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Integrated optical biosensor for in-line monitoring of cell cultures

Stéphanie Pasche*, Bernard Wenger, Réal Ischer, Marta Giazzon, Silvia Angeloni, Guy Voirin

CSEM (Centre Suisse d'Electronique et de Microtechnique) SA, Jaquet-Droz 1, Case Postale, CH-2002 Neuchâtel, Switzerland

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ABSTRACT

An analytical detection platform was developed to evaluate the induced toxicity in cell cultures exposed to foreign agents like growth factors or nanoparticles. Connecting a biosensing detection device to the cell culture flasks allows analyzing the composition of cell medium in real-time. The analysis relies on the quantification of inflammatory cytokines released by cells into the cell culture medium, by means of solid-phase immunoassays analyzed with the wavelength interrogated optical sensing (WIOS) instrument. A fluidic system for in situ measurements allows detecting cytokines in real-time, with a sensitivity of 1–100 ng/mL depending on the cytokine. In addition, integration of an in-line optical absorbance measurement unit, in combination with the standard AB cell proliferation assay, provides information on the cell viability in the culture. Fluidic connections between the cell culture flasks, the optical biosensor and the absorbance measurement unit simultaneously allow quantifying up to three cytokines (interleukin 8, interleukin 6 and the monocyte chemotactic protein), assessing cellular proliferation, and thus discriminating between naïve cells and cells exposed to foreign agents such as growth factors (tumor necrosis factor alpha) or nanoparticles. This analytical tool presents a high potential for assessing the cytotoxicity of nanoparticles and other chemicals in vitro.

1. Introduction

The increased presence of nanotechnology based materials in commercial products has brought up a need for setting up reliable methods to assess their potential toxicity with short deadlines and reasonable costs (Dobrovolskaia and McNeil, 2007). Whereas most reliable methods for toxicity evaluation rely on costly, in vivo experiments, in vitro assays present a promising screening method, in particular for the study of the toxicity of nanomaterials, defined as "nanotoxicity" (Sayes et al., 2007). Cell cultures offer a useful prescreening method to assess the toxicity of various external agents on cells (Hediger et al., 2000; Ressler et al., 2004). Most current in vitro cytotoxicity assays rely on the evaluation of cell viability, based on cell proliferation assays such as the AlamarBlue™ (AB) assay on cell metabolism changes such as the MTT (mitochondrial activity) or Neutral Red (detection of intact lysosomes), on the lactate dehydrogenase (LDH) assay for necrosis, or on the detection of apoptotic markers (Annexin V, Caspase-3) (Davoren et al., 2006; Kroll et al., 2009).

Alternatively, the cellular stress and inflammatory responses are used to evaluate cellular toxicity, and can be investigated with the detection of specific biological markers (Kroll et al., 2009). In particular, the release of cytokines by cells has been studied as marker of the cellular immune responses (Duschl, 2007; Lamoureux and Bradley, 2007; Pfaller et al., 2009). Cytokines are small (8–35 kDa), soluble polypeptides or glycoproteins that are mostly produced from white blood cells such as macrophages and monocytes, but can also be released by endothelial cells, keratinocytes and fibroblasts. Cytokines regulate the growth and function of immune cells during inflammation and in later immune responses (Lamoureux and Bradley, 2007; Pfäfflin and Schleicher, 2009). Therefore, the concentration of cytokines in medium, typically below 10 ng/mL, will increase in response to inflammation. Interleukin 8 (IL-8), interleukin 6 (IL-6) and the monocyte chemotactic protein-1 (MCP-1) are small cytokines involved as inflammatory markers (Wottrich et al., 2004). IL-8 (MW 8.5 kDa) exists in multiple isoforms (72 or 77 amino acids), and appears to form a homodimer in solution. IL-8 possesses chemotactic properties and thus belongs to the chemokine subclass of cytokines. The release of IL-8 is stimulated by other cytokines, such as IL-1α or IL-1β, or the tumor necrosis factor alpha (TNF-α), and reaches a peak concentration after 6–24 h. IL-6 (MW 21 kDa) – a 186 amino acid N-glycosylated monomer – is produced by a wide variety of cell types and can be induced by IL-1 and TNF-α. MCP-1 (MW 8.6 kDa) is a chemokine that promotes the migration of a large number of cell types to the injured site.

In vitro detection of secreted cytokines often relies on immunoassays, which are based on cytokine capture by a specific antibody, measuring cytokine levels rather than their bioactivity

* Corresponding author at: CSEM (Centre Suisse d'Electronique et de Microtechnique) SA, Department of Nanotechnology & Life Sciences, Jaquet-Droz 1, Case Postale, CH-2002 Neuchâtel, Switzerland. Tel.: +41 32 720 5540; fax: +41 32 720 5740.
E-mail address: stephanie.pasche@csem.ch (S. Pasche).

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(Whiteside, 2005). A sandwich immunoassay format has been used for the detection of IL-8, IL-6 or MCP-1 (Battaglia et al., 2005; Ida et al., 1992; Kajikawa et al., 1996; Kemmler et al., 2009; Liu et al., 2005; Pfäfflin and Schleicher, 2009; Tan et al., 2008; Yang et al., 2005). Sensitive detection of low levels of cytokines in biological medium can be achieved with enzyme-linked immunosorbent assays (ELISA), using suitable combinations of antibodies. However, ELISA tests are time consuming, and require multiple manipulations. Therefore, real-time detection of cytokines has been investigated with surface immobilized immunoassays detection on optical sensing instruments such as the surface plasmon resonance (SPR) or waveguide grating sensors (Battaglia et al., 2005; Kemmler et al., 2009; Yang et al., 2005). These techniques are sensitive to changes in the refractive index at the liquid–solid interface, and thus allow monitoring biomolecule adsorption at the sensor surface with high sensitivity (Cottier et al., 2006; Gauglitz, 2005; Homola et al., 1999). Combined with immunoassays, they allow the sensitive detection of cytokines. However, cytokines rarely act alone, and the assessment of the inflammatory response with cytokines requires multiplexed detection, providing a pattern of several involved cytokines (Kemmler et al., 2009; Liu et al., 2005; Negm et al., 2006; Nielsen and Geierstanger, 2004; Pfäfflin and Schleicher, 2009; Swartzman et al., 1999).

A label-free, optical detection device based on a waveguide grating coupling with wavelength modulation, the wavelength interrogated optical sensing (WIOS) instrument, was developed at CSEM (Cottier et al., 2003). Combining WIOS with immunoassays resulted in a sensitive and selective detection instrument to analyze traces of contaminants (Adrian et al., 2009a). In addition, the simultaneous detection of four analytes was demonstrated, opening up the sensor technology to multiplexing (Adrian et al., 2009b). Further, combining the instrument with adapted fluidics extended the use of WIOS for lab-on-chip applications (Suárez et al., 2009). We have previously shown that the WIOS technology, combined with a sandwich immunoassay, was suitable for the detection of IL-8, and allowed differentiating naïve from induced cells by analysis of the cell medium (Pasche et al., 2009). A significant advantage of the WIOS instrument over similar systems such as SPR or waveguide grating sensors is its ability to individually address eight functionalized zones for detecting biomolecule adsorption. This not only allows referencing the measurement to account for drift, but also detecting multiple cytokines in a single measurement. In addition, the device is small and easily fits on a bench top or in a cell incubator.

The scope of this work is to integrate the assay in a semi-automated system for monitoring cell cultures. Therefore, this study aims at the development of a tool to quantitatively assess the immune response of cells induced by foreign agents, such as nanoparticles, based on a preliminary study using ELISA (Pfaller et al., 2009). In-line monitoring of cellular stress in cell cultures relies on the detection of cytokines that are released by induced cells into the medium. The label-free, optical detection WIOS instrument is connected to the cell culture vials with fluidic channels, and allows analysis of the cell culture medium for cytokine content in real-time (Pasche et al., 2009). Detection relies on a sandwich immunoassay developed for the simultaneous detection of three cytokines, IL-8, IL-6 and MCP-1. In addition, an absorbance measurement unit in combination with the standard AB assay provides information on cell proliferation. The developed platform integrates the cell culture unit and two optical detection systems, with fluidic connections (Fig. 1). First results on the immune response of cells induced by rhTNF-α or by gold nanoparticles are presented to validate the platform as a tool to monitor cell cultures. This development offers a new tool for direct analysis in cell cultures. The prototype was developed within the European project DIPNA (http://dipna.eu), which aimed at developing an integrated

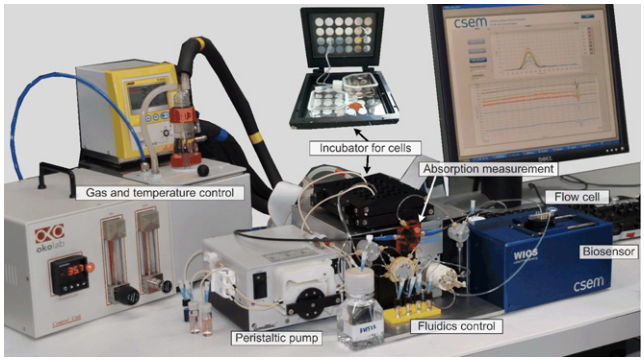

Fig. 1. The integrated platform allows in-line monitoring of cell cultures for the assessment of cellular toxicity of external agents. The elements of the platform comprise the cell culture unit, with its incubator (inset: measurement and reference cell culture flasks in incubator), the absorbance flow cell for cell proliferation measurement with AB, and the WIOS device with associated fluidics for cytokine detection with the immunoassay.

platform for the in vitro investigation of nanoparticle toxicity. A screening method for nanoparticle toxicity reduces the number of costly in vivo experiments and impacts favorably on occupational health safety.

2. Materials and methods

2.1. Chemicals

Chemicals, unless specified, were obtained from Sigma–Aldrich (Switzerland). Phosphate buffered saline (10 mM phosphate, 138 mM NaCl, 2 mM KCl), pH 7.4, was used as dilution buffer for the immunoassay. IL-8, IL-6 and MCP-1 recombinant human cytokines, as well as rhTNF-α were supplied by ImmunoTools (Germany). Human IL-8 (clone JK8-1, mouse IgG), human IL-6 (clone MQ2-13A5, mouse IgG) and human MCP-1 (clone 2H5, Armenian Hamster IgG) antibodies were used for capture in the immunoassays and supplied by BioLegend (USA). Polyclonal antibodies (rabbit IgG) used for detection were supplied by Peprotech (USA). Rabbit specific IgG (α-rIgG) used as secondary antibody was supplied by JacksonImmunoResearch (UK).

2.2. Immunoassay

Sandwich immunoassays were used for the sensitive detection of the three selected cytokines (IL-8, IL-6, MCP-1). The immunoassays were monitored with the wavelength interrogated optical sensing (WIOS) instrument (developed at CSEM SA, now commercialized at Dynetix AG, Switzerland). WIOS uses the evanescent field to probe changes in the refractive index at the surface of a waveguide grating upon adsorption of biomolecules (Cottier et al., 2003). Screening of the resonance wavelength allows real-time monitoring of the binding of unlabeled molecules on the waveguide grating surface, with a sensitivity of ~1 ng/cm^2. Tantalum oxide-coated, 17.8 mm × 17.8 mm glass waveguide chips were supplied by Oerlikon (Balzers, Liechtenstein). Each chip comprises three columns of eight waveguide grating pads, which can be addressed independently (Kunz and Cottier, 2006). The chips were functionalized with a photopolymerizable dextran layer (OptoDex®) by Arrayon Biotechnology SA (Neuchâtel, Switzerland). OptoDex® provides an adhesion layer for covalent binding of biomolecules, and at the same time prevents non-specific adsorption of biomolecules (Barie et al., 1998; Caelen et al., 2002). Each capture antibody is printed onto any of the eight distinct measurement zones on the chip using a NanoPlotter™ microdispenser (GeSim, Germany). The surface is exposed to

UV light for immobilization of the antibody on the chip. This enables simultaneous detection of several analytes. A flow cell that is placed on top of the waveguide for in situ measurements has been designed, and allows monitoring biomolecule adsorption in real-time. The flow cell comprises three independent fluidic channels (one for each column of the waveguide's grating pads) delimited with a soft polydimethoxysiloxane (PDMS) gasket.

For immunoassay, cell supernatant samples or calibration standards (cytokines diluted in cell medium) were flowed on top of the waveguide for 20 min in the stop-flow mode (multiple injection steps). After rinsing with PBS, detection antibodies were injected in the flow cell (20 μg/mL) for 20 min. After rinsing with PBS, α-rIgG (50 μg/mL) was injected in the flow cell for 20 min as secondary antibody, in order to amplify the response signal. The variation of the WIOS signal before and after incubation with α-rIgG was reported for the different samples. The active surface was regenerated after the immunoassay, with 5 min exposure to 3 M $MgCl_2$, 0.75 M HEPES containing 25% ethylene glycol (pH 3), thus enabling sequential measurements with a single chip (Ben-David and Firer, 1996).

2.3. Cell culture

A549 human bronchial epithelial cells (ATCC, LGC Promochem, Germany) were cultured in RPMI 1640, supplemented with L-glutamine (1.5 mM), penicillin (50 units/mL)/streptomycin (50 μg/mL), and 10% FCS (PAA Laboratories, Pasching, Austria), in 12.5 cm^2 BD Falcon™ cell culture flasks, seeding 250,000 or 400,000 cells per flask (5 mL). A549 cells produce a range of cytokines and chemokines upon suitable stimulation (Oostingh et al., 2008). The flasks were placed in a Basic WJ CO2 Microscope Stage Incubator from OKOLAB (Italy), which was connected to a gas and temperature control units. Cells were cultured at 37 °C and 5% CO_2. A549 cells were induced with 0, 20 or 300 ng/mL rhTNF-α, simulating the acute phase reaction. To evaluate the effect of nanoparticles on cells, 10 nm gold nanoparticles were added to the cell culture, at a concentration of 2.17×10^{10} particles/mL (36 pM). The nanoparticles were produced and supplied by the Institut Català de Nanotecnologia and Institut Català de Recerca I Estudi Avançats (Barcelona, Spain), in 2.2 mM sodium citrate. The effect of solvent without nanoparticles was also evaluated. Samples of the supernatant solution were collected after 6, 24 and 48 h for analysis.

2.4. Cell proliferation AlamarBlue™ (AB) assay

The AB assay incorporates a fluorometric and colorimetric cell proliferation indicator based on detection of metabolic activity (US Patent No. 5,501,959). The assay is designed to quantitatively measure the proliferation of various human and animal cell lines, bacteria and fungi, directly in the cell medium. The metabolic activity of growing cells results in the chemical reduction of AB, which causes a water-soluble oxidation–reduction (redox) indicator to change from an oxidized (nonfluorescent, blue (λ_{ox} = 600 nm)) form to a reduced (fluorescent, red (λ_{red} = 570 nm)) form. The AB assay is not destructive for cells, and was reported to be nontoxic for most cells. AB reagents were obtained from Invitrogen (CA, USA), and were added to the cell culture with a 1:10 volume ratio. For control measurements in cell cultures, absorbance data were collected after 6 h incubation, on a Tecan plate reader (Infinite M200). Absorbance was monitored at 570 and 600 nm. The percentage of AB reduction was calculated using Eq. (1) for the control assays (US Patent No. 5,501,959). The integrated device does not yet allow correcting for reference absorbance data, and thus displays a value corresponding to the ratio between the signals of two LEDs. Therefore, the ratio of the absorbance at 570–600 nm was considered in control measurements for comparison (Eq. (2)).

$$\%\text{Reduced} = \frac{\varepsilon_{ox}(\lambda_2)\cdot A(\lambda_1) - \varepsilon_{ox}(\lambda_1)\cdot A(\lambda_2)}{\varepsilon_{red}(\lambda_1)\cdot A'(\lambda_2) - \varepsilon_{red}(\lambda_2)\cdot A'(\lambda_1)} \cdot 100\% \quad (1)$$

$$\text{Ratio} = \frac{A(\lambda_1)}{A(\lambda_2)} \quad (2)$$

$A(\lambda_1)$, $A(\lambda_2)$, $A'(\lambda_1)$ and $A'(\lambda_2)$ are the absorbance values for the sample and for the reference at λ_1 = 570 nm and λ_2 = 600 nm, respectively. The extinction coefficients at λ_1 = 570 nm and at λ_2 = 600 nm are $\varepsilon_{ox}(\lambda_1) = 80{,}586\ M^{-1}\ cm^{-1}$, $\varepsilon_{ox}(\lambda_2) = 117{,}216\ M^{-1}\ cm^{-1}$, $\varepsilon_{red}(\lambda_1) = 155{,}677\ M^{-1}\ cm^{-1}$, and $\varepsilon_{red}(\lambda_2) = 14{,}652\ M^{-1}\ cm^{-1}$ for the oxidized and reduced form, respectively.

2.5. Instrument integration

For in-line cytotoxicity monitoring, a contaminated and a reference cell culture flasks were connected to the flow cell for proliferation measurement (AB), and to the WIOS instrument for cytokine detection (immunoassay), using syringe and peristaltic pumps, valves, connectors and Tygon™ (Upchurch Scientific) fluidic tubes for connections. The whole system comprises a cell culture unit, with supply of reagents to the cell culture, a cell proliferation measurement unit, with absorbance measurement, and a cytokine detection unit, with the WIOS instrument and fluidics for handling of detection reagents (Fig. 1). The fluidics and detection elements were connected to a data acquisition card (PCI-6024E, National Instrument). A program written in LabVIEW graphical language allowed controlling the fluidic pumps and valves, as well as the light absorption and WIOS detection systems. The LabVIEW interface is also used for recording experimental data as a function of time.

A basic WJ CO2 Microscope Stage Incubator from OKOLAB (Italy) was used for the cell culture. Temperature in the incubator was maintained with water circulating in jackets in the incubator plate, directly measured inside a reference flask and controlled by a software PID controller. CO_2 was mixed with air and supplied to the incubating chamber. Two cell culture chambers were placed in the incubator (inset Fig. 1): one to induce the cell culture with test agents, the other as control for reference. The caps of the cell culture flasks were replaced with silicone caps modified with syringe needles connected to Tygon™ tubes for liquid delivery or sampling. An aperture for air was protected with a 0.2 μm pore syringe filter (Millipore GV), to avoid contamination of the cell culture. Sterility was guaranteed by autoclaving all materials in contact with the cell culture, and maintaining cell culture media, tubings and culture flasks in a sterile environment. A peristaltic pump (Ismatec 4 channels) was used to deliver medium, toxic agents or AB into the cell culture, using Tygon™ fluidic tubes and 0.2 μm pore filters (Millipore GV). This design is compatible with the adaptation of a field-flow chamber for the delivery of contaminant from the environment to the cell culture. For analysis, cell medium was sampled from each cell culture flask using a linear CAVRO XC30 syringe pump (Tecan), through a CAVRO 6-port Smart Valve (Tecan) connected in series to a VICI C22 6-port/2 position injection valve, and delivered to the absorbance flow cell and to the WIOS analytical device. Reagents needed for the immunoassay were also delivered via the same valves. Samples and reagents were loaded via a 50 μL PEEK tube loop, using another syringe pump (CAVRO XC30, Tecan). The cell proliferation measurement was placed after the loop, allowing the analysis of cell medium after loading the loop. The device consists of an absorption flow cell chamber (FIAlab instrument FIA-Z-SMA-ULT), with an optical path length of 20 mm and a volume of 120 μL, a light source combining two LEDs from a tri-LED

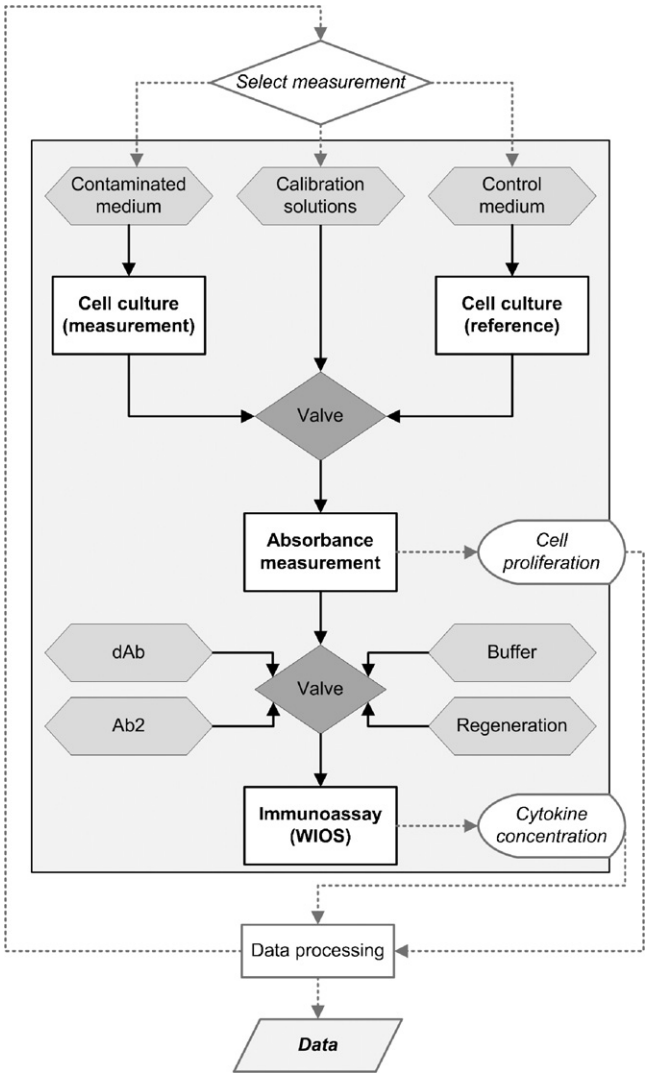

Fig. 2. Flow chart describing the sequence of operations of the system. A measurement cycle comprises selection of the sample (calibration solution, measurement or reference cell chamber), absorption measurement, immunoassay, and data processing. The cycle is repeated over the experiment time, switching between sample solutions. A system of fluidic pumps and valves allows dragging cell medium from the cell culture flask to the analytic devices, as well as distributing the detection reagents.

LATBg66b (Osram) – λ_1 = 528 nm (FWHM = 33 nm) and λ_2 = 617 nm (FWHM = 18 nm) – a photodiode for detection (SFH203, Siemens), and a home-designed electronic printed circuit board. The flow cell is connected to optical fibers to deliver and collect the transmitted light. Data is transferred to the computer through the same acquisition board used for the WIOS instrument, and the ratio between both LED signals (λ_1/λ_2) is recorded.

2.6. In-line measurement in cell cultures

A flow chart (Fig. 2) describes the sequence of operations of the system. A549 cell cultures were prepared in 12.5 cm^2 BD Falcon™ cell culture flasks (250,000 or 400,000 cells per flask in 5 mL complete medium) and placed in the portable incubator. Cells at early passages (four to eight) were used for each new experiment series. 20 or 300 ng/mL rhTNF-α was injected in one flask (sample), and the other cell culture was kept as reference (negative control). Both cell culture flasks were connected to the detection modules with an autoclaved, modified silicone cap integrating fluidic tubes. The cell culture medium was analyzed at different times (after 6, 24 and 48 h), sampling between 100 and 500 μL of medium from the culture, at a flow rate of 175 μL/min. The medium was replaced with fresh medium, using the peristaltic pump directly connected to the vials at a flow rate of 500 μL/min.

The presence of cytokines in the medium was analyzed with the WIOS immunoassay platform. IL-8, IL-6 and MCP-1 concentrations in the cell culture were quantified by comparing the response obtained in the samples with that of calibration standard solutions. The flow rate for immunoassays was set to 25 μL/min. The pumps and valves allowed alternating injection of sample solutions, reagents, regeneration solution and buffer. For injection, 100 μL of the selected solution was sampled to load the 50 μL loop of the injection valve at a flow rate of 175 μL/min. Switching the position of the valve from "loading" to "injection" mode, buffer was flowed through the fluidic system with a flow rate of 25 μL/min, driving the solution to the WIOS analysis chamber. Thus, switching the position of the 6-port valve allowed injecting 50 μL from each of the reservoirs. Injection of cell medium and detection reagents was performed in a "stop-flow" mode, renewing the WIOS chamber every 2 min with 8 μL of new solution. The regeneration solution was injected in a continuous flow mode.

Cell proliferation was evaluated after injecting 500 μL AB reagent into both the reference and the contaminated cell culture. After 6 h incubation, 500 μL of cell culture medium containing AB was pumped out through the valves at a flow rate of 175 μL/min, filling up the loop and, subsequently, the light absorption chamber. Absorption was measured at 570 and 600 nm, using two LEDs and a photodiode. The absorption signals were recorded on the computer, using LabVIEW.

3. Results and discussion

3.1. Immunoassay for the detection of cytokines

Simultaneous detection of three cytokines, IL-8, IL-6 and MCP-1, was achieved with the WIOS instrument, using a chip comprising different zones independently functionalized with the corresponding capture antibodies (Pasche et al., 2009). Monitoring of the immunoassay for cytokines consisted in (1) adsorption of the target cytokines on the immobilized capture antibodies, (2) recognition by the detection antibodies (dAb) – mixture of the three detection antibodies, α-IL8, α-IL6 and α-MCP1 – and (3) amplification with the secondary antibody (Ab2), α-rIgG. Finally, regeneration of the surface (4) allowed desorbing all adsorbed species, while maintaining a high number of active capture antibodies on the chip surface (Fig. 3a). Each regeneration cycle led to a loss of about 10% in WIOS signal, which could be accounted for serial experiments. The response signals for different cytokine concentrations in cell culture medium measured with WIOS allowed calibration for each cytokine (Fig. 3b). No cross-reactivity was observed between the different species, and the response signal for a mixture containing the three cytokines was comparable to each signal acquired independently (Pasche et al., 2009). The detection sensitivity varied for the three cytokines (1, 10 and 100 ng/mL for IL-8, IL-6 and MCP-1, respectively), due to differences in the affinities of the immunoreagents. The response signals obtained with cell culture supernatant samples from naïve and rhTNF-α-induced cells, as well as from cells exposed to gold nanoparticle, were compared to calibrated signals (Fig. 3b). Whereas clear distinction is observed for cells induced with both concentrations of rhTNF-α, in comparison with control cells, no particular release of any of the cytokines was detected in cells exposed to gold nanoparticles. This effect confirms what has already been demonstrated in another study (Pfaller et al., 2009).

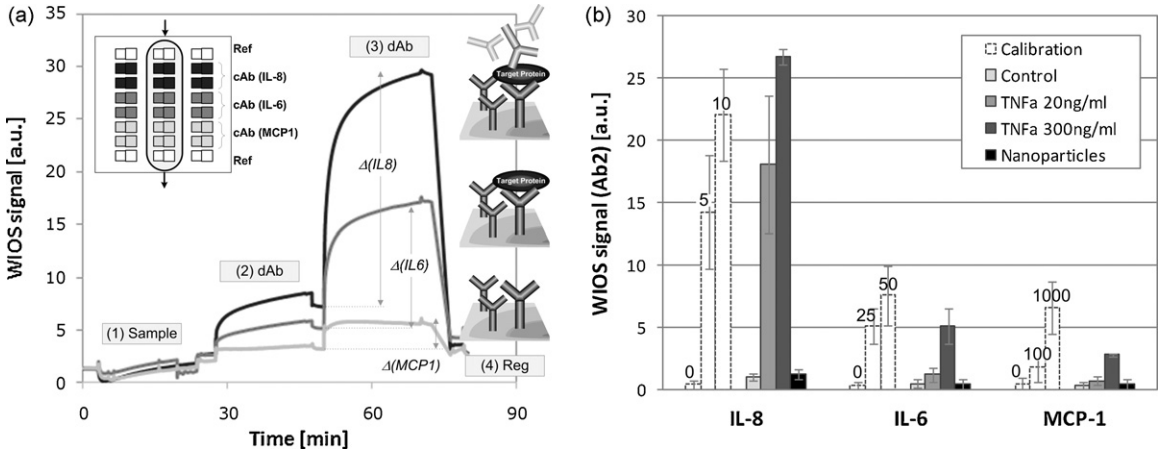

Fig. 3. (a) Immunoassay binding curve for the parallel detection of IL-8 (10 ng/mL), IL-6 (100 ng/mL) and MCP-1 (200 ng/mL), measured with the WIOS instrument, including incubation in cell medium (1), followed by exposure to the detection (dAb) (2) and, subsequently to the secondary antibody (Ab2) (3) and regeneration (4). Inset: schematic design of the optical chip for the immunoassay. (b) WIOS signal related to Ab2 obtained from cell cultures exposed to 0 (control), 20 and 300 ng/mL rhTNF-α, and to 10 nm gold nanoparticles, after 48 h, compared to calibration for 0–5–10 ng/mL IL-8, 0–25–50 ng/mL IL-6 and 0–100–1000 ng/mL MCP-1 in cell culture medium. Error bars correspond to the standard deviation between two to five measurements.

3.2. Cell culture

The viability of the cells in the customized incubator was verified in a first stage using a cell culture in standard conditions as reference. The behavior of A549 cells in the portable incubator system was compared to the normal conditions for cell culture, and showed that cells were not influenced by the culture conditions used in the integrated system. The behavior of cells was not influenced by the system, as long as sterility could be guaranteed. Cell proliferation of A549 induced and naïve cells was assessed using the AB assay. Involving addition of a nontoxic reagent to the cell culture, the non-destructive AB assay is compatible with in-line measurement for cell cultures. Cell proliferation was assessed by measuring changes in absorbance of the cell culture medium containing AB. However, leaving the AB reagents in contact with the cells for 24 h resulted in retraction and detachment of the cells from the surface, indicating some induced toxic effect (not shown). Consequently, reagents were only added in the medium 6 h prior the proliferation measurement, and the medium was renewed after measurement. Preliminary measurements were performed after 6, 24 and 48 h of cell culture, monitoring the absorbance at 570 and 600 nm (Fig. 4a). The absorbance spectra allowed calculating the chemical reduction of AB, which is directly related to the proliferation rate of cells (as in standard AB protocols, US Patent No. 5,501,959). The percentage of reduction of AB was calculated from Eq. (1), and compared to the ratio between the absorbance at 570 and 600 nm (Eq. (2)). Both evaluation methods led to similar results, and are therefore suitable to assess cellular proliferation (Fig. 4b). The proliferation of naïve cells and of cells exposed to 10 nm gold nanoparticles was compared over a period of 48 h. Cell proliferation seemed not to be affected for the first 24 h, but the presence of nanoparticles started to interfere with cells after 48 h of culture (Fig. 4b). Although the inflammatory response of cells did not seem to be influenced by the presence of gold nanoparticles in the medium, there seem to be an effect on cell proliferation. Therefore, the use of at least two analytical techniques is required to provide a more reliable picture of the toxicity induced by external agents on cells.

3.3. Cell culture monitoring

A prototype was developed for monitoring toxicity in cell cultures, based on the assembly of a cell culture unit and optical detection devices (Fig. 1). The breadboard system was designed with several functions: automated sampling in the cell culture, optical measurement of the cell proliferation with AB, and optical measurement of the concentration of cytokines (IL-8, IL-6, MCP-1) in a cell culture medium extract. The platform was developed to allow repeated analyses during the full extent of the cell culture period, and comparing the results with a reference cell culture flask, not exposed to any contaminant. Thus, fluidics including pumps and valves interfaced the cell culture with the analytical devices, allowing feeding and sampling of cell medium from and to the flask. The developed platform relies on standard laboratory equipment used for cell culture, fits on a bench, and can easily be transported (Fig. 1).

Assessment of cell toxicity in the system is based on cell proliferation and on the release of cytokines by cells. In this perspective, an absorbance measurement flow cell allowed cell proliferation measurement relying on the AB assay, monitoring the ratio of the signals of two LEDs (528 and 617 nm). For analyzing the release of cytokines by cells, the cell culture and reservoirs with immunoreagents were connected to the WIOS instrument. Both measurements were performed in the cell culture medium during the life of the cells, and did not interfere with each other. For the proliferation assay, the cells are not processed and only AB reagent is added to the cell culture. Relying on a sandwich immunoassay for cytokine monitoring eliminates any interference with the coloration of the medium, as the response signal is read in a subsequent step, performed in buffer. Also, matrix artifacts from the environment are removed by subtraction of the signal measured on a reference pad.

For testing the system, A549 cell cultures flasks with and without rhTNF-α were placed in the portable incubator, and connected to the detection modules with fluidic tubes. Cell medium was sampled at different times for analysis, over a cell culture period of 24–48 h. The concentration of cytokines in the medium was measured at different times; cell supernatant was pumped through the fluidic system to the WIOS flow cell after 6, 24 and 48 h of culture, where the sandwich immunoassay was monitored in real-time. The time for a complete immunoassay (including regeneration) was 90 min. Calibration was performed between sample measurements, with reference solutions containing known concentrations of IL-8, IL-6 or MCP-1 in cell culture medium. Repeated data logging allows recording the response signals for the sample and calibration solutions as a function of time (Fig. 2). An example of the mon-

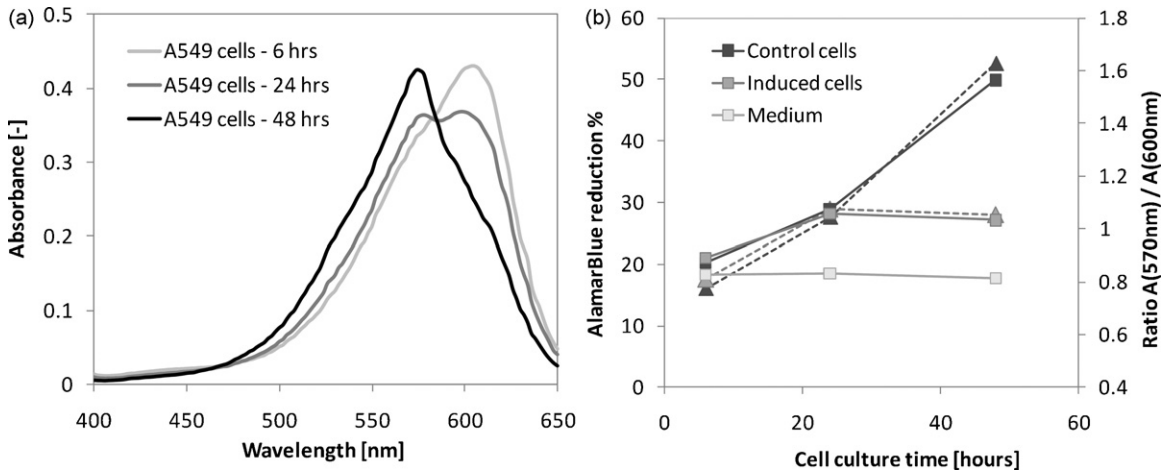

Fig. 4. Cell proliferation using the AlamarBlue™ assay. (a) Absorption spectra of the medium of A549 cell culture containing AB reagents, as a function of cell culture time (after 6, 24 and 48 h), showing a shift from the oxidized (λ_{ox} = 600 nm) to the reduced (λ_{red} = 570 nm) form of the indicator. (b) Monitoring of the chemical reduction of AB as a function of time, assessing the effect of 10 nm gold nanoparticles on cell proliferation. The standard reduction percentage calculated from Eq. (1) (dashed lines) is compared with the ratio of the absorbance at 570–600 nm (plain lines, Eq. (2)).

itoring of IL-8 is shown in Fig. 5, comparing the WIOS response signal related to the adsorption of the secondary antibody for each cell culture to an IL-8 standard (7 ng/mL). Thus, the WIOS signal measured for the samples can be directly compared to the calibration curve. The reported values were corrected for the loss due to regeneration, allowing for a 10% signal decrease in each cycle. Fig. 5 shows an increase in the release of IL-8 with culture time, and that cells induced with rhTNF-α release substantially more IL-8 than control cells, indicating an acute immune response of the induced cells.

Control and induced cells are shown in Fig. 6a and c, after 24 h of culture. At first sight, the induced cells seem to proliferate less than control cells. Quantitative assessment of cell proliferation was performed only at the end of the culture period, as the AB reagents seemed to be toxic for cells after 24 h exposure, and therefore did not lead to any reliable results. For instance, in Fig. 6d, AB was added to the cell culture after 18 h, and the medium was flowed after 24 h in the absorption flow cell. The ratio between the signals from the two LEDs (signal at λ_1 = 528 nm divided by the signal at λ_2 = 617 nm) provided qualitative information on the cell metabolic activity. The results confirmed that induced cells tend to proliferate less than control cells. In the case of induction of cells with rhTNF-α, the effect of induction was observed both on cell proliferation (AB absorbance), and on the increased release of cytokines by cells (immunoassay). However, cells exposed to gold nanoparticles only showed an effect on cell proliferation after 24 h, with no response from the selected cytokines. As a consequence the combination of two analytical techniques to assess cellular toxicity significantly improves the quality of the analysis, providing simultaneous information on cell viability and on the cellular immune response. This example confirms that multiplexed detection is required to provide a more accurate view of cellular toxicity. Not only different cytokines should be considered, but also various techniques reduce the uncertainty of cellular toxicity.

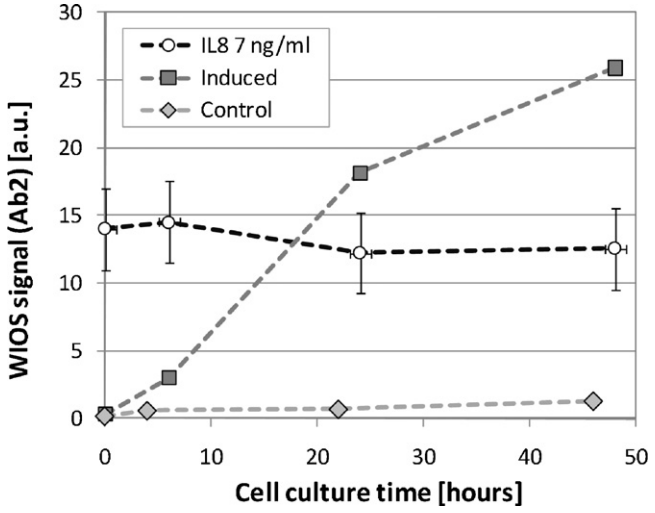

Fig. 5. Monitoring of the release of IL-8 by cells induced by 20 ng/mL rhTNF-α, compared to a reference cell culture (control), showing the WIOS signal related to Ab2 adsorption from the immunoassay. The WIOS response of cells is compared to the response of a calibration standard (7 ng/mL IL-8 in medium), measured between measurements. The signals were corrected to take into account the 10% signal decrease due to surface regeneration for each measurement cycle. Error bars calculated from the standard deviation on the same chip for 7 mg/mL IL-8 are displayed as indication, as single data points are represented on the graph.

This paper focused on the description of the developed platform, rather than providing information on the toxicity of nanoparticles on cells. The device is now available for deeper investigations of cellular toxicity, in particular to assess the toxic effect resulting from cell exposure to nanoparticles. The preliminary results presented here provide the basis for validating the integrated platform. Assessing cellular nanotoxicity requires deeper investigation, and possibly, new selection of cytokine markers. Nevertheless, a tool is now available for a systematic study to evaluate the effect of any external agent onto any adherent types of cells. In addition, 3D cell cultures could be easily implemented in the platform, as studies have shown that 3D cell cultures may present a more accurate model for in vitro toxicity assessment (Lee et al., 2009). Ultimately, the use of microfabricated chamber to better integrate cell culture, and combination with other, analytical techniques, e.g. based on electrical detection, would significantly improve the reliability of the actual platform (Hediger et al., 2000; Ressler et al., 2004). In its current state, the system thus benefits from considerable flexibility, as it relies on standard laboratory equipment, and can be combined with additional detection devices. Contamination in cell cultures is reduced in comparison with standard laboratory techniques used for quantifying biomarkers (e.g. ELISA), by relying on closed fluidic channels. Also, the shorter time per analysis (from 1 to 2 days to 2 h per analysis) in combination with programmable data acquisition allow real-time analysis. The platform thus brings in relevant

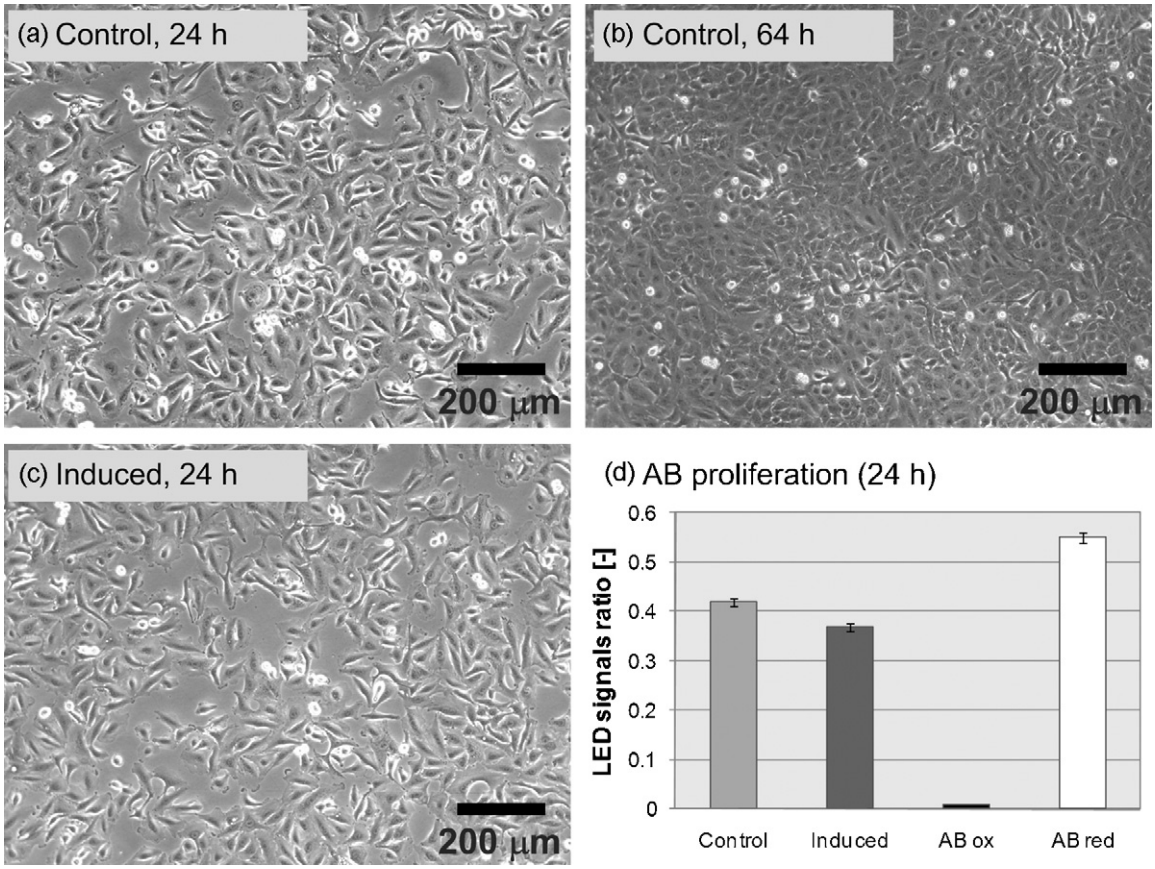

Fig. 6. Optical micrographs of A549 cells showing qualitative information on the cell viability in the customized cell incubator, after 24 h (a) and after 64 h (c) in culture, compared to cells induced with 20 ng/mL rhTNF-α (b). The proliferation of induced cells is slightly delayed. (d) Quantitative assessment of cell proliferation after 30 h, with the AB assay, calculated from the ratio of the signals from the two LEDs, and compared the signals obtained for the oxidized and for the reduced form of the AB indicator. The slower reduction of AB in induced cells confirms the influence of rhTNF-α on cell proliferation. An error of 2% was observed for each LED ratio measurements.

biological information in real-time, whereas existing commercial devices for monitoring cell cultures are limited to temperature, pH and gas (mostly O_2 and CO_2) measurement.

4. Conclusions

In conclusion, an analytical tool that allows monitoring the production of cytokines and cell proliferation in cell cultures have been developed and tested with adherent epithelial cells. The device combines a controlled cell culture unit with optical detection devices, interconnected with an automated fluidic system. The platform is transportable, fits on a laboratory bench, and relies on standard biological laboratory equipment. The actual design provides flexibility for adaptation to a field exposure chamber, pumping the liquid directly from the chamber into the cell culture, using a filter to prevent bacterial contamination, which would extend the application to the assessment of cellular toxicity on site.

A critical point of the device is to keep the cells healthy in an atypical environment, and maintain sterility in the cell culture. In addition, the cells had to be prepared freshly for each new experiment, in a biological laboratory. Therefore, whereas the device is now ready for monitoring cell cultures in a laboratory environments, further improvements are required to use the instrument on the field. The development of an integrated, ready-to use chip for cell culture, with adapted fluidics, would facilitate the use of the platform outside a laboratory environment. Further improvements include the extension to detect up to six cytokines in cell medium, and the use of a less toxic optical test for cell proliferation.

The integrated platform, combining cell culture, analysis of cell viability and in situ detection of cytokines in cell supernatant, offers a new tool to assess the cytotoxicity of nanoparticles, drugs, chemicals and other external agents in vitro, thus reducing the number of costly in vivo experiments. Further, the device could be combined with automated cell culture as control, or could be used in cell bioreactors for controlling the production.

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