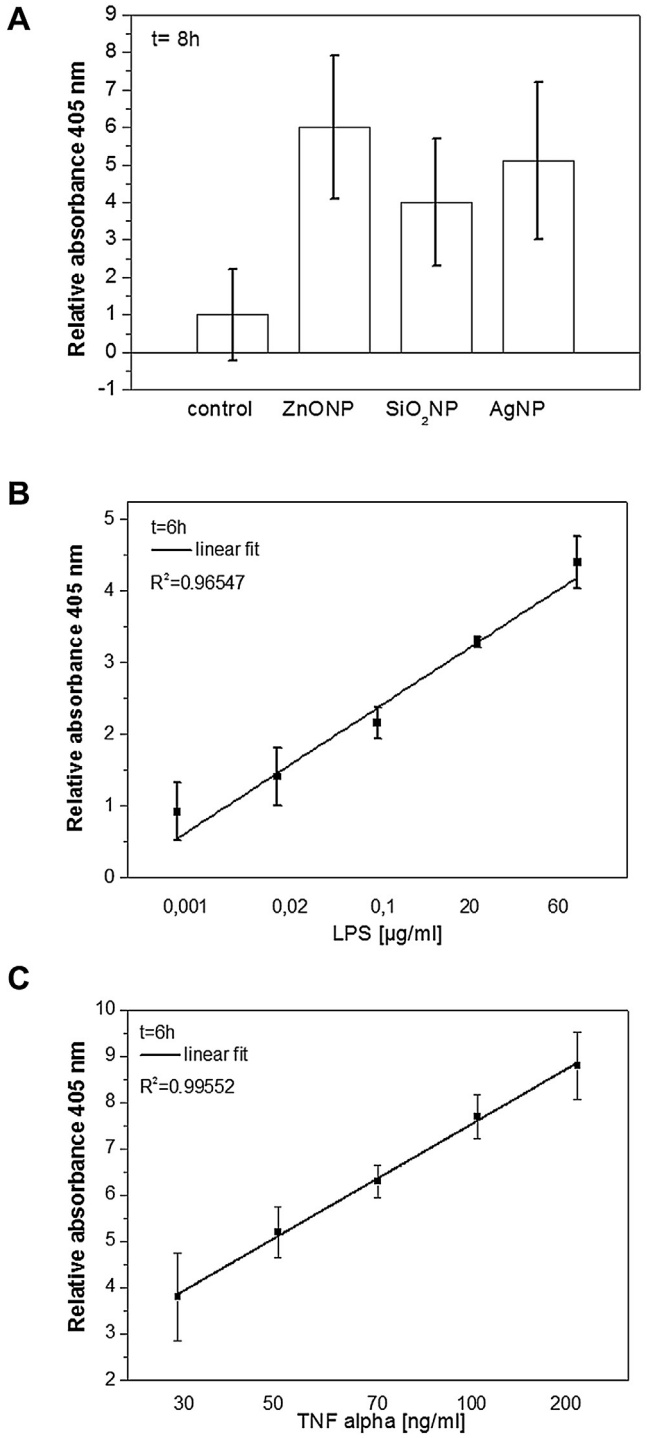

Fig. 1. Generation of NF-κB_HepG2 sensor cells. (A) Exposure of transiently transfected NF-κB_HepG2 cells with 15 μg/ml AgNP, 250 μg/ml SiO_2NP and 80 μg/ml ZnONP leads to induction of SEAP reporter activity. Control cells were unstimulated, transiently transfected cells (n = 4 ± SD). (B, C) Response of stably transfected NF-κB_HepG2 cells after stimulation with LPS and TNF-α with linear fitting (unstimulated control cells set to 1; n = 3 ± SD).

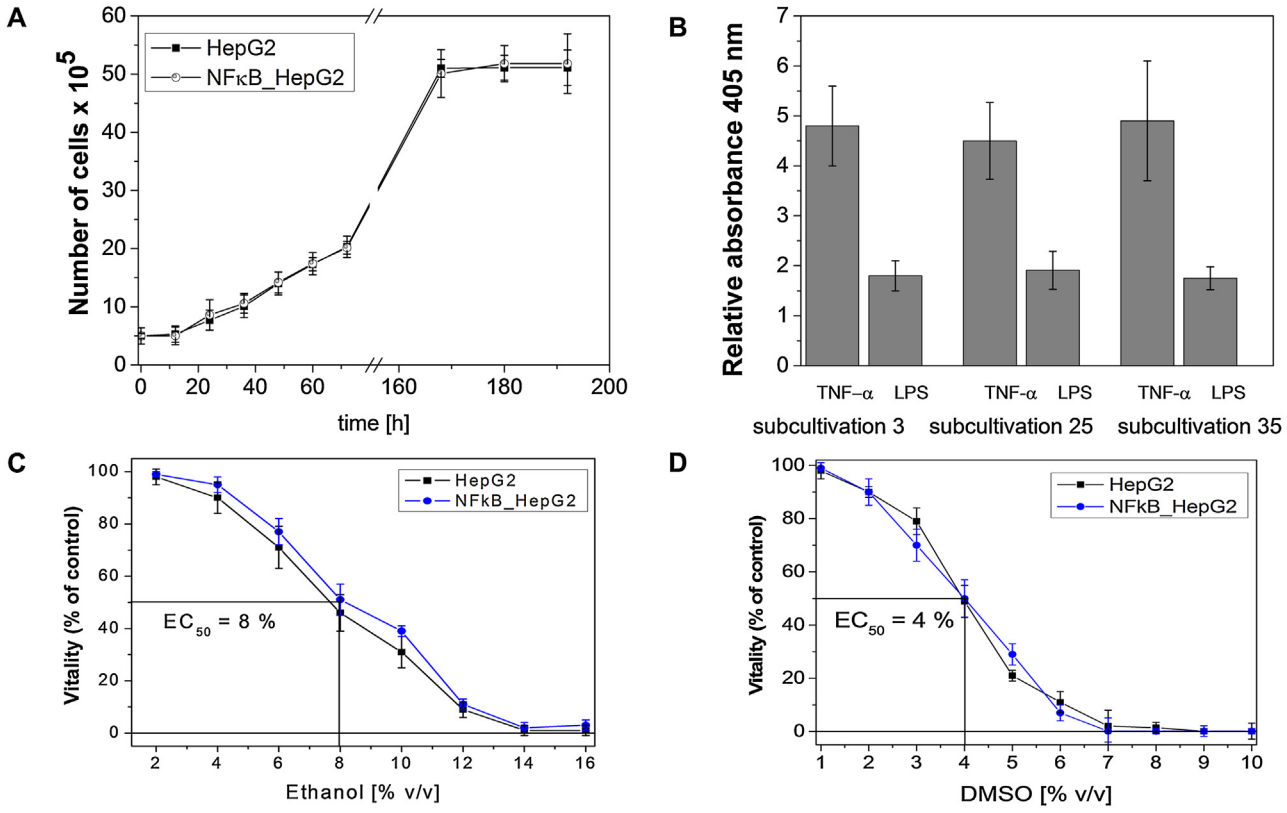

Fig. 2. Properties of NF-κB_HepG2 sensor cells. (A) Growth rates of untransfected HepG2 cells and NF-κB_HepG2 cells. Initial cell seeding was 5×10^5 cells per well; observation time was 192 h (n = 4 ± SD). (B) Investigation of the sensor cell stability after 3, 25 and 35 subcultivations. NF-κB_HepG2 cells exposed to 30 ng/ml TNF-α and 0.02 μg/ml LPS for 8 h (n = 3 ± SD). (C) Sensitivity of HepG2 cells and NF-κB_HepG2 cells to exposure with 2–16% v/v ethanol (n = 3 ± SD). (D) Sensitivity of HepG2 cells and NF-κB_HepG2 cells to exposure with 1–10% v/v DMSO (n = 3 ± SD).

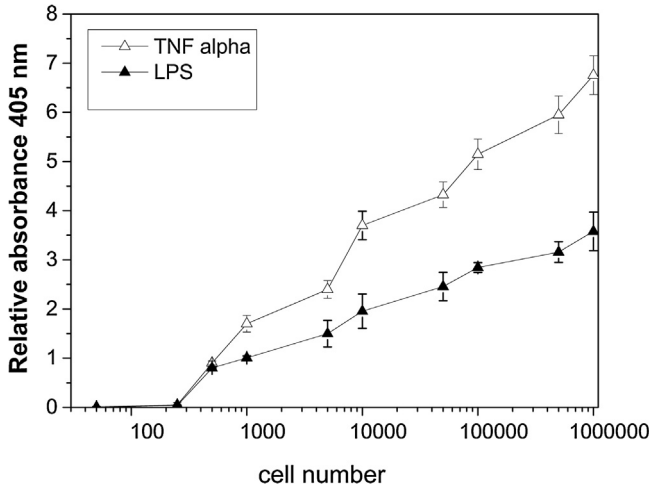

Fig. 3. SEAP reporter activity in NF-κB_HepG2 cells. Correlation of SEAP activity with NF-kB_HepG2 cell number after stimulation with 70 ng/ml TNF-α or 30 μg/ml LPS for 8 h (n = 5 ± SD).

Implementation of biosensor and 3D cell culture is anticipated to ensure more predictive results for *in vivo* studies, as 3D models come closer to *in vivo* conditions than conventional 2D monolayer culture, (Bokhari et al., 2007; Lan et al., 2010; Li et al., 2008; Pruksakorn et al., 2010). The morphology of spheroids formed by NF-κB_HepG2 cells in Matrigel is shown in Fig. 4. Our recently published data shown that HepG2 cells are more sensitive to exposure to10 nm AgNP, 25 nm ZnONP, and 10 nm SiO_2NP when growing in monolayer culture than in a 3D environment (Dubiak-Szepietowska et al., 2016). Cytotoxic effects of NPs on spheroid HepG2 growing in Matrigel under serum-deprived conditions (1% FBS) were observed with EC_{50} values of 15 μg/ml for AgNP, 80 μg/ml for ZnONP, and 250 μg/ml for SiO_2NP (Dubiak-Szepietowska et al., 2016). The transfected NF-κB_HepG2 cells were comparably sensitive to NPs exposure. The determined EC_{50} values of 20 μg/ml for AgNP, 82 μg/ml for ZnONP, and 280 μg/ml for SiO_2NP, suggesting that the transfected cells have properties similar to the parental HepG2 cells (Fig. 5).

Nevertheless, the NF-κB_HepG2 cells might signal the presence of cytotoxic NPs even before visible cell-damaging effects occurred. As shown in Fig. 6, sensor cell response in terms of increasing SEAP activity in the supernatant of cells increased with a delay of approximately 2 h after first exposure and a maximum was reached at 8–12 h. The observed response delay is consistent with the cells activating the NF-κB pathway and subsequently inducting gene transcription and translation of SEAP. Under the experimental conditions, the threshold concentrations of AgNP 3.75 μg/ml; SiO2NP 125 μg/ml and ZnONP 20 μg/ml were required to perceive a reproducible and significant response from the sensor cells after 2 h of exposure to the hazard. Application of lower doses require longer incubation times to observe a significant response of NF-κB_HepG2 cells. After 48 h incubation of sensor cells with nanomaterials was determined a detection limit of AgNP 0.1 μg/ml; for SiO_2NP, 90 μg/ml and ZnONP 5 μg/ml. Prolonged incubation produced similar results, suggesting that the analyzed nanomaterials do not induce pro-inflammatory response at lower concentrations in HepG2 cells. High concentrations of examined analytes (AgNP > 30 μg/ml; ZnONP > 160 μg/ml; SiO2NP > 500 μg/ml) or extended incubation times (>12 h) resulted in lower signals,

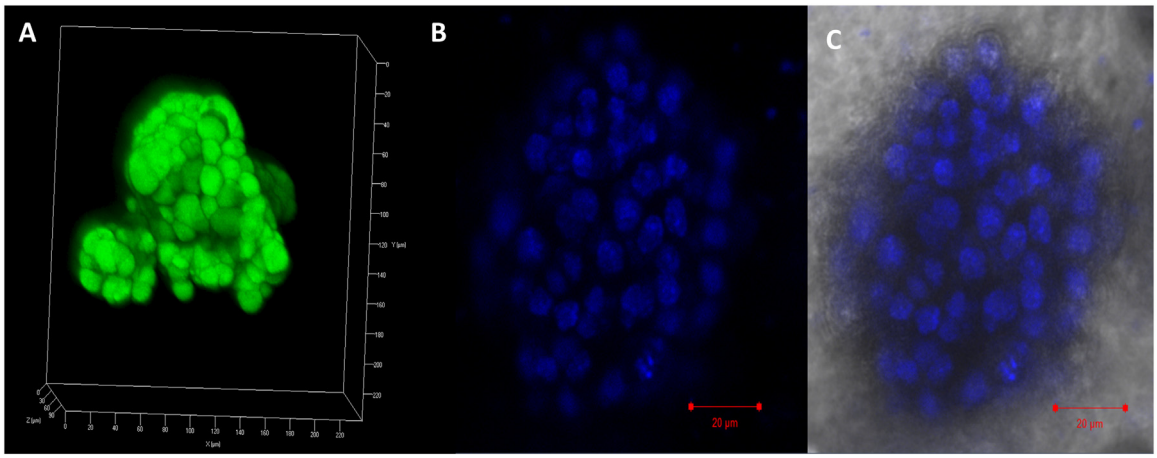

Fig. 4. Morphology of NF-κB_HepG2 spehoids in Matrigel. (A) Spheroid was stained with calcein AM to visualize viable cells (3D display). (B) Spheroid stained with DAPI to visualize cell nuclei (display of a single image in a frame mode). (C) Superimposed image of DAPI channel and transmitted light. Scale bars: 20 μm.

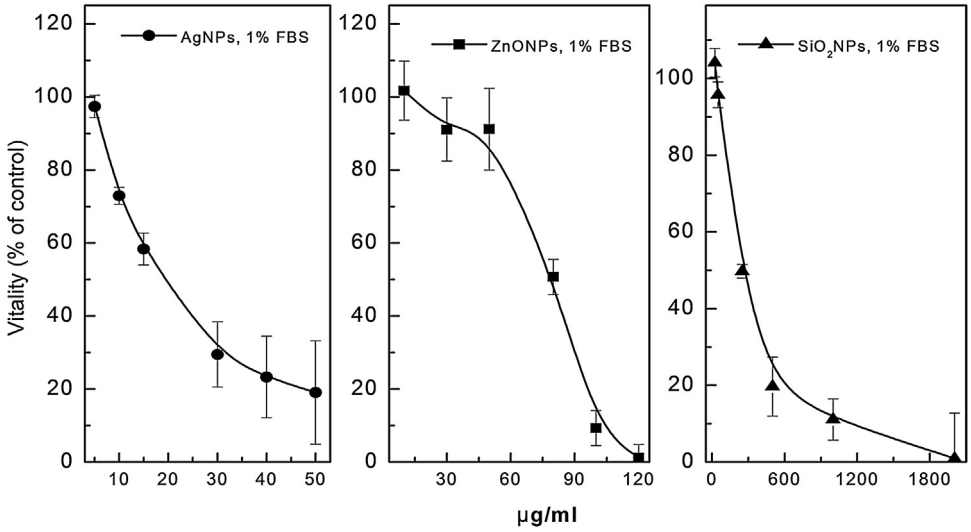

Fig. 5. Sensitivity of NF-κB_HepG2 sensor cells to exposure with nanoparticles. Cells were exposed to AgNP, ZnONP, and SiO_2NP at the indicated concentrations and cytotoxicity was determined using the Alamar blue assay (n=5 ± SD).

which may be either a result of inactivation of NF-κB or the progressing cell damage. Comparison of the data obtained from nanomaterials toxicity assessment applying sensor cells (Fig. 6) and commercially available assay (Alamar Blue; Fig. 5) displays higher sensitivity of developed system. All commercial assays based on the reduction of tetrazolium salts differentiate cells into death and viable. Even in the terminal phase of the toxicity (late apoptosis) cells still are able reduce tetrazolium salts and are counted as a viable cells. This explain long incubation time of toxic compounds with this kind of assays (usually >12 h). Therefore, NFκB_HepG2 cells seems to be attractive alternative for assessment toxicity of pro-inflammatory agents in very early stage and short incubation time. Moreover, modified reporter cells enable multiplex experiments, since readout performance don not require termination of cell culture and cells remain intact. Previously published observations suggested that Matrigel is the most suitable matrix to grow HepG2 cells in a 3D environment (Dubiak-Szepietowska et al., 2016) In our hands, measurements performed in a 3D milieu produced more robust data compared to measurements in 2D; however, the relative response of NFκB_HepG2 cells in the 3D environment was somewhat lower compared to monolayer cell culture (Dubiak-Szepietowska et al., 2016). This may be explained by higher resistance of cells to nanomaterials when grown in a 3D environment. Because, cells in native tissue create multilayer structures with hindered diffusion of chemicals and uneven exposure of cells to external stimuli, the 3D cell culture system mimics the native tissue better than conventional cell culture.

To confirm the induction of SEAP reporter activity in the sensor cells reflects activation of the cellular NF-κB pathway, the transcription level of the NF-κB inhibitor IκBα was determined. The IκBα gene is induced by NF-κB, generating a negative feedback loop (Bottero et al., 2003). IκBα protein enters the nucleus to bind and inactivate NF-κB. Thus, the more active the NF-κB pathway is, the more IκBα gene expression is observed (Bottero et al., 2003). This is an indirect measure of the NF-κB pathway activity. As shown in Fig. 7, IκBα transcript levels were strongly increased in a concentration-dependent manner 2 h after the first exposure of the cells to NPs. The IκBα gene induction was limited in time, but may well contribute the reduction of reporter protein activity by turning down the NF-κB pathway 8–12 h after exposure to the hazard.

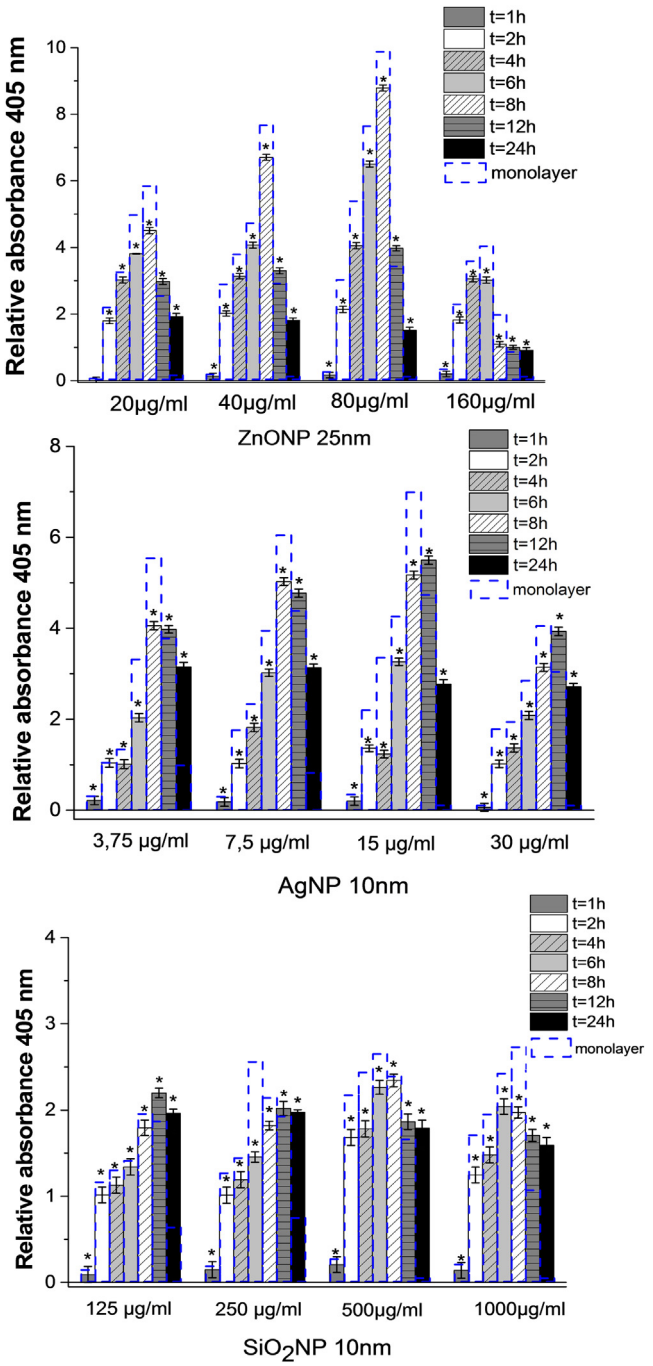

Fig. 6. Response of NF-κB_HepG2 cells to exposure to nanoparticles. Relative response of sensor cells in Matrigel after stimulation with ZnONP, AgNP and SiO_2NP at the indicated concentrations. (unstimulated control cells set to 1; n = 7 ± SD); * $p < 0.05$.

To further evaluate the NFκB_HepG2 sensor cells in basic screening, the cytotoxic potential of nanomaterials with different size and chemical composition was analyzed (Fig. 8A). In cell viability assays were tested concentrations up to 300 µg/ml for TiO_2NP 4–8 nm, 600 µg/ml for SiO_2NP 20 nm, 400 µg/ml for ZnONP 14 nm and 500 µg/ml for TiO_2NP 16–26 nm. Higher concentrations could not be tested because excessive precipitation of the nanomaterials interfered with the assay. The response of NFκB_HepG2 cells to the analytes increased in a dose-dependent manner (Fig. 8B). Both the cell viability tests and the sensor cell readout revealed that TiO_2NP 4–8 nm was the most toxic agent and the lowest toxicity was observed after stimulation the sensor cells with SiO_2NP 20 nm. Furthermore, sensor cells detected toxic effects of NPs at lower concentrations (10 µg/ml) and shorter incubation time. Interestingly the low dose of nanoparticles enhanced the metabolic activity of cells, suggesting that the low doses of some nanomaterials might activate intracellular repair and defensive mechanism. There are many efforts in understanding the effects of various NPs on cell viability and metabolism, but not much is known regarding the distinct molecular mechanisms of inflammation and cellular stress using low NPs concentrations. The new NF-κB_HepG2 biosensor can be applied in basic screening without exact knowledge of the interaction mechanisms between NPs and living systems. However, as the NF-κB pathway is an explicit marker for the activation of inflammatory pathways in cells, effects of test compounds on NF-κB_HepG2 cells may give a prediction on the pathological mechanism of observed toxicities.

The optical properties of some NPs may interfere with the endpoint measurement of absorbance in biochemical assays. Possible interference effects (such as surface plasmon resonance and quantum confinement) resulting from varying size, shape, surface modality, inter-particle interaction and composition make optical characterization of each NPs species essential. Because higher concentrations of NPs have greater probability of interfering with toxicity assays (Kroll et al., 2012; Ong et al., 2014), it is desirable to evaluate NPs toxicity at the lowest concentrations as possible. Therefore, assays designed to predict the cytotoxicity of NPs should be either very sensitive (such that NPs do not interfere with readout) or enable to remove the applied NPs before performing the assay of reporter activity. The NF-κB_HepG2 sensor cell assay was design to combine both because it is sensitive enough to provide readouts at low concentrations of NPs and it uses a secreted and soluble reporter protein, thus allowing to remove remaining NPs by centrifugation before performing the assay. Therefore, the NF-κB_HepG2 sensor cells with simple and fast protocol are highly dedicated for nanomaterials toxicity screening prior to any in vivo or environmental applications as an alternative for conventional viability assays based on reduction of tetrazolium slats or cellular membrane integrity. Nevertheless, CBB have some feature that might limit their application. Unlike to nucleic acid- or antibody-based methods, CBBs are unsuitable for the identification of the analyte. Activation of cell signaling pathways or cell death may be the results of exposure to multiple stimuli (Fig. A1–A2 in Supplementary material). Undoubtedly interaction of chemicals with living cells are complex and involved many intracellular pathways. Therefore, cell-based biosensors may give rather an answer if analyte is toxic in physiological range to the cells than what kind of components contain examined sample. Until now a biosensor based on mammalian cells which is specific to exactly one type of analyte was not described. NF-κB_HepG2 cells may provide an advanced screening system with simple procedure for companies that produce nanomaterials for accessing the risks posed by chemicals and providing proper safety information to their users.

4. Conclusions

The simulation of tissue conditions by growing the cells in a 3D environment, the use of a secreted reporter protein (human secreted alkaline phosphatase) that allows readout in a high-throughput setting without cell lysis and cell extraction from complex 3D milieu, the use of DNA control elements that reflect the activation of the NF-κB pathway in response to a plethora of external stimuli resulted in development a new whole cell-based biosensor with unique properties. The sensor cells based on transgenic NF-κB_HepG2 cells presented in this study enable the detection of cytotoxicity of a variety of nanoparticles with different

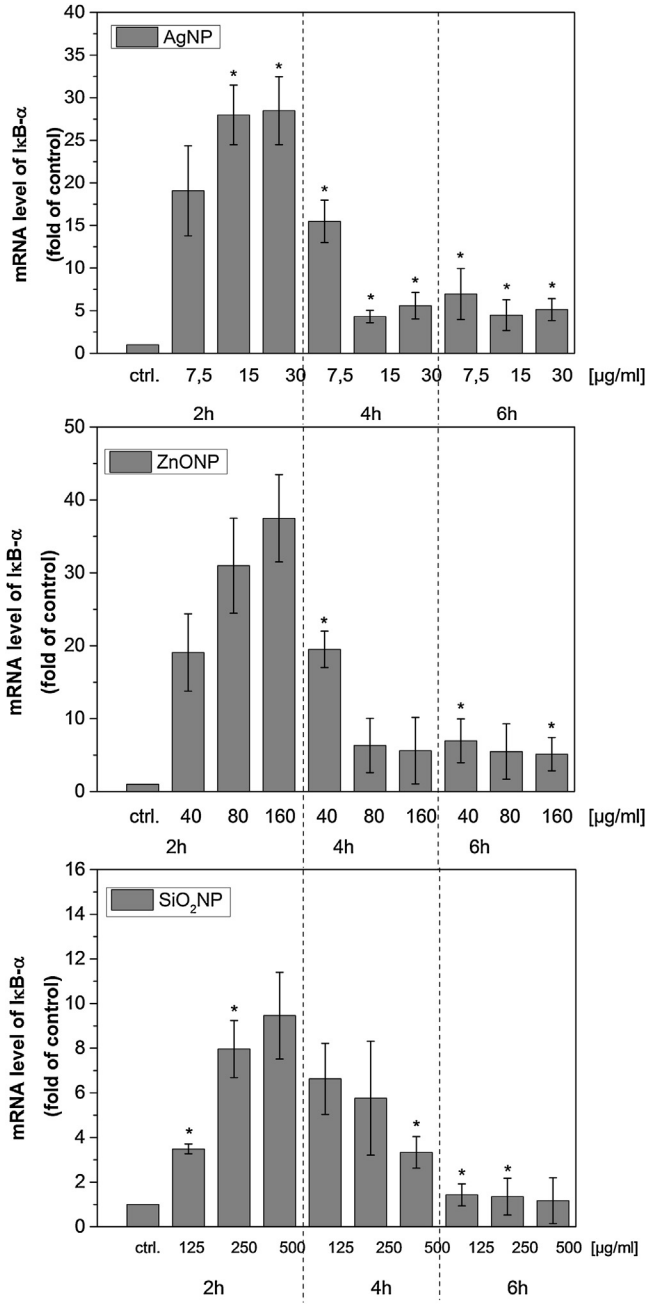

Fig. 7. Activation of the NF-κB in sensor cells after exposure to nanoparticles. The mRNA level of IκBα in NF-κB_HepG2 cells was determined by RT-qPCR after stimulation with ZnONP, AgNP and SiO_2NP at the indicated concentrations. The data are expressed as a fold change from basal levels in unstimulated control cells (n=6 ± SD); * $p < 0.05$.

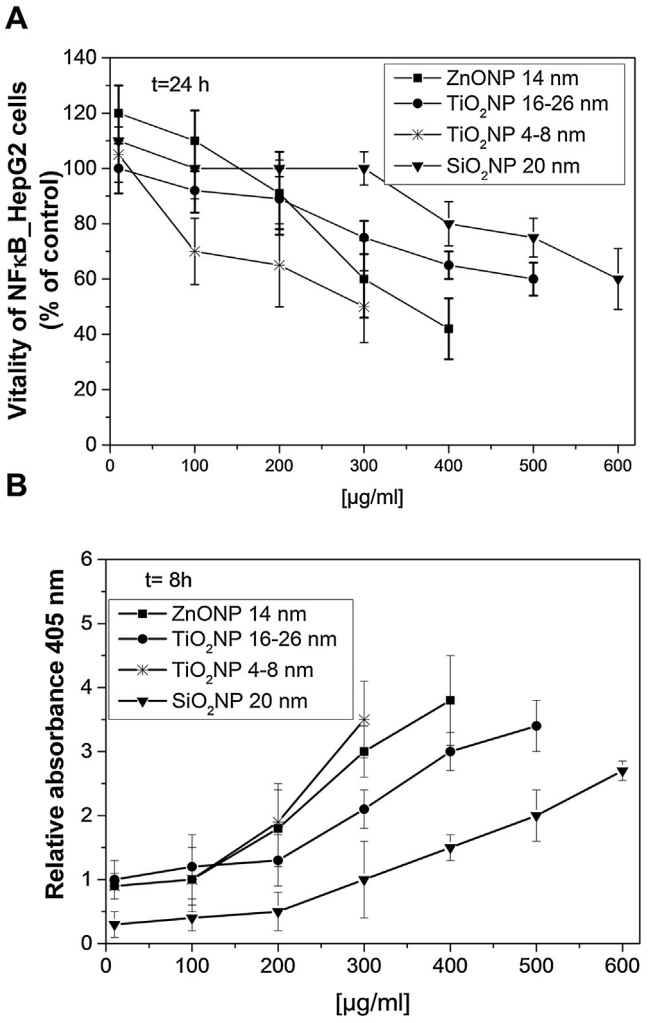

Fig. 8. Response of NF-κB_HepG2 spheroids to exposure to nanomaterials. (A) Cell viability after incubation of NF-κB_HepG2 cells in Matrigel for 24 h with ZnONP 14 nm, TiO_2NP 16–26 nm, TiO_2NO 4–8 nm and SiO_2NP 20 nm determined by Alamar Blue assay (n = 4 ± SD). (B) SEAP reporter response after cultivation of cells in Matrigel for 8 h with the same nanomaterials (unstimulated control cells set to 1; n=5 ± SD).

chemical composition, size, and tendency to agglomerate or cytotoxic potential. Sensor response is typically recorded at an early stage of cytotoxic effects, usually before detectable decrease of cell viability is observed with conventional assays or even cell death is observed microscopically. The NF-κB_HepG2 cells may be used to monitor long term cellular stress and inflammation at low concentration of chemicals. Furthermore, the ability to monitor transcriptional regulators in real time after exposure with nanomaterials greatly enhances the understanding of cellular regulation and control of their toxic mechanisms. Conventional gene expression analysis usually engages measuring single-time-point with techniques such as northern blots, reverse transcription polymerase chain reaction (RT-PCR) or DNA microarrays. Taking advantage of these techniques, dynamics can only be approximated by assembling average results from separate cell populations, one for each time point. Therefore, presented sensor cells seem to be a perfect solution to control over the time activation of specific intracellular pathway (NF-κB) after exposure of cells to nanoparticles. Additionally developed human-cell based models for toxicity testing may reflect with the great efficiency living tissue and give highly predictive results to simulate the *in vivo* situation. Implementation of a whole cell biosensor based on human cells in a three dimensional milieu and its application for screening of nanoparticle toxicity has not been investigated before. Therefore, the presented novel biosensor may be applied to a wide diversity of potentially hostile compounds in a basic screening to provide initial warning of adverse effects and trigger subsequent analysis and remedial actions.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

The authors thank Dr. Günter Brader from Austrian Institute of Technology GmbH in Tulln (Austria) for valuable discussions and advice in quantitative PCR and transfection of HepG2 cells. This study was financed by the European Union (SAMOSS, FP7-PEOPLE-2013-ITN).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tox.2016.09.012.

References

Banerjee, P., Bhunia, A.K., 2009. Mammalian cell-based biosensors for pathogens and toxins. Trends Biotechnol. 27 (3), 179–188.
Bellezzo, J.M., Britton, R.S., Bacon, B.R., Fox, E.S., 1996. LPS-mediated NF-kappa beta activation in rat Kupffer cells can be induced independently of CD14. Am. J. Physiol. 270 (6), G956–G961.
Bennett, M., Shern, J., Kahn, R., 2015. In: Meyerkord, C.L., Fu, H. (Eds.), Reverse Two-Hybrid Techniques in the Yeast Saccharomyces Cerevisiae. Protein–Protein Interactions, vol. 1278. Springer, New York, pp. 433–446.
Berger, J., Hauber, J., Hauber, R., Geiger, R., Cullen, B.R., 1988. Secreted placental alkaline phosphatase: a powerful new quantitative indicator of gene expression in eukaryotic cells. Gene 66 (1), 1–10.
Bokhari, M., Carnachan, R.J., Cameron, N.R., Przyborski, S.A., 2007. Novel cell culture device enabling three-dimensional cell growth and improved cell function. Biochem. Biophys. Res. Commun. 354 (4), 1095–1100.
Bonizzi, G., Karin, M., 2004. The two NF-κB activation pathways and their role in innate and adaptive immunity. Trends Immunol. 25 (6), 280–288.
Bottero, V., Imbert, V., Frelin, C., Formento, J.L., Peyron, J.F., 2003. Monitoring NF-kappa B transactivation potential via real-time PCR quantification of I kappa B-alpha gene expression. Mol. Diagn. 7 (3–4), 187–194.
Bouwmeester, T., Bauch, A., Ruffner, H., Angrand, P., Bergamini, G., Croughton, K., Cruciat, C., Eberhard, D., Gagneur, J., Ghidelli, S., Hopf, C., Huhse, B., Mangano, R., Michon, A., Schirle, M., Schlegl, J., Schwab, M., Stein, M.A., Bauer, A., Casari, G., Drewes, G., Gavin, A.C., Jackson, D.B., Joberty, G., Neubauer, G., Rick, J., Kuster, B., Superti-Furga, G., 2004. A physical and functional map of the human TNF-[alpha]/NF-[kappa]B signal transduction pathway. Nat. Cell Biol. 6 (2), 97–105.
Bulich, A.A., Isenberg, D.L., 1981. Use of the luminescent bacterial system for the rapid assessment of aquatic toxicity. ISA Trans. 20 (1), 29–33.
Chen, P., Kanehira, K., Taniguchi, A., 2013. Role of toll-like receptors 3, 4 and 7 in cellular uptake and response to titanium dioxide nanoparticles. Sci. Technol. Adv. Mater. 14 (1), 015008.
Chen, J., Zhang, J., Cao, J., Xia, Z., Gan, J., 2016. Inflammatory MAPK and NF-(B signaling pathways differentiated hepatitis potential of two agglomerated titanium dioxide particles. J. Hazard. Mater. 304, 370–378.
Chen, P., 2012. Detection of DNA damage response caused by different forms of titanium dioxide nanoparticles using sensor cells. J. Biosens. Bioelectron. 03 (05) .
Chouteau, C., Dzyadevych, S., Chovelon, J.M., Durrieu, C., 2004. Development of novel conductometric biosensors based on immobilised whole cell Chlorella vulgaris microalgae. Biosens. Bioelectron. 19 (9), 1089–1096.
Chouteau, C., Dzyadevych, S., Durrieu, C., Chovelon, J.M., 2005. A bi-enzymatic whole cell conductometric biosensor for heavy metal ions and pesticides detection in water samples. Biosens. Bioelectron. 21 (2), 273–281.
Cullen, B.R., Malim, M.H., 1992. Secreted placental alkaline phosphatase as a eukaryotic reporter gene. Methods Enzymol. 216, 362–368.
Dean, R.L., 2002. Kinetic studies with alkaline phosphatase in the presence and absence of inhibitors and divalent cations. Biochem. Mol. Biol. Educ. 30 (6), 401–407.
Dubiak-Szepietowska, M., Karczmarczyk, A., Jönsson-Niedziółka, M., Winckler, T., Feller, K.H., 2016. Development of complex-shaped liver multicellular spheroids as a human-based model for nanoparticle toxicity assessment in vitro. Toxicol. Appl. Pharmacol. 294, 78–85.
Dusinska, M., Boland, S., Saunders, M., Juillerat-Jeanneret, L., Tran, L., Pojana, G., Marcomini, A., Volkovova, K., Tulinska, J., Knudsen, L.E., Gombau, L., Whelan, M., Collins, A.R., Marano, F., Housiadas, C., Bilanicova, D., Halamoda Kenzaoui, B., Correia Carreira, S., Magdolenova, Z., Fjellsbø, L.M., Huk, A., Handy, R., Walker, L., Barancokova, M., Bartonova, A., Burello, E., Castell, J., Cowie, H., Drlickova, M., Guadagnini, R., Harris, G., Harju, M., Heimstad, E.S., Hurbankova, M., Kazimirova, A., Kovacikova, Z., Kuricova, M., Liskova, A., Milcamps, A., Neubauerova, E., Palosaari, T., Papazafiri, P., Pilou, M., Poulsen, M.S., Ross, B., Runden-Pran, E., Sebekova, K., Staruchova, M., Vallotto, D., Worth, A., 2015. Towards an alternative testing strategy for nanomaterials used in nanomedicine: lessons from NanoTEST. Nanotoxicology 9, 118–132.
El-Said, K.S., Ali, E.M., Kanehira, K., Taniguchi, A., 2014. Molecular mechanism of DNA damage induced by titanium dioxide nanoparticles in toll-like receptor 3 or 4 expressing human hepatocarcinoma cell lines. J. Nanobiotechnol. 12, 48.
Eom, H.-J., Choi, J., 2010. P38 MAPK activation, DNA, damage, cell cycle arrest and apoptosis as mechanisms of toxicity of silver nanoparticles in jurkat T cells. Environ. Sci. Technol. 44 (21), 8337–8342.
Fang, Z., Kuang, X., Zhang, Y., Shi, P., Huang, Z., 2015. A novel HAC1-based dual-luciferase reporter vector for detecting endoplasmic reticulum stress and unfolded protein response in yeast Saccharomyces cerevisiae. Plasmid 79, 48–53.
Ghosh, S., Karin, M., 2002. Missing pieces in the NF-kappaB puzzle. Cell Adhes. Migr. 109 (0092-8674 (Print)).
Guillouzo, A., Corlu, A., Aninat, C., Glaise, D., Morel, F., Guguen-Guillouzo, C., 2007. The human hepatoma HepaRG cells: a highly differentiated model for studies of liver metabolism and toxicity of xenobiotics. Chem. Biol. Interact. 168 (1), 66–73.
Guo, L., Dial, S., Shi, L., Branham, W., Liu, J., Fang, J.-L., Green, B., Deng, H., Kaput, J., Ning, B., 2011. Similarities and differences in the expression of drug-metabolizing enzymes between human hepatic cell lines and primary human hepatocytes. Drug Metab. Dispos. 39 (3), 528–538.
Hayden, M.S., Ghosh, S., 2008. Shared principles in NF-kappaB signaling. Cell 132 (3), 344–362.
He, X., Young, S.-H., Schwegler-Berry, D., Chisholm, W.P., Fernback, J.E., Ma, Q., 2011. Multiwalled carbon nanotubes induce a fibrogenic response by stimulating reactive oxygen species production, activating NF-κB signaling, and promoting fibroblast-to-myofibroblast transformation. Chem. Res. Toxicol. 24 (12), 2237–2248.
Hofmann, U., Michaelis, S., Winckler, T., Wegener, J., Feller, K.H., 2013. A whole-cell biosensor as in vitro alternative to skin irritation tests. Biosens. Bioelectron. 39 (1), 156–162.
Hofmann, U., Priem, M., Bartzsch, C., Winckler, T., Feller, K.H., 2014. A sensitive sensor cell line for the detection of oxidative stress responses in cultured human keratinocytes. Sensors (Basel) 14 (7), 11293–11307.
Hyun-Jeong Eom, J.C., 2009. Oxidative stress of CeO2 nanoparticles via p38-Nrf-2 signaling pathway in human bronchial epithelial cell, beas-2B. Toxicol. Lett. 187 (2), 77–83.
Karin, M., Lin, A., 2002. NF-kappaB at the crossroads of life and death. Nat. Immunol. 3 (3), 221–227.
King, K.R., Wang, S., Irimia, D., Jayaraman, A., Toner, M., Yarmush, M.L., 2007. A high-throughput microfluidic real-time gene expression living cell array. Lab Chip 7 (1), 77–85.
Kroll, A., Pillukat, M.H., Hahn, D., Schnekenburger, J., 2012. Interference of engineered nanoparticles with in vitro toxicity assays. Arch. Toxicol. 86 (7), 1123–1136.
Lan, S.-F., Safiejko-Mroczka, B., Starly, B., 2010. Long-term cultivation of HepG2 liver cells encapsulated in alginate hydrogels: a study of cell viability, morphology and drug metabolism. Toxicol. In Vitro 24 (4), 1314–1323.
Li, C.L., Tian, T., Nan, K.J., Zhao, N., Guo, Y.H., Cui, J., Wang, J., Zhang, W.G., 2008. Survival advantages of multicellular spheroids vs. monolayers of HepG2 cells in vitro. Oncol. Rep. 20 (6), 1465–1471.
Liu, X., Sun, J., 2010. Endothelial cells dysfunction induced by silica nanoparticles through oxidative stress via JNK/P53 and NF-κB pathways. Biomaterials 31 (32), 8198–8209.
Liu, F., Nordin, A.N., Li, F., Voiculescu, I., 2014. A lab-on-chip cell-based biosensor for label-free sensing of water toxicants. Lab Chip 14 (7), 1270–1280.
Millan, J.L., 1986. Molecular cloning and sequence analysis of human placental alkaline phosphatase. J. Biol. Chem. 261, 4023.
Nakashima, K., Shibasaki-Kitakawa, N., Miyamoto, T., Kubo, M., Yonemoto, T., Shuler, M.L., 2013. Production of human secreted alkaline phosphatase in suspension and immobilization cultures of tobacco NT1 cell. Biochem. Eng. J. 77, 177–182.
Nishanth, R.P., Jyotsna, R.G., Schlager, J.J., Hussain, S.M., Reddanna, P., 2011. Inflammatory responses of RAW 264.7 macrophages upon exposure to nanoparticles: role of ROS-NF-κB signaling pathway. Nanotoxicology 5 (4), 502–516.
Oeckinghaus, A., Ghosh, S., 2009. The NF-kappaB family of transcription factors and its regulation. Cold Spring Harb. Perspect. Biol. 1 (4), a000034.
Ong, K.J., MacCormack, T.J., Clark, R.J., Ede, J.D., Ortega, V.A., Felix, L.C., Dang, M.K., Ma, G., Fenniri, H., Veinot, J.G., Goss, G.G., 2014. Widespread nanoparticle-assay interference: implications for nanotoxicity testing. PLoS One 9 (3), e90650.
Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Res. 29 (9), e45.
Prasad, R.Y., McGee, J.K., Killius, M.G., Suarez, D.A., Blackman, C.F., DeMarini, D.M., Simmons, S.O., 2013. Investigating oxidative stress and inflammatory responses elicited by silver nanoparticles using high-throughput reporter genes in HepG2 cells: effect of size, surface coating, and intracellular uptake. Toxicol. In Vitro 27 (6), 2013–2021.
Pruksakorn, D., Lirdprapamongkol, K., Chokchaichamnankit, D., Subhasitanont, P., Chiablaem, K., Svasti, J., Srisomsap, C., 2010. Metabolic alteration of HepG2 in scaffold-based 3-D culture: proteomic approach. Proteomics 10 (21), 3896–3904.
Schütze, S., Potthoff, K., Machleidt, T., Berkovic, D., Wiegmann, K., Krönke, M., 1992. TNF activates NF-κB by phosphatidylcholine-specific phospholipase C-induced Acidic sphingomyelin breakdown. Cell 71 (5), 765–776.
Shing, W.L., Heng, L.Y., Surif, S., 2013. Performance of a cyanobacteria whole cell-based fluorescence biosensor for heavy metal and pesticide detection. Sensors (Basel) 13 (5), 6394–6404.
Singh, J., Mittal, S.K., 2012. Chlorella sp. based biosensor for selective determination of mercury in presence of silver ions. Sens. Actuators. B 165 (1), 48–52.

Sniadecki, N.J., 2010. Minireview: a tiny touch: activation of cell signaling pathways with magnetic nanoparticles. Endocrinology 151 (2), 451–457.
Soboleski, M.R., Oaks, J., Halford, W.P., 2005. Green fluorescent protein is a quantitative reporter of gene expression in individual eukaryotic cells. FASEB J. 19 (3), 440–442.
Stepkowski, T.M., Brzoska, K., Kruszewski, M., 2014. Silver nanoparticles induced changes in the expression of NF-kappaB related genes are cell type specific and related to the basal activity of NF-kappaB. Toxicol. In Vitro 28 (4), 473–478.
Taniguchi, A., 2010. Live cell-based sensor cells. Biomaterials 31 (23), 5911–5915.
Vopálenská, I., Váchová, L., Palková, Z., 2015. New biosensor for detection of copper ions in water based on immobilized genetically modified yeast cells. Biosens. Bioelectron. 72, 160–167.
Wang, B., Buck, M., 2012. Customizing cell signaling using engineered genetic logic circuits. Trends Microbiol. 20 (8), 376–384.
Wang, T.H., Hui, G.H., Deng, S.P., 2010. A novel sweet taste cell-based sensor. Biosens. Bioelectron. 26 (2), 929–934.
Wilkening, S., Stahl, F., Bader, A., 2003. Comparison of primary human hepatocytes and hepatoma cell line Hepg2 with regard to their biotransformation properties. Drug Metab. Dispos. 31 (8), 1035–1042.
Xing, J.Z., Zhu, L., Gabos, S., Xie, L., 2006. Microelectronic cell sensor assay for detection of cytotoxicity and prediction of acute toxicity. Toxicol. In Vitro 20 (6), 995–1004.
Ye, S.-F., Wu, Y.-H., Hou, Z.-Q., Zhang, Q.-Q., 2009. ROS and NF-κB are involved in upregulation of IL-8 in A549 cells exposed to multi-walled carbon nanotubes. Biochem. Biophys. Res. Commun. 379 (2), 643–648.
Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., Madden, T., 2012. Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. BMC Bioinf. 13, 134.