

Cite this: *Lab Chip*, 2011, **11**, 2551

www.rsc.org/loc

PAPER

Monitoring cellular stress responses to nanoparticles using a lab-on-a-chip†

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Received 25th March 2011, Accepted 26th May 2011

DOI: 10.1039/c1lc20256a

As nanotechnology moves towards widespread commercialization, new technologies are needed to adequately address the potential health impact of nanoparticles (NPs). Assessing the safety of over 30 000 NPs through animal testing would not only be expensive, but it would also raise a number of ethical considerations. Furthermore, existing *in vitro* cell-based assays are not sufficient in scope to adequately address the complexity of cell–nanoparticle interactions including NP translocation, accumulation and co-transport of *e.g.* allergens. In particular, classical optical/fluorescent endpoint detection methods are known to provide irreproducible, inaccurate and unreliable results since these labels can directly react with the highly catalytic surfaces of NP. To bridge this technological gap we have developed a lab-on-a-chip capable of continuously and non-invasively monitoring the collagen production of primary human fibroblast cells (NHDF) using contactless dielectric microsensors. Human dermal fibroblast cells are responsible for the maintenance of soft tissue integrity, are found throughout the human body and their primary function is collagen expression. We show that cellular collagen production can be readily detected and used to assess cellular stress responses to a variety of external stimuli, including exposure to nanoparticles. Results of the study showed a 20% and 95% reduction of collagen production following 4 hour exposure to 10 $\mu\text{g mL}^{-1}$ gold and silver nanoparticles (dia. 10 nm), respectively. Furthermore a prolonged perfusion of sub-toxic concentrations (0.1 $\mu\text{g mL}^{-1}$) of silver NP reduced NHDF collagen production by 40% after 10 h indicating increased NP take up and accumulation. We demonstrate that the application of microfluidics for the tailored administration of different NP treatments constitutes a powerful new tool to study cell–nanoparticle interactions and nanoparticle accumulation effects in small cell populations.

Introduction

Nanomedicine, defined as the application of nanotechnology to healthcare, is a relatively new field that promises to facilitate biomedical research and significantly improve medical care.^{1,2} Nanomedicine consists of nanodiagnostics, targeted drug delivery and regenerative medicine and is expected to greatly impact the prevention, reliable diagnosis and treatment of disease.³ Nanoscale technologies such as colloidal gold, quantum dots semiconductor crystals, and iron oxide crystals capable of targeting different cells and extracellular components in the body have been used for some time in the delivery of drugs, genetic material, and diagnostic agents.^{4,5} In fact, magnetic nanoparticles (NPs) have been employed in the diagnosis of liver and spleen tumors using magnetic resonance tomography⁶ since the 1990s due to their ability to separate, isolate and detect cells or

molecules such as proteins, peptides and DNA.⁷ More recently, however, NPs are being injected intravenously for the specific delivery of chemotherapeutic agents to identify and eliminate cancer cells.⁸ Overall, clinical applications of NPs have improved detection sensitivity in medical imaging, raised therapeutic effectiveness through targeted drug delivery and decreased the associated side effects of drugs. Although their clinical benefits are clearly established and exploited, limited attempts in the evaluation of their potential undesirable long-term effects have been made. For instance, it is still unknown whether the same properties that make NPs appealing in nanomedicine could also be harmful when interacting with healthy cells.⁹ Due to their extensive use and projected increase in world-wide production, the safety evaluation of NPs should be given greater priority in order to establish adequate risk assessment protocols and to set up an industrial “Code of Conduct” for consumer protection.

To date, no reliable testing methods exist to assess the safety of NPs for a variety of reasons. For instance, *in vivo* models have low validity because of the intrinsic genetic differences between, *e.g.* rodents and humans, and because LD₅₀ (lethal dose 50%) values for acute toxicities do not address the effects of long term exposure. In terms of *in vitro* testing, it is well known that NPs

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c1lc20256a

can interact with organic dyes such as those commonly employed in cell-based assays, thus rendering most cell-based assays useless. Furthermore, *in vitro* viability assays are generally performed under physiologically irrelevant conditions^{9,10} because most nanotoxicological studies use cell lines that exhibit cellular phenotypes that are significantly different from those found in native human tissue.

To adequately assess the potential health impacts of engineered NPs we have developed a lab-on-a-chip capable of monitoring *ex vivo* living human skin fibroblast cells (NHDF). Primary cell cultures were used because they are more likely to display natural phenotypes, thus mimicking *in vivo* situations.¹¹ Human fibroblasts were selected as indicator cells for nanotoxicological studies because they are found throughout the human body due to their inherent ability to maintain the integrity of soft connective tissue. NHDF are able to modify their microenvironment by producing various components of the extracellular matrix (ECM) and are involved in a number of important biological processes including wound healing, immune responses, inflammation, angiogenesis and connective tissue construction.¹ For example, during the inflammatory phase of the wound healing process, fibroblasts proliferate, accumulate and form a new, temporary ECM by excreting collagen and fibronectin fibers to increase the strength of the damaged tissue.^{12,13} Since ECM deposition represents a primary biological function, the biosynthesis of proteins forming the ECM is constitutively expressed in fibroblasts. Constitutive genes are generally responsible for regulating basic cellular processes and continuous expression is therefore required to maintain normal cell function. Consequently, changes in collagen production can thus be associated with an altered fibroblast phenotype. For instance, work performed by Korin *et al.*¹⁴ and Wang *et al.*¹⁵ demonstrates that ECM production increases when *in vitro* cultured human fibroblasts are exposed to elevated shear force. Additionally, a recent publication by Liu *et al.*¹⁶ describing *in vivo* wound healing processes indicated that the application of silver nanoparticles as a disinfecting agent could cause a significant decrease in the expression of collagen I, further attesting that collagen expression is indicative of the health status of fibroblasts.¹⁷

Although many studies have shown that cellular phenotypes are best studied using single cell analysis, the study of multicellular systems is of higher physiological relevance in the field of toxicology. Cell–cell communication has a considerable influence on the cellular life cycle where the interplay of cells is known to significantly alter their behavior.¹⁸ Consequently, in the present work, we have investigated the collagen production rate of small cell populations exposed to NPs using a lab-on-a-chip. In the context of cell analysis, we have chosen to integrate various interdigitated capacitors (μ IDCs) for contactless dielectric sensing. These sensors are embedded in the microfluidic biochip platform and are capable of continuously and non-invasively monitoring collagen secretion by NHDF. Dielectric sensing is a label-free and non-destructive method that has been traditionally used to measure polarization responses of low dielectric materials such as (bio)polymers, proteins or cells exposed to radio-frequency electrical fields.^{19–21} We have recently reported the integration of high-density micro-interdigitated capacitors (μ IDCs) in various microfluidic biochips for the non-invasive

monitoring of microbial biofilms, virus contamination and liposomes.^{22,23} In a previous work we have also demonstrated that contactless dielectric measurements provide stable and non-drifting signals over long periods of time due to complete sensor insulation and physical removal from the liquid sensing environment.^{24,25} Therefore dielectric microsensors are ideally suited for detecting both viability²⁶ (e.g. cell membrane integrity) and collagen (biopolymer) production rates of fibroblast cells because any alteration in the intrinsic dielectric properties of both the cell membrane and extracellular matrix composition can be readily identified.^{27,28}

Microfluidic systems are generally used to perform cell sampling, trapping and sorting as well as cell analysis involving cell treatment, transport and cultivation in the presence of concentration or temperature gradients or varying shear force conditions.^{29–31} In the present work, however, microfluidics is combined with dielectric sensing to create a microdevice displaying *in vivo* characteristics. Microfluidics is used to recreate a low stress *in vivo*-like microenvironment³² through the use of uniform and biocompatible surfaces, physiologically relevant shear force conditions and appropriate cell-volume to liquid-volume ratios. Furthermore, microfluidic control over nutrient supply, waste removal, and constant temperature provides the necessary environmental conditions for cells to display their natural phenotypes.^{33,34} In the present work we also use microfluidics to expose fibroblast cells to NPs to study nanoparticle–cell interactions and nanoparticle accumulation effects. Size, size distribution, particle shape, agglomeration state and surface charges of nanoparticles are determined because they could account for possible adverse biological responses. Furthermore, 3-dimensional computational fluid dynamic (CFD) is employed to accurately estimate nanoparticle distribution and movement in microchannels. Performance evaluation of the presented microfluidic device involves the assessment of NHDF collagen production rates under different environmental pressures including increased shear force conditions, diminished nutrient supply and exposure to pharmaceutical compounds such as acetaminophen and valinomycin. Finally, the well-known nanomaterials gold and silver were used to demonstrate the ability of the collagen-based detection method to assess the stress response of fibroblasts to various nanoparticle treatments.

Experimental section

Lab-on-a-chip design and fabrication

The entire lab-on-a-chip system consists of a microfluidic biochip located in an aluminium fixture connected *via* tubing to external valves, injection ports and a syringe pump (KDS 250, KdScientific, USA). The microfluidic biochip was fixed using spring loaded connectors integrated within two printed circuit boards providing shielded connection to the potentiostat (VMP3, BioLogic, France). The microfluidic biochip shown in Fig. 1a consisted of a 30 × 30 mm² quartz glass substrate (Borofloat®) containing the electronic sensors and a 15 × 25 mm² soft polymer polydimethylsiloxane (PDMS) from Dow Corning in Germany (Sylgard 184) based microfluidic. The microfluidic channel layout comprised of four individual addressable proliferation chambers of approx. 100 nL volume. Electrodes, leads, as well as

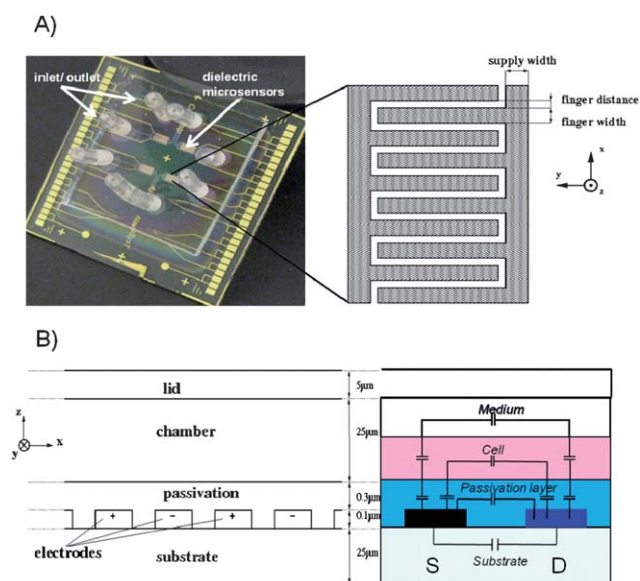


Fig. 1 (A) Picture shows the microfluidic biochip consisting of PDMS microchannels bonded to a glass bottom substrate and includes four sample inlets and wastes as well as contactless dielectric microsensors located underneath monitoring chambers. The dielectric microsensors (inset) consist of a high-density μ IDC (200 fingers of 5 μ m width that are spaced apart by 5 μ m). (B) Schematic representation of the measurement principle using passivated interdigitated electrode structures as dielectric microsensors.⁴⁹

contact pads, were fabricated by first sputtering a 10 nm titanium seed layer followed by a 100 nm gold layer onto the pre-patterned substrates. The high-density interdigitated capacitors (μ IDCs) consisting of 200 fingers of 5 μ m width a space were covered by a 300 nm insulating silicon nitrate (SiN_x) layer deposited *via* low temperature plasma enhanced chemical vapor deposition (PECVD, Oxford Plasmalab 80). Microfluidic master molds were fabricated by photolithography using the epoxy resin SU8-2050 (height of 30 μ m) patterned on top of a silicon wafer. Microfluidic channels were formed by pouring a mixture (10 : 1) of PDMS (Sylgard 184) silicone elastomer base and curing agent over the epoxy master molds while polymerization was allowed to take place over a period of 4 hours at 70 $^\circ\text{C}$ following a 10 min degassing step in a vacuum chamber. The soft polymer fluidic was removed from the master and cut to the appropriate size followed by extensive cleaning using isopropanol and deionized (DI) water. The pre-cut PDMS microfluidic was aligned under a microscope and put in contact with the bottom substrate immediately after oxygen plasma treatment (Diener Electronics) (30 s at 40 W). A curing time of 24 hours at 50 $^\circ\text{C}$ was implemented to allow complete cross-linking between the activated PDMS and SiN_x surfaces.

Microchannel surface modification procedure

To create uniform surface properties favorable for cell adhesion within our hybrid material system, both the hydrophobic PDMS polymer and the SiN_x passivation were chemically modified. In a first step, contact angle measurements using a goniometer (ramé-hart, USA) were employed to optimize the modification

procedure for both substrates. Next, 1 M HCl was pumped at 1 $\mu\text{L min}^{-1}$ for 5 min through the microfluidic channel system to hydrolyze the PDMS surface. This was followed by 1 M NaOH treatment (5 min at 1 $\mu\text{L min}^{-1}$) to deprotonate both surfaces. In a second step, 2% (3-aminopropyl) trimethoxysilane (APTS 97%, Sigma-Aldrich, 281778, Germany) solution in methanol containing 0.15 M acetic acid was pumped for 5 min at 1 $\mu\text{L min}^{-1}$ through the microfluidic channels. This was followed by extensive rinsing using methanol. Finally, the dried chip was cured at 80 $^\circ\text{C}$ for 15 min. To further modify the proliferation chamber the crosslinker bis(sulfosuccinimidyl)suberate (BS_3), 0.5 mM in PBS, and the peptide sequence RGDS, 100 μM in PBS, were pumped through the chamber for 5 min each.

Cultivation of mammalian cells

NHDF cell cultures were obtained from PromoCell (C-12300) and cultivated in 25 cm^2 cell culture flasks (PAA, Austria) at 37 $^\circ\text{C}$ in a 5% CO_2 humidified atmosphere using DMEM high glucose medium with stable glutamine (PAA, E15-883, Austria) supplemented with 10% FCS (PAA, A15-101). When reaching confluency, cells were counted (approx. $0.5 \times 10^5 \text{ cells cm}^{-2}$) and split (1 : 2) twice a week using 0.25% trypsin EDTA (fisher scientific) at 37 $^\circ\text{C}$ for enzymatic cell removal. Cell number was determined after addition of 50 μL Trypan blue (Sigma Aldrich, Germany) solution to 200 μL cell suspension using a haemocytometer (10 μL aliquots). During cell cultivation on chip the medium was supplemented with 25 mM Hepes buffer (PAA, S11-001, Austria) and 1% gentamicin (PAA, P11-004, Austria).

Contactless dielectric measurements

Initially, the microfluidic biochip was chemically sterilized for 30 min using 70% EtOH at 2 $\mu\text{L min}^{-1}$ flow rate, followed by sequentially pumping sterile DI water for 4 h and medium for at least 2 h through the microfluidic channels to remove any residual alcohol from the microchannels. At this time, medium flow was stopped while NHDF cells were seeded into the monitoring chambers by gently sucking a 10 μL cell suspension *via* the waste port using an externally located syringe. As soon as 80–100% confluence was reached, cell seeding was stopped and cells were allowed to adhere for 20 min in the absence of fluid flow. This was followed by media perfusion through activation of fluid flow to remove dead or non-adherent cells. Flow rates were adjusted to 100 nL min^{-1} to ensure total medium replacement of each proliferation chamber every 60 s. Cell attachment, movement and viability as well as collagen production were monitored using a multichannel potentiostat (VMP3/P-01, Princeton Applied Research) consisting of 15 independently addressable channels. The four μ IDCs were addressed simultaneously by continuously scanning 101 frequencies (5 to 500 kHz) applying $\pm 75 \text{ mV}$ (V_{pp} 150 mV) between the finger electrodes. Absolute impedance ($|Z|$), phase angle (θ), real part ($\text{Re}(Z)$) and imaginary part ($-j \times \text{Im}(Z)$) of the impedance were continuously recorded during experimentation.

Nanoparticle preparation and characterization procedures

The gold and silver NPs used in this study were purchased as colloidal solutions from PlasmaChem. Gold NP colloid solution

(PL-Au-S20-5 mg, PlasmaChem, Germany) had a concentration of 0.05 mg mL⁻¹ and a nominal average particle size of 20 nm diameter while silver NP (PL-Ag-S10-10 mg, PlasmaChem, Germany) had 0.1 mg mL⁻¹ sized 10 nm. Prior to NP analysis, the obtained particle suspensions were centrifuges and resuspended in DI-water, DMEM medium without FCS and PBS buffer containing 10% FCS. All three NP suspensions were subjected to 20 min sonication prior 6 hours sedimentation period. Next, 1 mL aliquots of the supernatant were subjected to dynamic light scattering (DLS) and zeta potential measurements using a Zeta Sizer (Malvern) to estimate particle size, size distribution and surface charges. Additionally, NP samples were also examined by transmission electron microscopy (TEM) in order to determine shape and agglomeration status of NPs.

Computational fluid dynamic (CFD) simulations

A 3-dimensional computation fluid dynamics (CFD) simulation of a monitoring chamber was performed to analyze the movement of nanoparticles through the chamber and assess the influence of the gravitational forces exerted on them. A polyhedral mesh with approximately 2.4 million elements was created to model the geometry of the chamber. The implicit unsteady solver for laminar flows of the commercial CFD program *Starccm+* was used. A multi-fluid Euler–Euler model including diffusion and gravitation was employed to simulate the dispersed flow, since the inertial forces of the NPs are negligible due to their small size and the dispersed phase can thus be represented by a fluid phase.³⁵ The diffusion constant of the NPs was determined using the Stokes–Einstein relation: $D = k_b T / (6\pi\eta r)$, where k_b is the Boltzmann constant, T is the temperature, η is the dynamic viscosity of the fluid and r is the radius of the particle. The inlet boundary was realized as a constant-velocity inlet and a pressure-outlet boundary was utilized for the outlet. All other surfaces were implemented as no-slip walls. Fluid dynamic simulations for a set of different inlet velocities were performed in order to cover the same range of flow rates used in our experiments. Initially, both Reynolds and Peclet numbers describing flow regimes and particle transport phenomena in fluids were determined to characterize microfluidic conditions present during NP treatment. The Reynolds number of a flow between two parallel plates is given by $Re = u2h/\nu$, where u is the mean fluid velocity, h is the distance of the plates, and ν is the kinematic viscosity of the fluid. This assumption is valid since the width of the chamber (2000 μ m) is much greater than the chamber height (30 μ m). The Peclet number (Pe) given by $Pe = (6\pi\rho u^2 r^3 \lambda^2) / (kT)$ represents the ratio of inertial to diffusive forces acting on a spherical particle,³⁵ where ρ , r , k and T are the density of mass of the fluid, the radius of the particle, the Boltzmann constant, and the absolute temperature, respectively. The relative analyte size λ is defined as $\lambda = 2r h^{-1}$.

Based on nanoparticle size determinations obtained from light-scatter (DLS) measurements simulations were performed for silver nanoparticles using 100 nm (see Fig. S3†) for size distribution of Ag NP in medium. A suspension of 100 μ g silver particles in 1 mL fluid was used for the simulations, which corresponds to a mass fraction of approximately 0.0001. The molecular diffusion coefficient (D) of 100 nm Ag particles can now be determined by the Stokes–Einstein equation $D = (kT)/(6\pi\eta r)$. The diffusion constant was found to be 4.36×10^{-12} m² s⁻¹ and a gravitational

constant of 9.81 m s⁻² was used. The mass density of the silver particle and of the fluid was 10 490 kg m⁻³ and 980 kg m⁻³, respectively. Using the above calculated values, CFD simulations are applied to investigate microfluidic flow and pressure profiles and the distribution of the silver particles inside the chamber. The nanoparticles were applied to the chamber at time step $t = 0$ s by defining a particle mass fraction at the inlet surface. The time step size Δt of the unsteady solver varied with the fluid velocity and ranged from 2.0×10^{-3} s to 1.0 s (see Table 3).

Limitation: The model did not take into account particle forces caused by van der Waals interaction and electrostatic double layers. Furthermore, the diffusion constant was assumed to be location-independent, not taking into consideration the change of the diffusive properties of particles close to the chamber walls.

Immunofluorescence staining of collagen fibers

NHDF cells were seeded into microfluidic chambers as described in the above section and allowed to adhere to the chip surface and fluid flow (100 nL min⁻¹) was activated after 20 min. In a series of experiments, cell populations were stained for collagen expression after 3 h, 24 h and 48 h. The staining procedure was performed at room temperature unless otherwise indicated. Following PBS washing, cells were fixed using PFA solution (4% paraformaldehyde (PROLABO, 88794.295, VWR, Germany) in PBS, pH 7.4) for 10 min followed by two washing steps with PBS prior 20 min addition of blocking buffer (2% bovine serum albumin (Applichem, A6588) in PBS). The rabbit anti-human collagen I–V (Abcam, ab36064) was diluted 1 : 10 using a blocking buffer and was incubated overnight at 4 °C (for controls no primary antibody was applied). Following a rinsing step with blocking buffer, a 1 : 200 dilution of rhodamine conjugated goat anti-rabbit IgG antibody (Abcam, ab6718, UK) was added for a period of 1 h. After a 10 min rinsing step, the samples were placed in the Vectashield® mounting medium (H-1000). Images were analyzed using ImageJ software.

Cytotoxicity testing using nanoparticles

The XTT assay was employed as a reference method to assess cell viability following 22 h exposure to increasing gold and silver NPs. The XTT solution was prepared immediately before use by dissolving XTT sodium salt (Applichem, Germany A2240, 1 mg mL⁻¹) and phenazine methosulfate (Applichem A2212, 10 μ g mL⁻¹) in a phosphate buffer (saline). In a 6-well microtiter plate, 2×10^6 cells were seeded in the absence and presence of increasing NP concentrations ($n = 3$) and incubated for 22 hours at 37 °C in a 5% CO₂ atmosphere. At this point 500 μ L of the XTT solution was added to each well and incubated for an additional 2 hours in the CO₂ incubator. Next, absorption of the supernatant was measured using a UV-VIS spectrophotometer at 450 nm and 690 nm wavelengths.

Since nanoparticles are also known to accumulate at mitochondrial membranes³⁶ the effect of gold and silver NPs on the mitochondrial membrane potential (MMP) of NHDFs was also determined using the cationic fluorescent dye tetramethylrhodamine ethyl ester perchlorate (TMRE, Sigma-Aldrich, 87917, Germany). In healthy cells, the lipophilic dye enters the

negatively charged mitochondria where it accumulates and emits a fluorescent light. In contrast, the collapse of the MMP in apoptotic cells results in the dispersion of TMRE leading to a general loss of fluorescence.³⁷ In this context, confluent NHDF cells were stained using 25 nM TMRE dye (diluted in culture medium from a 25 μM stock in DMSO : ethanol = 1 : 1) for 10 min at 37 °C. Once the staining solution was removed, a fresh culture medium containing NPs was added. After 24 h NP exposure fluorescence images were taken (rhodamine filterblock) and average fluorescence intensities ($n = 4$) were determined for each concentration using ImageJ software (see Fig. S5†).

Results

Characterization of lab-on-a-chip system for cell analysis

An important aspect of cultivating cells on chip is the optimization of the microfluidic environment including surface biocompatibility, applied flow rates and nutrient supply, and apparent shear force conditions. The ability of normal human dermal fibroblast cells to adhere, spread and proliferate on functionalized glass surfaces was visually inspected. In a series of experiments, various cell adhesion promoters such as the peptides REDV, RGD and RGDS linked to different organosilanes including APTS, MPTS and MPS were employed. Fibroblast adhesion and cell population doubling in the presence of RGDS linked *via* succinimide ester (BS3) chemistry to aminopropyl-trimethoxysilane (APTS) modified glass slides were found to be similar to those of standard cell culture plates, and were thus used for subsequent experiments. Next, contact angle measurements were conducted to further confirm uniform APTS coverage on both SiN_x (bottom) and PDMS (side walls and top) surfaces of the hybrid microfluidic biochip. Contact angles for droplets of DI water layered on top of PDMS decreased from 104° to 63° and increased from 43° to 67° for SiN_x covered glass substrates as reported in the literature (see the ESI, Fig. S1†).³⁸ Once surface biocompatibility was established, the influence of applied electrical potential on sensor performance was investigated since higher applied AC voltages normally increase the sensitivity of the contactless dielectric microsensors by reducing the signal to noise ratio. However, it is also possible that the elevated electric flux within the biological material could interfere with the intrinsic membrane potentials of mammalian cells, thus leading to unwanted cellular stress during cell analysis. The effects of increasing AC voltages on cell adhesion were thus investigated and results indicated that an applied voltage (V_{pp}) of 150 mV (AC ± 75 mV) yields lowest noise contributions (5170 ± 5 Ohm for $n = 20$ at 160 kHz) while still maintaining optimum cell viability (see Table S1†).

Subsequent analyses employed the optimized measurement conditions described above and compared contactless impedance signals obtained from healthy and dead cells. In previous studies we demonstrated that the complete isolation and passivation of electrode structures of μIDC eliminates ohmic (faradaic) contributions leaving the imaginary part dominating the overall bio-impedance signal.^{23,39} This means that the classic impedance equation of $Z = R + jXC$ can therefore be simplified to $Z \approx jXC$ or $1/j\omega C$ making contactless dielectric measurements particularly sensitive to capacitive changes occurring along cell membranes

and cellular structures.²⁴ Fig. 2a shows impedance–time and phase angle traces of fibroblast cell cultures seeded to 100% confluence into the microfluidic monitoring chambers. Following a 60 min cell attachment period, microfluidic flow was activated (100 nL min^{-1}) to remove excess dead or unattached NHDF cells from the monitoring chambers. Impedance signals of cells that had been exposed to a lethal dose of phenol ($600 \mu\text{g mL}^{-1}$ for 30 min) prior to cell seeding showed no significant signal variation. Meanwhile, impedance traces of healthy fibroblasts decreased steadily over the entire 42 h cultivation period. A linear signal decrease of $-16.0 \pm 0.2 \Omega \text{ h}^{-1}$ was obtained after complete sensor coverage was visually confirmed at approx. 10 hours. These results demonstrate that the contactless dielectric microsensors cannot only readily distinguish between live and dead cells (offset of absolute impedance directly after cell seeding) but are also capable of continuously monitoring dynamic cell attachment and spreading processes as seen in Fig. 2b. While the initial signal decrease is largely caused by increased sensor coverage, it is possible that the observed linear impedance decrease in the absence of significant cell proliferation events (NHDF doubling time is of 72 h to 96 h) is mainly attributed to ECM (*e.g.* collagen) production and excretion. This notion was further supported by visual inspection during cell removal procedures and confirmed by collagen immunofluorescence staining tests as seen in Fig. 3. Visual inspection of cell removal using trypsin showed that individual NHDF cells were trapped in bundles of fibers (see arrow in Fig. 3a ii), while the addition of the collagen degrading enzyme collagenase IV allowed the complete removal of cellular material (data not shown). Additionally, on-chip immunostaining of collagen fibers showed a linear increase in collagen excretion by

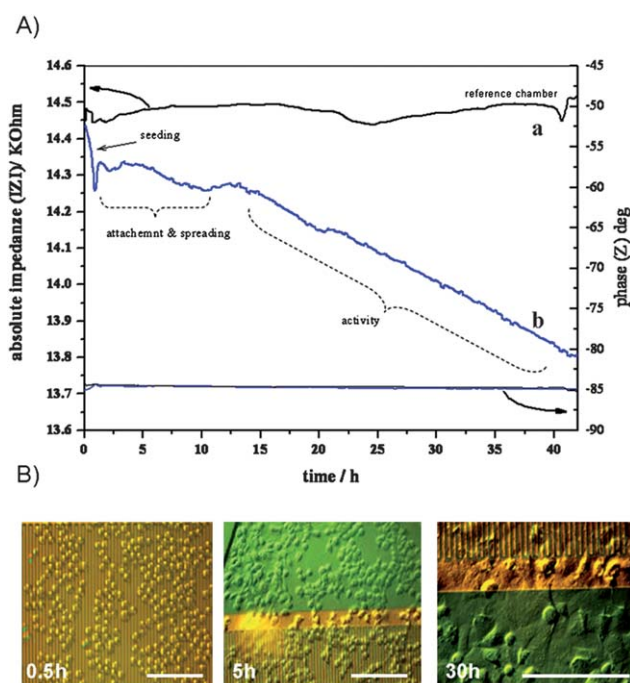


Fig. 2 (A) Impedance–time traces (at 70 kHz) of (a) dead that have been pre-treated with $600 \mu\text{g mL}^{-1}$ prior to cell seeding and (b) live fibroblast cell culture on chip. (B) Pictures taken after 0.5 h, 5 h and 30 h after cell seeding show NHDF seeding density, adhesion and spreading processes during cell cultivation. White scale bars represent 200 μm .

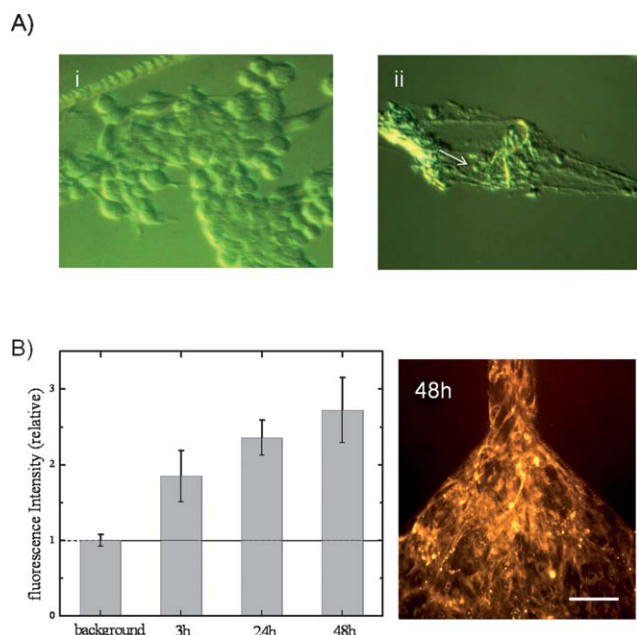


Fig. 3 (A) Phase-contrast microscope pictures of fibroblasts taken after (i) 24 h on chip cultivation, (ii) following a 5 min trypsin treatment. (B) NHDF collagen expression on chip over 48 h detected by immunofluorescence using antihuman antibody against collagen types I to V and stained using a rhodamine-conjugated secondary Ab (orange). Graph shows linear time dependency of detected fluorescence intensities ($n = 4$). Scale bar represents 200 μm .

NHDF cells over a period of 48 hours as seen in Fig. 3b (see also the ESI, Fig. S2†). To confirm sensor sensitivity to collagen, commercially available collagen monomers were precipitated (air-dried) above the biosensors and recorded impedance values listed in Table 1. The significant signal changes in the presence of increasing concentrations of precipitated collagen monomers indicates that the integrated contactless dielectric microsensors are also able to quantify collagen concentrations.

The effects of reduced serum supply and exposure to well-known cytotoxins on collagen excretion were investigated in subsequent experiments since NHDF collagen synthesis is also dependent on nutrient supply/health status of the cell population.⁴⁰ Although serum reduction from 10% to 1% FCS in the media did not show any adverse effects within the first 12 hours of cultivation, significant signal decreases were observed during the following 9 hours leading to a stagnation of impedance

Table 1 Background subtracted impedance signals obtained at 100 kHz for increasing concentrations of precipitated (air dried) collagen monomers

Collagen monomer concentration/ $\mu\text{g mL}^{-1}$	Averaged normalized impedance values ^a $\Delta\text{IZI}/\text{Ohm}^{-1}$	Absolute error ($n = 9$) $\text{SD}/\text{Ohm}^{-1}$
0	0	+/-0
1	138	+/-3
10	583	+/-2
100	571	+/-1
1000	652	+/-3

^a Average is taken from nine replicate measurements.

signals over the remaining 40 hours. Fig. 4a clearly reveals three distinct cell population responses during a 62 hour starvation period. After an initial adaptation process and constant collagen production, NHDF cells formed visible cell agglomerates (see inset of Fig. 4a) that resulted in a massive alteration in dielectric cell properties, reduced sensor coverage and eventual termination of collagen production. Next, cell population response to the presence (i) and absence (ii) of nutrient supply (no activation of flow) is shown in Fig. 4b. The rapid decline in collagen production and resulting signal stagnation after three hours indicates that in the absence of fluid flow, the NHDF cell population reacts to the limited availability of nutrients. To further investigate the effect of reduced health status of NHDF cells on collagen production, two cytotoxic compounds were added to on-chip cell cultivation. Fig. 4c shows impedance–time traces of (i) untreated, (ii) 5 mM acetaminophen, (iii) 1 μM valinomycin and (iv) in the absence of cells (background). The decreasing impedance slopes of (i) $-11.2 \pm 0.1 \Omega \text{ h}^{-1}$, (ii) $-9.3 \pm 0.1 \Omega \text{ h}^{-1}$ and (iii) $-3.3 \pm 0.1 \Omega \text{ h}^{-1}$ clearly point at the large influence that external stimuli can have on cellular collagen production.

Since NHDF cells are also known to react *in vivo* to increased shear forces present *e.g.* when tissue is damaged and during the wound healing processes,⁴⁰ on-chip NHDF cell cultures were also exposed to increased shear force conditions. The corresponding average fluid velocity u_{avg} in the monitoring chamber during cell cultivation in the presence of 100 nL min^{-1} flow rate is $27.8 \mu\text{m s}^{-1}$ exhibiting a maximum fluid velocity u_{max} of $55.6 \mu\text{m s}^{-1}$ at the center of parabolic flow profile. The analytically estimated wall shear stress in the growth chamber (rectangular cross section of $2000 \times 30 \mu\text{m}$) is approx. 5.5 mPa or $0.055 \text{ dyn cm}^{-2}$. This shear stress is well below reported physiological shear stress conditions in blood vessels starting at approx. 1 dyn cm^{-2} .^{41,42} Consequently, we increased flow velocities by a factor of 100 to visualize cellular responses under natural blood flow conditions. Fig. 5a shows pictures of a NHDF population taken after 24 hours in the presence of $27.8 \mu\text{m s}^{-1}$ and $2780 \mu\text{m s}^{-1}$ fluid flow velocities where the formation of cell clusters was visible under prolonged increased shear force conditions. In an attempt to further investigate the effect of increased shear forces on collagen production, a NHDF cell population was consecutively treated with four short fluid pulses with the designated hundred fold velocity. Surprisingly, impedance–time traces shown in Fig. 5b revealed different cell detachment and resulting re-attachment processes following four consecutive fluid pulses. While the first fluid pulse prompted a small signal increase (cell detachment) followed by a gradual signal recovery over a period of six hours, the consecutive three fluid pulses induced immediate cell detachment from the surface. Re-attachment periods in the presence of the last three fluid pulses steadily increased from 2.2 h to 3.7 h and then to 5.1 h, respectively. This means that, while collagen fibers can help to prevent significant cell loss during fluid pulses, multiple cell detachment events reduce the ability of NHDF cells to re-attach to the sensor surface.

Nanoparticle transport characteristics in microchannels

The actual size of Ag and Au nanoparticles (NPs) in the cell culture medium had to be determined prior to estimating NP

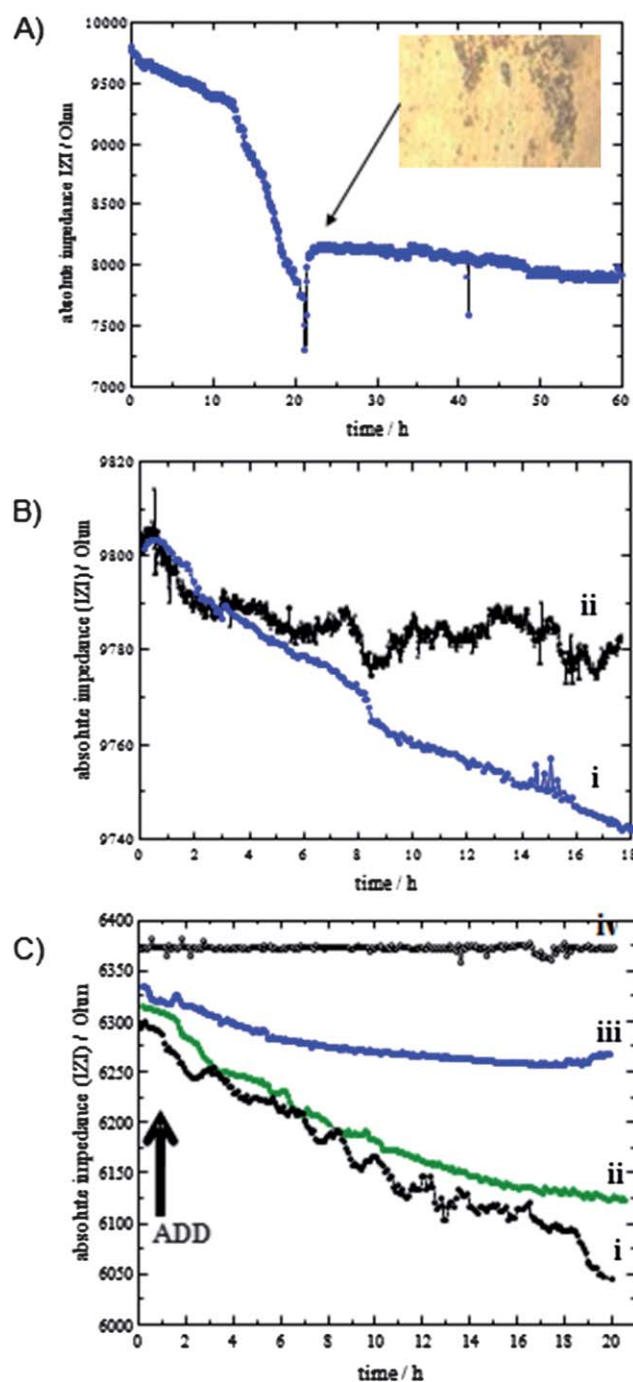


Fig. 4 (A) Impedance–time traces (at 90 kHz) of confluent NHDF population under serum restricted conditions containing 1% FBS in the cell culture medium. Inset shows picture taken after 20 h cultivation featuring visible cell aggregation events. (B) Impedance–time traces (at 90 kHz) of NHDF cell populations in (a) the presence of 100 nL min^{-1} medium supply and (b) absence of fluid flow. (C) Impedance–time traces (at 110 kHz) using four contactless dielectric microsensors in the absence (iv) and presence of (i) confluent NHDF cell cultures exposed to 5 mM acetaminophen (line ii) and 1 μM valinomycin (line iii) over 20 hour time period.

transport characteristics under laminar flow conditions. Although often overlooked in nanotoxicological research, the determination of material characteristics is crucial for a sound

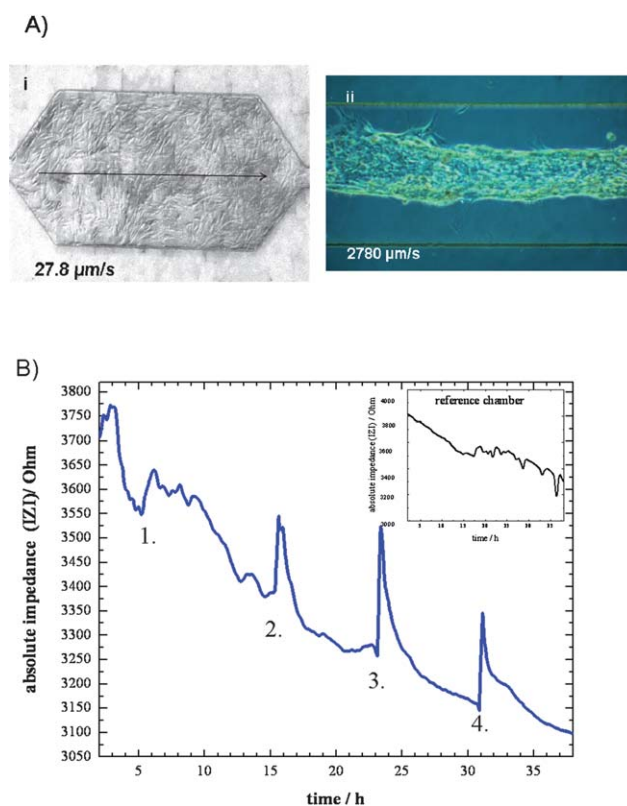


Fig. 5 (A) Phase-contrast microscope pictures of two microfluidic NHDF cell cultures in the presence of (i) low shear force conditions ($0.055 \text{ dyn cm}^{-2}$; chamber width 1 mm) and (ii) under prolonged (24 h) increased fluid mechanical shear forces (5.5 dyn cm^{-2} ; chamber width $100 \mu\text{m}$). (B) Impedance–time traces (at 220 kHz) show response of NHDF cell culture exposed to four subsequent high fluid pulses of $5.5 \text{ dyn cm}^{-2} \text{ s}^{-1}$. Inset shows impedance–time traces of NHDF culture in reference chamber where cells are continuously perfused at $0.055 \text{ dyn cm}^{-2}$.

understanding of NP interaction with medium components and biological substances, such as proteins and cells.⁴³ It is important to note that, due to the absence of accredited reference nanomaterials and limited information provided by industry regarding synthesis routes and detergents used, nanotoxicological studies also need to provide information on nanomaterial size, size distribution, agglomeration state and surface charges. Consequently, we employed dynamic light scattering (DLS) measurements and transmission electron microscopy (TEM) to analyze commercially available 20 nm gold and 10 nm silver nanoparticles dissolved in DI water and cell culture medium. Results listed in Table 2 clearly show a solvent dependent size distribution and agglomeration behavior, while the observed shifts in surface potentials in the presence of 10% serum (FCS) further indicates protein adsorption at the NP surface (see also the ESI, Fig. S3†).

Based on the above results, transport characteristics of 100 nm Ag NP agglomerates were calculated using CFD simulations to estimate particle flow profiles, mass fractions and concentration gradients in the microfluidic channels. The Reynolds numbers listed in Table 3 were obtained using a kinematic viscosity of $10^{-6} \text{ m}^2 \text{ s}^{-1}$. Even for the maximum flow rate of $50 \mu\text{L min}^{-1}$ the resulted Reynolds number of 0.833 was clearly in the laminar

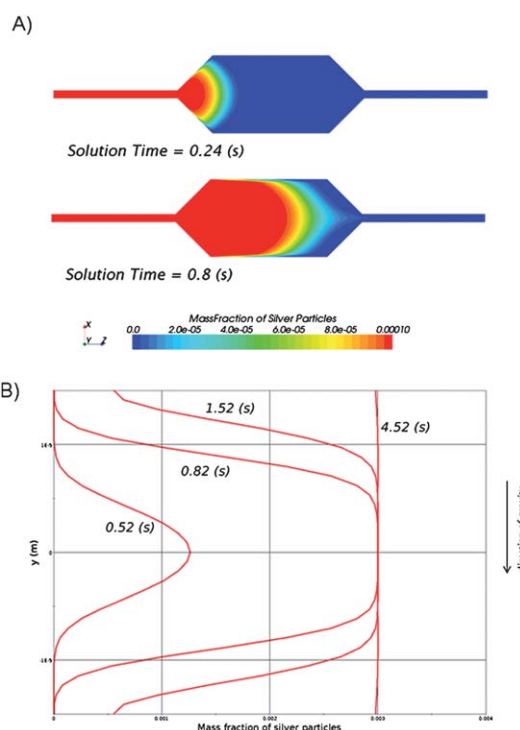
Table 2 Nanoparticle characterization using DLS measurements to assess size, size distribution and zeta potential of silver and gold nanoparticles

Solvent	Gold NP (20 nm dia.)		Silver NP (10 nm dia.)	
	Size (distribution)	Zeta/ mV pH	Size (distribution)	Zeta/ mV pH
Water	38 nm (87%), 444 nm (9%)	−42 7.8	47 nm (72%), 6 nm (26%)	−43 7.9
Medium	60 nm (61%), 10 nm (36%)	−13 7.7	129 nm (94%), 14 nm (6%)	−11.6 7.7

domain. The calculated Peclet number of Ag particle aggregate having a radius $r = 50$ nm ($\rho = 980$ kg m^{−3} and $T = 298$ K) under different flow conditions ranged from 4.81×10^{-12} to 1.20×10^{-6} (see Table 3). Since $Pe \ll 1$ for all flow rates, the influence of NP motion based on inertial forces, such as the tubular pinch effect,⁴⁴ can thus be ignored. Due to the low Reynolds and Peclet numbers ($\ll 1$) under experimental conditions, NP motion through a microchannel can be described in terms of advection and diffusion. The molecular diffusion coefficient (D) of 100 nm Ag particles is 4.36×10^{-12} m² s^{−1} and average nanoparticle resident time is approximately 110 s using a flow rate of 100 nL min^{−1}. Nanoparticle distribution profile estimated for a flow rate of 10.0 μ L min^{−1} is shown in Fig. 6 where silver particles are carried by the parabolic flow into the middle of the chamber and rapidly (4.52 s) establish a homogeneous particle distribution (see also the ESI, Fig. S4†). Although the establishment of a homogeneous Ag nanoparticle distribution in the presence of 100 nL min^{−1} flow requires 192 s, no negative influence of gravity on the homogeneity of particle distribution takes place. The simulation results indicate that microfluidics is ideally suited to transport NPs uniformly above the cell monolayer.

Microfluidic collagen-based assessment of nanoparticle toxicities

Nanotoxicological reference experiments were initially performed using a conventional XTT viability assay and a mitochondrial membrane potential stain (TMRE) in a microtiter plate format to determine the cytotoxic effects of increasing concentrations of Au and Ag NPs on fibroblasts (see ESI, Fig. S5a†). While exposure to Au nanoparticles did not show any significant adverse effect, both cytotoxicity assays revealed a similar dose–response relationship in the presence of Ag nanoparticles. Furthermore, significant particle agglomeration

**Fig. 6** (A) Computational fluid dynamic simulations showing mass fraction of silver nanoparticles at a horizontal plane position for two time steps. (B) Mass fraction of 100 nm silver particles shown at a vertical plane positioned at the centre of the chip's chamber. The mass fraction is shown for four time steps.

within dead cells was visually detected indicating active NP uptake by fibroblast cells. In follow-up continuous flow microfluidic cell chip experiments, NHDF collagen production was continuously and non-invasively monitored during Ag and Au NP exposure. Fig. 7a shows bioimpedance–time traces of NHDF collagen production rates in the presence of 10 μ g mL^{−1} Au and Ag NP, respectively. Here, both cell chambers were seeded with NHDF to above >90% confluence and fluid flow (u_{av} of ~ 27.8 μ m s^{−1}) carrying a constant stream of NPs was activated 30 min after cell loading. While the administration of 10 μ g mL^{−1} Ag NPs resulted in drastic impedance decrease within 2 hours, the application of Au NPs showed no or limited measurable effect on NHDF collagen production over a 24 hour period. We believe that the observed 85% reduction in NHDF collagen excretion rate points at a significant population stress response due to the continuous Ag NP uptake by fibroblasts. To further

Table 3 Computation fluid dynamics of nanoparticle distribution under laminar flow regimes

Simulation parameter	Unit	Calculated values for flow rates/ μ L min ^{−1}			
		0.1	1.0	10	50
Inlet velocity	m s ^{−1}	2.77×10^{-4}	2.77×10^{-3}	2.77×10^{-2}	1.39×10^{-1}
Avg. velocity in chamber	m s ^{−1}	2.77×10^{-5}	2.77×10^{-4}	2.77×10^{-3}	1.39×10^{-2}
Re number		1.66×10^{-3}	1.66×10^{-2}	1.66×10^{-1}	8.33×10^{-1}
Pe number		4.81×10^{-12}	4.81×10^{-10}	4.81×10^{-8}	1.20×10^{-6}
Time step (Δt)	s	1	10^{-1}	10^{-2}	2×10^{-3}
Avg. NP resident time	s	108.3	10.8	1.08	0.217
Time to homogeneous distribution	s	192	31.2	5.5	1.8

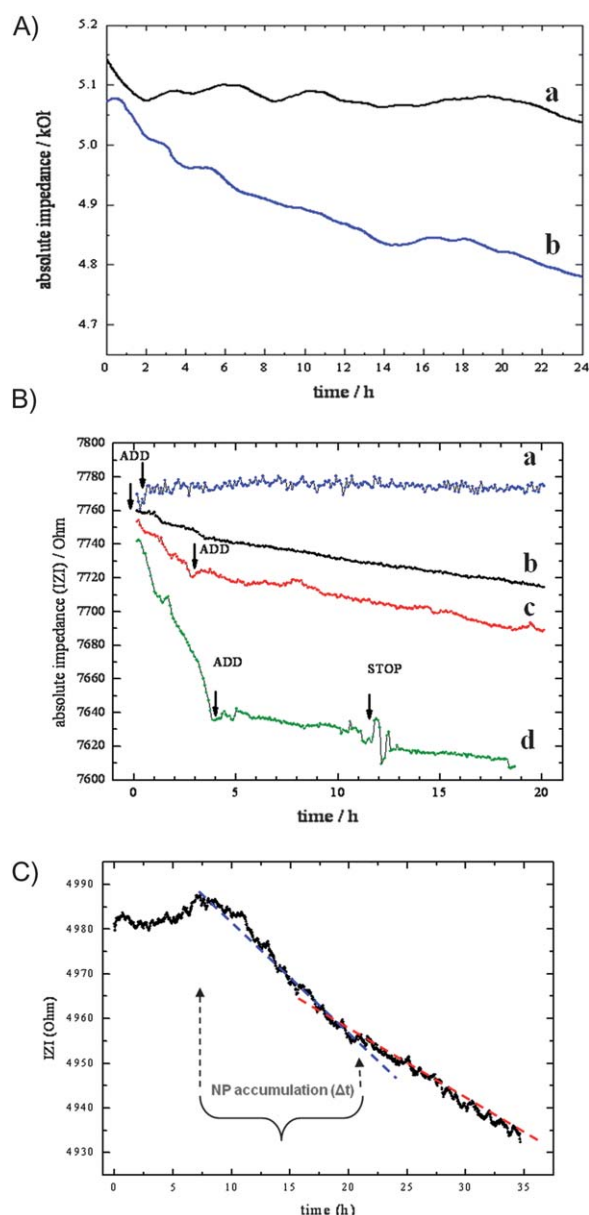


Fig. 7 (A) Impedance–time traces (at 160 kHz) of NHDF collagen production rates in the presence of a constant supply ($27.8 \mu\text{m s}^{-1}$) of $10 \mu\text{g mL}^{-1}$ gold and silver NPs. (B) Impedance–time traces (at 100 kHz) of NHDF collagen production rates containing $1 \mu\text{g mL}^{-1}$ silver NP added (a) to an empty chamber and (b–d) at different time points to the NHDF cell populations. In trace (d) after 12 h silver NP medium was exchanged with standard culture medium. (C) Impedance–time trace (at 160 kHz) of NHDF collagen production rate in the presence of $0.1 \mu\text{g mL}^{-1}$ silver nanoparticles over 24 hours.

investigate the effects of NP treatment on collagen production under microfluidic flow conditions, Ag NP concentrations were further reduced to $1 \mu\text{g mL}^{-1}$ and $0.1 \mu\text{g mL}^{-1}$ in subsequent experiments. Impedance–time traces of three NHDF cell populations treated with $1 \mu\text{g mL}^{-1}$ Ag NP for varying exposure times are shown in Fig. 7b. NHDF responses following NP administration at (b) $t = 0$ h, (c) $t = 3$ h and (d) $t = 4$ h showed similar impedance slopes of $-2.0 \pm 0.1 \Omega \text{ h}^{-1}$ over the remaining

NP exposure period, while in the absence of NHDF cells (a) the addition of $1 \mu\text{g mL}^{-1}$ Ag NPs caused a marginal increase in absolute impedance values. Since the contactless microsensors are also able to detect NP induced dielectric changes in the media, cellular collagen production prior, during and after NP administration was also investigated. The removal of Ag NPs from the medium supply after an 8 hour exposure time period (impedance–time trace d of Fig. 7b) did not show any signal recovery over the final six hours cultivation, thus pointing at the negative health effects of Ag nanoparticle uptake. Final nanotoxicological lab-on-chip measurement investigated accumulation effects using the non-cytotoxic concentration of $0.1 \mu\text{g mL}^{-1}$ Ag NP (see Table 4). Results displayed in Fig. 7c demonstrate that prolonged Ag NP up-take reduces fibroblast collagen production rates by about 50%, thus indicating that the internalized Ag NP concentration has reached a critical level after approx. 10 hours.

Conclusions

The assessment of cellular stress responses to NPs is of great relevance in the field of nanotoxicology where a large number of nanoparticles and different nanomaterials are being developed for *in vivo* clinical applications. However, no technology exists to adequately measure the health effects of nanoparticles on human cells⁴⁵ due to the many limitations associated with current endpoint detection methods.⁴⁶ Also, the majority of cell-based assays employ immortalized cell lines that are cultivated under static conditions, thus displaying phenotypes that are significantly different from those found in primary cell cultures. To address this technological void we have developed a lab-on-a-chip that combines contactless dielectric sensing with microfluidics to create a microdevice that features *in vivo* characteristics by minimizing cellular stress levels during cell cultivation.⁴⁷ The chip consists of four chambers (100 nL) designed to hold small cell populations ranging from 10^2 to 10^3 in size. The ability to cultivate small cell populations is that, in principle, small populations display more homogeneity, thus maintaining more stable conditions during experimentation. Another advantage of the chip design is that it allows the controlled application of similar cell numbers where complete sensor coverage can be readily achieved by applying cell seeding densities $>90\%$. Another benefit of the current assay is that it employs primary cells that maintain constant cell numbers during long-term measurements because they stop proliferating due to contact inhibition.⁴⁸ Since cell numbers are kept constant, the developed microfluidic cell analysis platform is capable of determining cell viability through the label-free detection of collagen production of normal human dermal fibroblast (NHDF) cells using four integrated interdigitated capacitors (μIDCs). In fact, we have demonstrated that on chip cultured primary NHDF cells continuously excrete collagen fibers in large quantities over a period of three days. Even in the presence of increasing flow velocities ranging from $28.7 \mu\text{m s}^{-1}$ to $278 \mu\text{m s}^{-1}$, NHDF collagen production appears to be marginally influenced by elevated fluid mechanical shear forces. We have also demonstrated that extended starvation periods (1% FCS) terminated cellular collagen synthesis altogether and that in the presence of two well known cytotoxins 5 mM acetaminophen and $1 \mu\text{M}$

valinomycin the collagen production rate decreased to 85% and 30%-remaining activity, respectively.

The microfluidic cell analysis platform was evaluated using commercially available Au and Ag nanoparticles that had been extensively characterized prior to testing. The characterization of NPs included dynamic light scatter (DLS) and transmission electron microscopy (TEM) measurements of commercially available 20 nm Au and 10 nm Ag NPs. Data revealed that in the presence of cell culture medium, both Au and Ag nanoparticles agglomerated to 50 nm and 100 nm, respectively. Based on the size of the nanoparticles, CFD simulations were performed to calculate the particle flow profiles of 100 nm Ag NP agglomerates, including time-dependent mass fractions and concentration gradients in the presence of laminar fluid flow conditions. CFD simulations demonstrated that NP distribution in the microchannels is uniform and independent from gravity. This means that NPs are transported homogeneously through microchannels, thus allowing for the controlled application of defined particle concentrations and exposure times. Precise control over concentration and exposure time is conducive to reproducible and reliable nanoparticle treatments.

Reference testing using standard *in vitro* cell-based assays confirmed that the newly developed collagen based cell assay can be used to assess the toxicity of NPs. Unlike static cell culture systems, the cell chip continuously supplies adherent cells with a homogeneous stream of NPs thus facilitating possible accumulation effects. The assessment of accumulation effect is important because it could result in increased cytotoxicity that overwhelms cellular defences over time. The fact that the newly developed microfluidic cell analysis platform can mimic realistic cell–nanoparticle interactions and assess repeated low exposure levels over time means that it is ideally suited to address safety concerns of regulatory agencies in the fields of labour law and consumer protection.

Acknowledgements

Financial support of the ZIT (Center of Innovation and Technology, Vienna Austria) is greatly acknowledged. Micro-fabrication was carried out in clean rooms of the Center of Micro- and Nanostructures, Vienna University of Technology.

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