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Effects of microenvironmental factors on assessing nanoparticle toxicity

Suyeong Seo,[ab] Ji Eun Lee,[c] Kangwon Lee *[de] and Hong Nam Kim *[afgh]

Nanoparticles, such as dust or fine particles produced from diverse sources, are regarded as hazardous materials to human organs, and the interest in understanding their biological mechanisms and evaluating the cellular response of nanoparticles is growing. The toxicity of nanoparticles is determined by several factors associated with not only their own properties, but also the surrounding microenvironmental factors of the cell. However, owing to its simplicity, *in vitro*-based evaluation of nanomaterial toxicity has been conventionally performed using a monolayer cell sheet cultured on a stiff surface in a static environment. Although 2D-based static models efficiently provide rapid assay results, the cytotoxicity of nanomaterials is frequently over- or underestimated due to the low consideration of the microenvironmental factors observed *in vivo*. To overcome the issue of low predictivity of conventional toxicity assays, many researchers have replicated some physiological factors when monitoring cellular responses induced by nanoparticles. In this review, we classify these microenvironmental factors and summarise their effects on cellular cytotoxicity evaluated in *in vitro* models that recapitulate the physiological microenvironment. Cellular toxicity upon exposure to nanoparticles was found to be different in the presence of physiological conditions, including vascular geometries, fluidic conditions, cyclic stretching, physical barriers, and cell–cell interactions; in some cases, these results are more like those observed *in vivo*. Our results imply that the consideration of microenvironmental factors is indispensable to provide more useful and reliable information than that provided by conventional 2D-based static *in vitro* testing platforms.

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Environmental significance

Air pollution has been considered a global issue that needs to be solved for a long time. As fine dust or nanoparticles penetrate the body and dysregulate our organ system, interest in their entry and toxic mechanisms has grown significantly. To predict their potential risk accurately and reliably, physiological factors (physical forces, cellular crosstalk, surrounding matrix, *etc.*) in the body must be considered because these nanoparticles show physiological stimuli-dependent localisation and toxicity. Therefore, many researchers have assessed the toxic effects of nanoparticles using *in vivo* physiology-mimetic platforms. This review is expected to be useful for anyone interested in toxicology studies of environmental pollutants.

1. Introduction

As technologies mature and industry evolves, the risk posed by environmental factors, such as fine particles from various sources, has been rising throughout the world.[1] In recent times, tiny particles with critical dimensions of a few to a few hundred nanometers, also called nanoparticles, have been increasingly considered as one of the main environmental toxicants in industrialised society.[2,3] As these hazardous fine particles in the ambient atmosphere can invade human organs *via* the respiratory organs or skin and ultimately cause diseases, research on the effect of nanometer-sized particles and their toxic mechanism has been performed.[4,5] Several studies have shown that environmental toxicants can induce apoptosis and autophagy at the individual cell level.[6–8] As cells are damaged, the structure and functions of an organ

[a] *Brain Science Institute, Kore Institute of Science and Technology (KIST), Seoul 02792, Republic of Korea. E-mail: hongnam.kim@kist.re.kr; Tel: +82 2 958 5617*
[b] *Program in Nano Science and Technology, Graduate School of Convergence Science and Technology, Seoul National University, Seoul 08826, Republic of Korea*
[c] *Center for Theragnosis, Biomedical Research Division, Kore Institute of Science and Technology (KIST), Seoul 02792, Republic of Korea*
[d] *Department of Applied Bioengineering, Graduate School of Convergence Science and Technology, Seoul National University, Seoul 08826, Republic of Korea. E-mail: kangwonlee@snu.ac.kr; Tel: +82 31 888 9125*
[e] *Research Institute for Convergence Science, Seoul National University, Seoul 08826, Republic of Korea*
[f] *Division of Bio-Medical Science & Technology, KIST School, Korea University of Science and Technology (UST), Seoul 02792, Republic of Korea*
[g] *School of Mechanical Engineering, Yonsei University, Seoul 03722, Republic of Korea*
[h] *Yonsei-KIST Convergence Research Institute, Yonsei University, Seoul 03722, Republic of Korea*

are gradually destroyed, and the disruption of organ-level function ultimately affects the whole body through the circulatory system.[9]

According to previous studies, nanoparticles have a more profound effect on cell viability compared to large-sized particles, since they can easily penetrate human tissue, be widely distributed throughout the body and be taken up by cells.[10] When evaluating the toxicity of nanoparticles, the characteristics of nanoparticles have been considered a critical factor due to their physiochemical property-dependent toxicity.[11] These properties include the material comprising the particles, their size and shape, and their surface chemistry.[12–14] For example, silver nanoparticles, which are known to induce more severe damage to cells than nanoparticles composed of other materials, showed higher toxicity when the particle size was smaller.[15] In the case of rod-shaped gold nanoparticles tested in a mouse model, the uptake of nanorods in the brain decreased as the particle size increased, while accumulation in other organs increased.[16]

In terms of surface chemistry, surface modification with polyethylene glycol (PEG) or polyethyleneimine (PEI) increases the uptake of nanoparticles by cells.[17] Because the combination of material composition, size, shape, and surface chemistry is extremely diverse, complete toxicity evaluation of diverse nanoparticles cannot be performed using *in vivo* models.[18]

In vitro models have been widely used as an alternative approach to animal models to assess the toxicity of engineered and air-pollution-related nanoparticles.[19,20] Despite the urgent need for precise estimation of nanoparticle-mediated toxicity, conventional 2D-based cell sheet models frequently show limited ability in toxicity evaluation due to the lower consideration of the physiological characteristics (cell–cell and cell–matrix interactions) of human organs.[21,22] As invading particles circulate through the bloodstream,[23,24] they are exposed to several physiological factors in the dynamic physical environment, including fluidic shear stress, cyclic stretching, physical transport barriers, and multicellular interactions.[25–27] These factors are known to significantly influence the movement and distribution of invading particles in the body. Additionally, the effect of particulate matter that can be inhaled through the respiratory system differs depending on breathing patterns, airway structure, and age.[28] This means that these physiological factors and individual characteristics must be reflected in *in vitro* studies to accurately predict the toxicity of nanoparticles.[29] The flow pattern can be determined by vascular structures, such as various diameters, curvatures, and branched paths, and can eventually influence the location of nanoparticles in the bloodstream and their uptake in the vascular endothelium.[30,31] Additionally, the 3D matrix surrounding the cells can hinder the diffusion of nanoparticles by physically and chemically trapping them within the matrix.[32,33] Therefore, physiological factors have been regarded as indispensable factors in determining the distribution of nanoparticles in the blood vessels or organs, as they affect particle–cell interactions.[30,34] For these reasons, several microfluidic devices mimicking *in vivo* environments have been designed and utilised for toxicity screening.[35–38] These devices allow monitoring of the interaction between nanoparticles and cells exposed to mechanical stress (fluidic shear stress and cyclic stretching) or cultured within the 3D matrix space.

In this review, we summarise the effects of physiological factors on the toxicity of various types of nanoparticles. Recent results indicate that mimicking physiological factors in the tissue microenvironment can enhance the prediction of the uptake and toxicity of nanoparticles. First, we discuss the limitations of current toxicity testing models. We then summarise recent experimental results that show the different uptakes and toxicities of nanoparticles in the presence of physiological stimuli. We believe that microenvironment-mimetic testing models may be used to predict the potential risk of nanoparticles more efficiently in *in vivo* models.

2. Nano–bio interactions *in vivo*: exploring the behaviour of nanoparticles

It is well known that nanoparticles interact with biological molecules upon entering the body due to their unique properties of nano-level size and large surface-to-mass ratio.[39] The physicochemical properties of nanoparticles affect the process of their penetration into the organ and ultimately induce changes at cellular and molecular levels. For example, smaller nanoparticles are prone to enter deep into the respiratory system[40] and induce enhanced uptake and severe damage to cells.[17,41–43] Since the nano–bio interaction can lead to cellular dysfunction, including dysregulated immune response and redox reactions, the following aspects in the nano–bio interactions need to be understood in order to evaluate the biological reactivity of the NPs.

2.1 Protein–nanoparticle corona (PNC) formation

Even though environmental nanoparticles enter human organs, they do not maintain their original surface characteristics. *In vivo*, proteins interact with nanoparticles and form a protein corona.[44,45] Once the NPs come into the bloodstream consisting of cells, their initial properties could be changed since proteins, such as serum albumin and apolipoproteins, aggregate on the surface of the nanoparticle to form a nanoparticle–protein corona.[46] As the properties of nanoparticles can be influenced by bound proteins in blood or serum, biochemical characterization of the particle–protein corona has been conducted by using various methodologies to investigate the structural and functional transformation.[39,47,48] 'Synthetic identity', which is intentionally designed properties in the synthesis process of nanoparticles, is transformed to 'biological identity' with changed values of size and surface charge.[49,50] There are two

types of protein coronas formed *via* a competitive binding governed by protein abundance, binding affinity, and exposure time: transient soft corona and stable hard corona.[51–54] Soft corona formation occurs within a short exposure time and features a composition of highly abundant proteins *via* transient low affinity interaction. If it exists in a static environment, over time, it could reach an equilibrium state and harden. In this procedure, weakly bound proteins are replaced with proteins with higher affinity, forming a hard corona.[51,52,55] However, the presence of physiological factors such as fluid flow and cellular metabolism delays or inhibits the protein corona from reaching equilibrium.[53,56]

Nanoparticles that have entered the body dynamically evolved due to sequential exposure to different molecules in a protein-rich environment.[39] Since the biological identity could cause an unexpected response including the behaviour and distribution of NPs and cellular interaction like uptake, the effect of physiological factors on the identity of NPs needs to be considered in order to predict their cytotoxicity.[46,57–59]

2.2 Effect of physicochemical properties of nanoparticles on PNC formation

Protein corona formation is significantly influenced by the synthetic identity of nanoparticles themselves, such as composition, surface chemistry, shape, size, radius of curvature, and hydrophobicity.[49,60] The physical properties of particles, such as surface area, shape, and size, are closely related to each other. Since most of the proteins in blood serum are smaller than NPs, proteins can easily bind to the surface of the particles.[46] It was reported that in the case of gold NPs, the relation between the size of particles and the bound amount of human serum albumin (HSA) was proportional.[61] Bosellli *et al.* used gold NPs with a diameter from 2 up to 5 nm and then showed that smaller NPs did not form a particle–protein corona.[62] The surface area of NPs closely associated with the shape also influences the protein binding. The large surface area of NPs enables serum proteins to adsorb onto the surface of NPs by providing a large contact area.[46] Rafaela *et al.* showed that a larger amount of proteins bound to AuNSs (gold nanostars) than AuNRs (gold nanorods) due to their higher surface area,[63] and Moustaoui *et al.* reported that spherical-shaped gold NPs showed higher binding affinity than branched-shaped NPs.[64] In addition to the surface area and shape, the radius of curvature of NPs also influences the protein binding affinity. The NPs possessing a large radius of curvature, which is directly related to size and shape, feature a planar surface which provides a wide area for protein binding.[46]

The chemical properties of nanoparticles also affect their interaction with blood proteins. Generally, the cell membrane has many negatively charged groups on the surface, while a small positively charged portion also exists.[65] Therefore, positively charged nanoparticles are known to more easily penetrate the cytoplasm.[66] Anionic particles have a tendency to favourably bind to a protein having an isoelectric point (pI) higher than 5.5, while cationic particles prefer to absorb a protein with a pI value lower than 5.5.[50] The bandgap of the nanoparticle is known to correlate with the toxicological impact on cellular and whole animal levels.[67] When proteins are attached to the surface of particles due to several factors, they have a larger body and uneven surface with changed physiochemical properties.[39] The accumulation of proteins increases the size of NPs by 3–35 nm and makes the particles more negative with changed surface charge in the range from −10 to −20 mV.[50,55,68–70]

The complexity of the cell microenvironment also regulates nanoparticle–tissue interactions. The porosity of the extracellular matrix (ECM) is a critical factor in the transport of nanoparticles through the tissue.[71] The basement membrane has a more highly crosslinked and dense matrix structure than the interstitial matrix.[71] In the case of the ECM with small pore size, large particles are physically obstructed while small particles are allowed to be transported.[72] The local charge of the ECM controls the penetration of nanoparticles through the pores *via* repulsive electrostatic interactions.[73]

2.3 Impact of PC formation on cellular interactions

In addition to changes in the bare particles, structural changes also occur in the bound proteins, such as abnormal unfolding and epitope formation, which eventually initiate fibril formation or an unexpected immune response.[74–77] These conformational changes of bound proteins on the corona may affect its interaction with other proteins and cells associated with cellular signalling and uptake pathways.[39,78] For example, apolipoproteins, which are often identified in the protein corona as bound proteins, are known to facilitate receptor-mediated endocytosis.[39,49] Lunov *et al.* reported that enrichment of serum in media changed the kinetics of uptake of polystyrene NPs by macrophages from clathrin-mediated endocytosis to phagocytosis.[79] Deng *et al.* confirmed that fibrinogen in blood plasma bound to the negatively charged-NPs and then unfolding of bound proteins occurred, which resulted in the activation of THP-1 cells to secrete high levels of inflammatory cytokines.[80]

Exposure to nanoparticles induces the production of reactive oxygen species (ROS), which results in many toxicological effects in humans.[81,82] Therefore, ROS production has been used as an indicator of cellular and tissue damage.[83,84] The NPs entering the biological fluid interact with contact components and then produce or scavenge reactive oxygen species (ROS). Interestingly, animal models and cellular models showed different degrees of ROS production depending on the physicochemical properties of the nanoparticles.[85,86] The ROS-generating ability of NPs can be utilized in nanomedicines to kill targeted cells (*i.e.*, cancer cells) for therapeutic purposes; however, it could induce toxicity to the nontargeted normal cells.[87] For example, zinc oxide (ZnO) NPs induced the production of ROS and zinc

ions, and then induced toxicity to human umbilical vein endothelial cells (HUVECs).[88] Gaharwar *et al.* reported that the intracellular uptake of iron oxide (IO) NPs induced a morphological change of lymphocytes with increased levels of ROS and DNA damage followed by cell death.[89] Silver (Ag) NPs, palladium (Pd) NPs, palladium–iridium (Pd@Ir) NPs, graphene oxide (GO), and graphene quantum dots (GQDs) also showed ROS-generating ability.[90–94]

These findings led to the development of strategies to prevent protein aggregation around the NPs. To inhibit nonspecific binding of proteins to NPs, NPs are commonly coated with polyethylene glycol (PEG), which is called 'PEGylation' for the stealth effect, for longer circulation in blood.[39,95,96] The other approach, siliconate treatment, is also used to block protein adsorption.[39,97] However, despite all these efforts, the protein aggregation around the NPs still remains challenging.[98] Therefore, understanding the reactions at the nano–bio interface in the procedure of corona formation is essential in predicting and assessing the unexpected cytotoxicity of NPs. As mentioned above, because the characteristics of nanoparticles are extremely diverse, the method of interaction cannot be simply explained. In this review, we focus on the effects of the cellular microenvironment on the toxicity of nanoparticles rather than their intrinsic properties.

3. Conventional experimental models for toxicity evaluation of nanoparticles

In recent years, environmental toxicity has been largely assessed using two representative models: 2D cell culture and *in vivo* animal models.[36,99] In the case of 2D cell culture models, cells were cultured in a well plate with controlled cell numbers and subsequently exposed to nanoparticles suspended in the culture media. This 2D-based cell culture system is easy to carry out and allows high-throughput screening, depending on the particle type and concentration.[100] As a more advanced version, the Transwell™ system could be utilised to track the damage in the cell monolayer and to allow cell–cell interactions through the pores.[101] However, these 2D-based systems only present (1) a static environment in which the culture media remain stationary throughout the culture period, (2) limited cell–cell interactions, especially for direct physical contact, and (3) a limited number of cell types in a single culture vessel.[102] Owing to the static environment, the introduced particles are often deposited over the cultured cells, and thus toxicity is frequently overestimated due to excessive exposure to the cell membrane. In terms of the physical cell–cell interaction, the cultured cells in the well plate usually make cell–cell contact in two dimensions, and the Transwell™ system does not allow direct cell–cell contact through the long and shallow pores. Furthermore, 2D cultured cells could not induce a concentration gradient of cytokines and form a physical barrier that inhibits particle penetration. Thus, cytokines and particles can access cells without limits.[103,104]

Animals have been widely used as *in vivo* models in such studies.[19,37] They have strong potential for mimicking the circulation of toxicants across the bloodstream, accumulation in a certain tissue, phagocytosis in the tissue by the immune cells, and excretion through the urinary system.[37] Furthermore, in addition to cell-level toxicity, the loss of organ-level function can also be evaluated using animal models.[105] To test particle toxicity, an animal model, usually a murine or a rat model, is exposed to aerosols that contain airborne particles for several weeks and allowed to inhale particle-bearing aerosols through the respiratory system.[106] Finally, the animals were sacrificed for end-point analysis, such as accumulation in a certain organ, tissue damage depending on the exposure duration, and loss of organ function over prolonged exposure.[107] Despite the promising ability of animal models, they also have several limitations, including (1) the inability of real-time monitoring, (2) inherent differences in human organ physiology, (3) different responses to external stimuli, and (4) the impossibility of decoupling of various experimental conditions to unravel the underlying mechanisms.[108]

To address these issues, multiple types of microphysiological systems have recently been developed and applied in toxicology studies.[29,109–111] Microphysiological systems refer to tissue models engineered to mimic the physiological aspects of human tissue. Microphysiological systems include (1) a cell spheroid that mimics tight cell–cell interaction in the 3D tissue, (2) a microfluidic system that can recapitulate the fluidic environment observed in the circulation system, and (3) an organ-on-a-chip model that presents a tissue-like mechanical environment to the co-cultured cells, as well as the fluidic environment.[112–114] By recapitulating the various aspects of human tissues, the transport and accumulation of particles, particle–cell interactions, and the number of cell-secreted cytokines could be monitored in real time. Thus, the mechanism of particle-mediated tissue damage can be evaluated with the help of microphysiological systems.[35,36,38,109,115–118] In the following sections, the important aspects of the microphysiological systems are reviewed, and the mechanisms of their effects on toxicity testing are described.

4. Important engineering aspects in modeling *in vivo*-like toxicity effects

In this section, the physiological stimuli that may affect the toxicity testing results are described, with a particular focus on their bio-mimicking aspects (Fig. 1).

4.1 Vascular geometry

There is a complex vascular network in the body that provides a path for circulating cells and transporting essential molecules for metabolic processes.[119] Blood vessels vary in

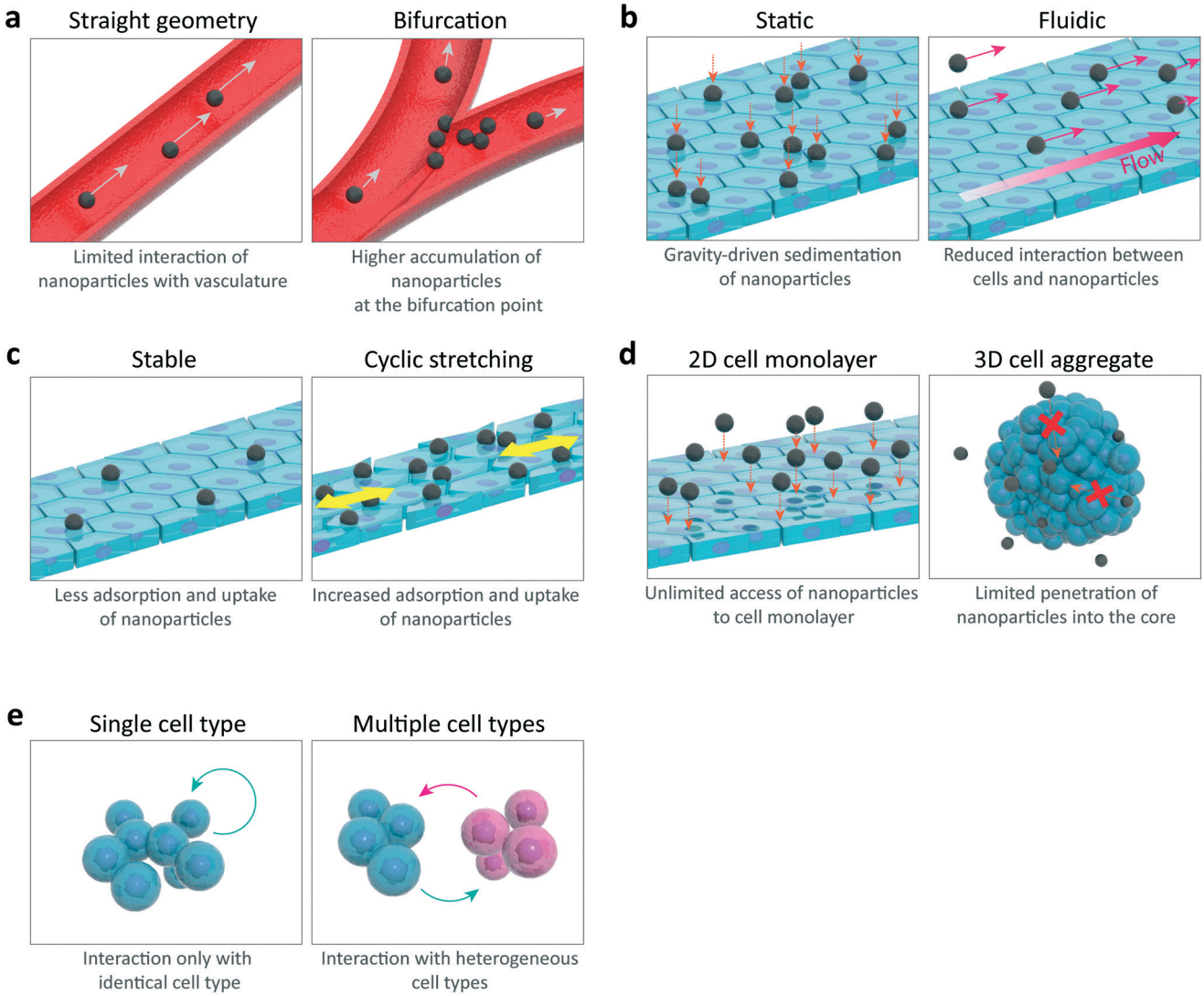

Fig. 1 Graphical summary of physiological factors that can affect nanoparticle-mediated toxicity testing. (A) Effect of vascular geometry; accumulation of nanoparticles increases near the branched or curved vessel due to the disturbed stream. (B) Effect of fluidic conditions; the fluidic environment reduces the interaction between cultured cells and nanoparticles. (C) Effect of cyclic stretching; the cyclic stretching environment increases the adsorption and uptake of nanoparticles. (D) Effect of physical transport barrier; the dense cell aggregate prevents the entry of nanoparticles into the deep core and thereby limits the toxic effect only at the cells located at the surface of cell aggregates. (E) Effect of cell–cell interaction; the co-culture of heterogeneous cell types facilitates soluble factor-mediated cell–cell interaction.

structure, size, and physiological/pathological conditions. These factors are considered to be critical factors that influence the distribution and localisation of nanoparticles circulating in the body.[120] The blood vessel network consists of various vessel shapes: straight, curved, and branched. Depending on their vascular geometry, the blood flow pattern changes, which consequently affects particle accumulation.[14,121] A higher accumulation of nanoparticles occurs at the intersection of the parent and daughter vessels in which the blood streamline is changed. Along the branched site, the blood flow is disturbed by a low wall shear stress, which facilitates the binding of particles to the surface of the vessel.[121–124] Gomez-Garcia *et al.* verified the relationship between the vascular type, blood flow pattern, and particle distribution in the zebrafish vasculature[31] (Fig. 2A). Nanoparticles were preferentially localised to the branched site, where the wall shear stress was the lowest. On the other hand, in a straight vessel, laminar flow enhances the force parallel to the flow and eventually decreases the interaction between the endothelium and nanoparticles. Tan *et al.* simulated the binding capabilities and adhesion patterns of two kinds of nanoparticles (spherical and rod-shaped), depending on the local vascular structure and flow conditions.[30] Except for the high flow rate, the density of bound nanoparticles was higher in the branched point than in the straight channel. The effect of vascular structure on the interaction of the endothelium with circulating particles was also demonstrated by Prabhakarpandian *et al.*[123] (Fig. 2B). The high binding affinity of particles at the branched site has been proven in both experimental and simulation studies.

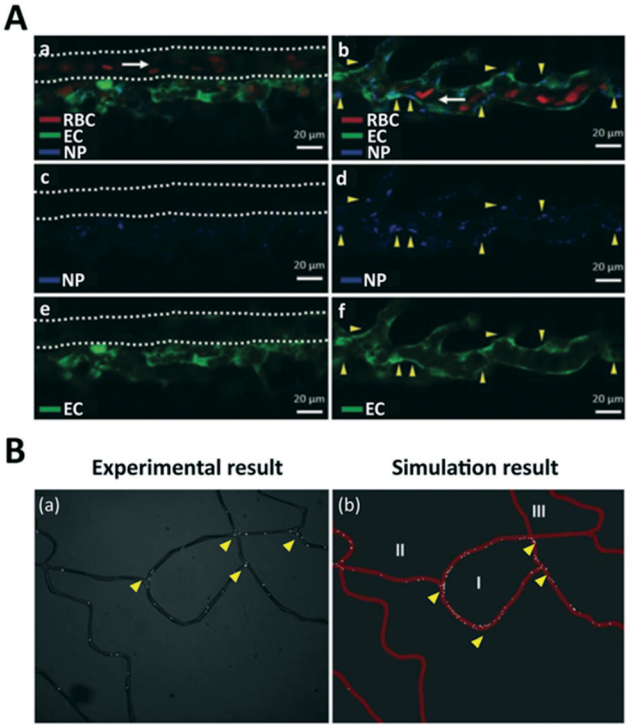

Fig. 2 The effects of vascular geometry on the uptake of nanoparticles. (A) Zebrafish embryos were exposed to 200 nM carboxylate-coated polystyrene NPs for 60 minutes. Accumulation of nanoparticles was compared in the two types of blood vessels: the dorsal aorta (a, c, and e) and ventral vein (b, d, and f). A higher accumulation of NPs was observed at the branching or curved regions (yellow arrows) in the vein. Reproduced from Gomez-Garcia *et al.*[31] with permission from *Nanoscale*, copyright 2018. (B) Anti-P-selectin conjugated 2 μm particles (yellow arrows) were preferably deposited near the bifurcations compared to the linear region of synthetic microvascular networks. The adhesion points of nanoparticle can be estimated by using computational simulation of the shear rate and the simulation results are well matched with the experimental results. Reproduced from Prabhakarpandian *et al.*[123] with permission from *Biomed Microdevices*, copyright 2008.

Additionally, vascular physiological/pathological conditions also influence the distribution and localisation patterns of nanoparticles. In pathological conditions such as atherosclerosis and vascular malformation, the vessels show a disorganised and irregular structure, with pores or plaque disturbing the blood flow. These diseased conditions of the endothelium are known to alter the blood flow profile, wall shear stress, and pressure.[120,125,126] Many researchers have investigated the preferential accumulation of nanoparticles in angiogenic vessels, such as tumor-angiogenic tissue, due to their increased leakiness.[127–130] This means that the vascular characteristics of individuals must be considered to completely predict the toxicity of nanoparticles. When nanoparticles are infused and circulate in the body, they interact with blood in a dynamic environment. Within the suitable range of fluidic shear stress and residence time, the circulating nanoparticles can interact with the endothelium.[131] Although the vascular structure is one of the critical factors influencing the nanoparticle distribution, many studies have focused on the study of particle geometries, including size, shape, and surface charge. Since the vascular geometries also determine the bloodstream and residence time of nanoparticles within the vessel or tissue, the physical properties of the vessel must be replicated in developing a predictive platform to reliably predict the toxicity of nanoparticles.

4.2 Fluidic conditions

In the human body, tissues such as the blood vessels, lymphatic vessels, heart, and kidney are consistently exposed to fluidic conditions due to the flow of blood and urine.[132] In addition to the fluidic-shear-stress-sensitive tissues, most tissues induce interstitial flow through the tiny space between the cells, which is relatively slow (~0.1 dyn cm^{-2}) compared to the flow velocity of blood and lung (1–30 dyn cm^{-2}).[133] Under fluidic conditions, floating particles are continuously supplied and cleared along with the body fluid. Under such conditions, the dwelling times of the particles in the unit volume of tissue are quite limited, and the particle–cell interaction is also suppressed. However, in the 2D static culture system, the suspended particles are introduced into the confined volume and ultimately settle over the cultured cell monolayer within a few hours. This sedimentation of particles increases the chances of particle–cell interaction and prohibits their clearing.[38] Therefore, cells exposed to particles under static conditions may exaggerate the toxic effects of particles compared to the fluidic culture model.

A study by Mahto *et al.* demonstrated the effect of fluidic culture conditions on the toxicity of quantum dots (QDs)[134] (Fig. 3A). In this study, the mouse embryonic fibroblast cell line, BALB/3T3, was exposed to QDs (core/shell: CdSe/ZnSe) under both static and flow conditions for a total exposure of 12 h. Cells showed remarkable differences in viability, with values of 30% and 75% upon exposure to 40 pM QDs under static and fluidic conditions, respectively. Furthermore, the cells showed more deformed morphology when exposed to QDs under static conditions than under fluidic conditions. Rothbauer *et al.* confirmed the importance of fluidic conditions by real-time monitoring of impedance change.[135] When the H441 lung papillary adenocarcinoma cell line was exposed to 240 μg mL^{-1} of toxic silica nanoparticles (AmSil30) under static and flow (4 and 40 μL min^{-1}, respectively) conditions, the impedance drop increased as the flow rate increased, indicating cell proliferation. However, under static conditions, the impedance did not increase or decrease, suggesting that there was no proliferation in this case. These results imply the crucial role of fluidic stimulation in the estimation of nanoparticle-induced toxicity.

In addition to cell viability, fluidic conditions also affect the cellular uptake of nanoparticles. When human umbilical vein endothelial cells (HUVECs) were exposed to negatively charged CdTe QDs, cellular uptake was enhanced in the presence of fluidic shear stress[136] (Fig. 3B). Interestingly,

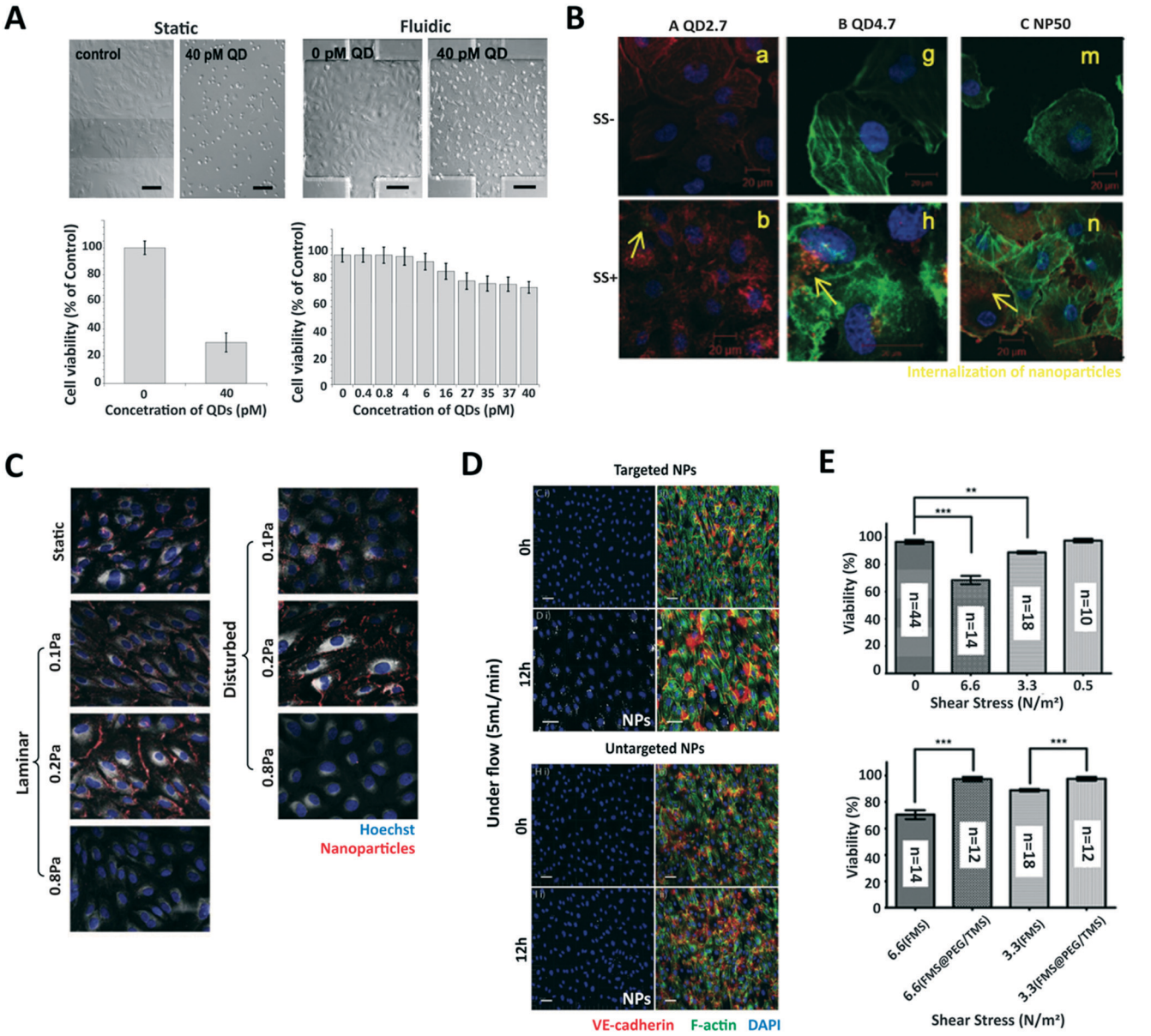

Fig. 3 The effects of fluidic conditions on the interaction between nanoparticles and cells. (A) When the fibroblast cells were exposed to quantum dots, morphological change of cells and cytotoxicity were more significant under static conditions. QDs reduced cell viability in a dose-dependent manner under fluidic conditions. Reproduced from Mahto *et al.*[134] with permission from *Biomicrofluidics*, copyright 2010. (B) The internalization of particles (QD2.7, QD4.7, and NP50, yellow arrows) was observed only in the presence of shear stress. Reproduced from Samuel *et al.*[136] with permission from *Int J Nanomed*, copyright 2012. (C) Nanoparticle–cell association differed depending on the magnitude of shear stress and flow pattern. A high accumulation of nanoparticles was observed in the disturbed region at a low level of shear stress. Above the threshold, particles were not localized on the cell membrane due to the short contact time caused by high flow velocity. Reproduced from Gomez-Garcia *et al.*[31] with permission from *Nanoscale*, copyright 2018. (D) The uptake of targeted gold nanoparticles into the endothelial cells was decreased under fluidic conditions due to insufficient residence time on the cell membrane. Reproduced from Chen *et al.*[131] with permission from *Adv. Mater*, copyright 2020. (E) The cytotoxicity of mesoporous silica nanoparticles to the human endothelial cells under different shear stress conditions. When the endothelial cells were exposed to the nanoparticles, high shear stress (6.6 N m^{-3}) induced notable cell death. Surface modification of nanoparticles (FMS@PEG/TMS) inhibited the nonspecific interaction with endothelial cells and thus decreased the cytotoxicity. Reproduced from Kim *et al.*[149] with permission from *Anal Chem*, copyright 2011.

when the fluidic shear stress rates were varied at 0.05, 0.1, and 0.5 Pa, the maximum cellular uptake was observed under the low fluidic shear stress of 0.05 Pa; this enhanced cellular uptake was not induced by treatment with tumour necrosis factor-α (TNF-α) or a low concentration of Triton X-100 solution. Gomez-Garcia *et al.* exposed the nanoparticles to endothelial cells (ECs) under static conditions and the presence of wall shear stress to study the effect of the flow pattern (laminar or disturbed flow) and wall shear stress (0.1 Pa, 0.2 Pa, and 0.8 Pa) on the nanoparticle distribution[31]

(Fig. 3C). A higher accumulation of NPs was observed at low shear stress under laminar and disturbed conditions, suggesting that increased shear stress decreased the contact time between NPs and the endothelial surface and NP–cell adhesion probability. These results emphasise the crucial role of fluidic conditions in the estimation of the cellular toxicity and uptake of cytotoxic nanoparticles.

Nanoparticles can be actively transported across the endothelium through endocytosis, during which they bind to specific receptors on the ECs to be internalised. This endocytic transport procedure is mediated by a specific signalling process and requires sufficient ATP to support cellular uptake.[137] The two major mediators of endocytic transport are clathrin and caveolae, which are polymerized around the cell membrane and independently engaged in the formation of endocytic vesicles and their transport.[138,139] Nanoparticles bind to receptors on the plasma membrane of ECs and are engulfed through the formation of clathrin- or caveolae-coated vesicles. Once the clathrin-coated vesicles are internalised into the cell, the coating is expelled, and the vesicles fuse with early endosomes to be transported. Meanwhile, caveolae-coated vesicles merge with the caveosome, which protects these complexes from lysosomal degradation.[140] Caveolae-mediated endocytosis is associated with mechanical stress derived from blood flow due to its mechanosensitivity. Caveolae, which are an assembly of caveolins and cavins, are linked to actin *via* actin-binding protein.[141,142] When ECs are exposed to fluidic shear stress, the actin filaments that play a crucial role in the distribution and organisation of caveolae align along the direction of the flow. Under flow conditions, caveolae biogenesis was observed, and its density increased. Additionally, signalling pathways, including those of tyrosine kinase receptors, integrins, and caveolae associated with the endocytic process, have also been reported to be activated.[143,144] For example, shear force was reported to activate the RhoA/ROCK signalling pathway, which is one of the intracellular signalling pathways associated with actin rearrangement. When the endothelium was treated with Y-27632 under dynamic conditions, the uptake of nanocarriers was inhibited without actin remodeling.[145] Additionally, when the dynamin-dependent endocytic pathway was hindered by inhibiting the function of dynamin-GTPase, the uptake of cationic nanoparticles was observed to decrease.[146]

Furthermore, previous studies have shown that fluidic shear stress enhances the production of ATP required for endocytosis.[147] Bodin *et al.* demonstrated that the release of ATP was elevated in the presence of shear stress, and high levels of fluorescent dye accumulated on the stimulated cells. When the level of cellular ATP was depleted by blocking the action of phosphoinositide 3-kinase, the uptake was inhibited only under static conditions.[148] These results indicate that mechanical stress induces the production of ATP from cells and facilitates the uptake of nanoparticles.

However, it seems that the uptake and toxicity of nanoparticles are significantly dependent on their surface chemistry, even under fluidic conditions. Chen *et al.* engineered a microfluidic chip integrated with a peristaltic pump and monitored the uptake of the designed nanoparticles under flow conditions of ref. 131 (Fig. 3D). An increased flow rate induced a reduction in the cellular uptake of nanoparticles due to decreased residence time in the endothelium. However, functionalized particles showed stronger binding affinity and resisted flow when compared to untargeted particles. Kim *et al.* demonstrated decreased cell viability as the fluidic shear stress increased from 0 to 6.6 N m^{-2} in the case of unmodified mesoporous silica nanoparticles[149] (Fig. 3E). However, in the case of highly organo-modified mesoporous silica nanoparticles, no significant toxicity to ECs was observed, even under flow conditions. These results indicate that it is critical to consider the *in vivo* physiological characteristics and properties of nanoparticles for designing nanomaterials such as drugs or diagnostic markers for biomedical applications.

4.3 Cyclic stretching

Lung tissues are always exposed to airborne particulate matter. For this reason, lung tissue has long been of interest to researchers studying inhalation toxicity. Lung tissue was exposed to cyclic expansion and shrinkage. When we take a closer look at the microscale tissue structure, the alveoli undergo constant cyclic stretching and release. Such a lung-specific dynamic microenvironment was recapitulated in the study by Huh *et al.*, in which they adapted microfluidic technology to fabricate miniaturised lung tissue models[25] (Fig. 4A). They cultured human alveolar epithelial cells and microvascular ECs attached to either side of a porous polydimethylsiloxane (PDMS) membrane, a type of silicone rubber. As PDMS has elastic mechanical properties, cyclic strain can be applied to the cell-bound membrane, and the cells attached on opposing sides of the membrane remained attached and even elongated following the cyclic strain of the porous membrane. When 12 nm silica nanoparticles were introduced into the alveolar epithelium side in the absence of cyclic strain, the production of ROS did not change. However, when the cyclic strain (10% at 0.2 Hz) was applied in parallel with the introduction of nanoparticles, a significant amount of ROS was generated. They confirmed that such cytotoxicity was attributed to the increased uptake of nanoparticles in the presence of cyclic strain, and this uptake was also confirmed in an *ex vivo* mouse lung model. Other research also demonstrated that the cyclic stretch induces the production of reactive oxygen species, and the increased level of reactive oxygen species ultimately enhances the permeability of cell membranes by activation of NF-κB and extracellular signal-regulated kinase (ERK)[150] (Fig. 4E). Huh *et al.* modeled drug toxicity-induced pulmonary edema by using an identical stretchable lung-on-a-chip microdevice.[151] Interestingly, upon exposure to interleukin-2 (IL-2) through the engineered endothelium, barrier permeability was notably increased in the presence of 10%

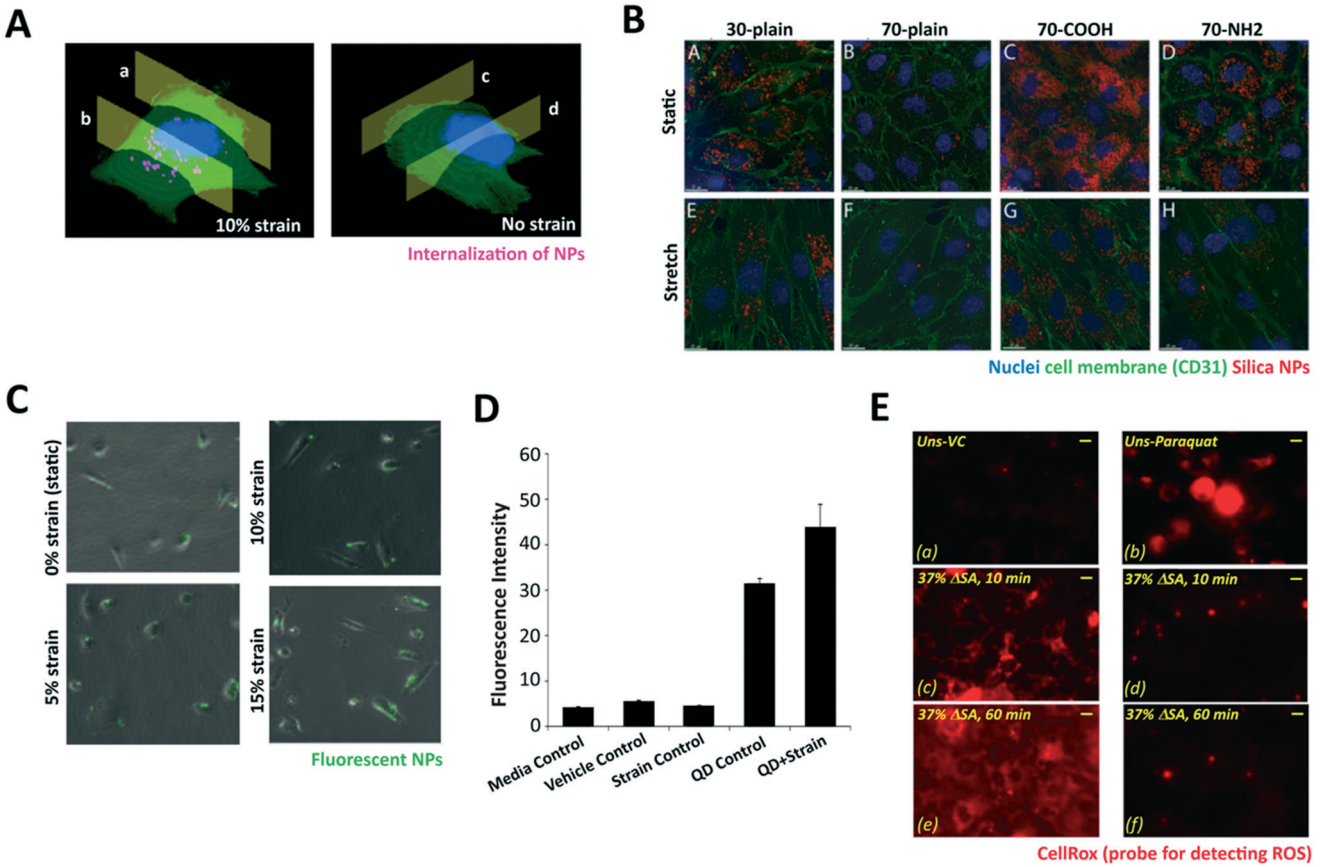

Fig. 4 The effects of cyclic stretching on the interaction between nanoparticles and cells. (A) Application of physiological mechanical force (10% strain) facilitated the internalization of fluorescent nanoparticles into the human pulmonary microvascular endothelial cells. Reproduced from Huh *et al.*[25] with permission from *Science*, copyright 2010. (B) Stretched cells showed more elongated morphology and hindered the cellular uptake of silica nanoparticles. Reproduced from Freese *et al.*[153] with permission from *Part Fibre Toxicol*, copyright 2014. (C) Nanoparticles were preferably localized within the cell cytosol when exposed to the cyclic strain. Higher cellular uptake of NPs was monitored at a higher strain level. Reproduced from Hu *et al.*[154] with permission from *Nanomater*, copyright 2015. (D) The highest uptake of quantum dots to human epidermal keratinocytes (HEK) was observed when the cells were exposed to 10% cyclic strain. Reproduced from Rouse *et al.*[155] with permission from *Toxicol in Vitro*, copyright 2008. (E) Production of reactive oxygen species was detected by using a fluorescent dye (CellRox). The reactive oxygen species increased in alveolar epithelial cells when they were stretched by 37% in surface area ((a) – vehicle control, (b) – positive control, (c) and (e) – stretched and treated with VC, (d) and (f) – stretched and treated with superoxide scavenger tiron). Reproduced from Davidovich *et al.*[150] with permission from *Am J Resp Cell Mol*, copyright 2013.

cyclic strain compared to the case of no strain. This result was corroborated by those of Stucki *et al.* who demonstrated an increase in permeability of small molecules, metabolic activity, and cytokine secretion by using a diaphragm-based lung-on-a-chip device, compared to non-strained cases.[152] Collectively, these results indicated that simulating cyclic stretching is a critical factor for modeling the normal and disease states of the human lung tissue as well as the testing of environmental toxicity.

Cyclic stretching was also observed in blood vessel structures exposed to heart-induced cyclic pressure variations. Furthermore, inhaled particulate matter circulates throughout the body through the bloodstream. Freese *et al.* tested whether cyclic stretching of the blood vessel endothelium may affect the damage upon nanoparticle exposure[153] (Fig. 4B) and found that, although the cytotoxic effects were not significantly different under static and dynamic stretching conditions (5% strain, 1 Hz), the internalisation of nanoparticles was decreased in the presence of cyclic stretching. They confirmed that the decreased internalisation of nanoparticles was due to decreased endocytosis. However, opposite results have been reported for carboxylate-modified fluorescent polystyrene nanoparticles. Hu *et al.* showed that the uptake of carboxylate-functionalized polystyrene nanoparticles in bovine aortic ECs increased as the strain increased from 0% to 15% (ref. 154) (Fig. 4C). They also found that once the cells were stretched, the increased uptake of nanoparticles lasted for 13 h, returning to a normal uptake rate 13 h after stretching. Rouse *et al.* demonstrated that human epidermal keratinocytes showed increased uptake of QD nanoparticles under cyclic strain[155] (Fig. 4D). As a result of the increased uptake of QDs, the cells showed decreased viability. Several mechanisms have been proposed to explain the cyclic strain-

induced changes in nanoparticle uptake. One study suggested that the addition of strain increased cytokine production and cell membrane permeability, resulting in irritation and increased uptake of nanoparticles.[155] Although little is known about the underlying mechanisms of nanoparticle uptake under cyclic stretch conditions, replicating the physiological conditions is indispensable not only for toxicology studies of nanoparticles but also for drug delivery evaluation in the future.

4.4 Physical barriers: extracellular matrix and tight cell–cell junction

Two factors can be considered as physical barriers to the movement of nanoparticles in the tissue: ECM and cell–cell junctions. When nanoparticles enter the body through the skin or lung, they encounter the ECM, which is composed of glycosaminoglycans (GAGs) and various fibrous proteins such as collagen, elastin, fibronectin, and laminin. Glycosaminoglycans (GAGs), which are a major constituent of the native ECM, are negatively charged and hydrophilic, with a high affinity for water molecules. Collagen, which is an abundant fibrous protein that accounts for 30% of the total protein mass of animals, organises into the hollow interfibrillar space around the cells, ultimately facilitating the diffusion of nutrients and wastes.[156] In addition to collagen, other fibrous proteins, including elastin and fibronectin, also mediate cell behaviour, growth, differentiation, and attachment to the surrounding ECM by providing a binding site to the integrins of the cell. Since the composition of these matrix proteins differs depending on the tissue, the heterogeneous physicochemical properties of the ECM are one of the factors that determine tissue specificity.[156,157]

As mentioned above, since the native ECM is characterised by a complex and charged network with a small pore size, the distribution of nanoparticles in the body is affected by the physiochemical properties of particles (shape, size, and charge) and the cell microenvironment (pore size and local charge of the ECM). The diffusion of the nanoparticles can

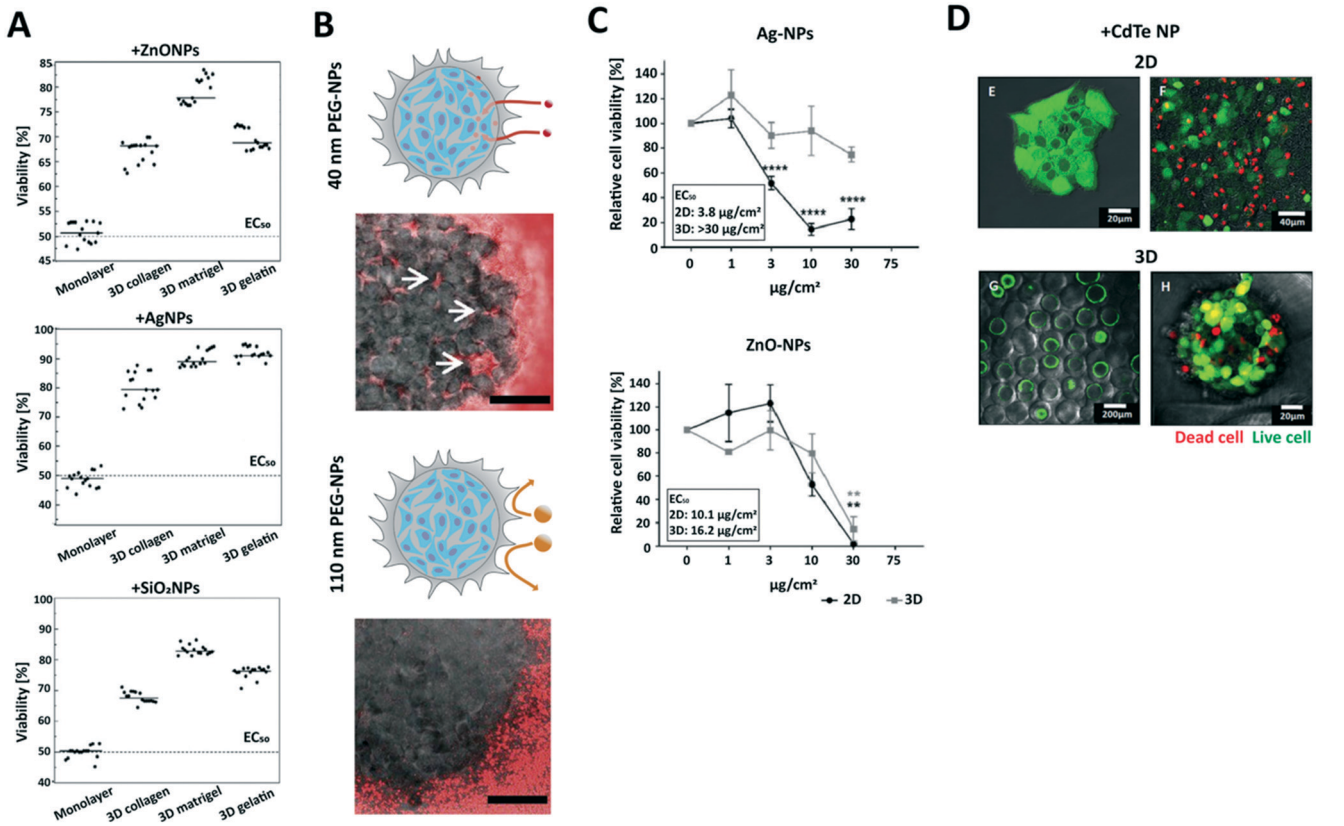

Fig. 5 The effects of the physical barriers on the penetration of nanoparticles into the cell aggregates. (A) HepG2 cells cultured in the 3D scaffold showed lower cytotoxicity of nanoparticles than the 2D monolayer culture did. Different toxic effects were observed in the 3D-cultured spheroids depending on the matrix types. Reproduced from Dubiak-Szepietowska *et al.*[161] with permission from *Toxicol Appl Pharmacol*, copyright 2016. (B) The penetration of PEGylated gold NPs into the interstitial space of the tumor spheroid depends on the particle size. Only small particles with a diameter of 40 nm entered the spheroid. Reproduced from Albanese *et al.*[168] with permission from *Nat Commun*, copyright 2013. (C) The cytotoxic effect of Ag-NPs and ZnO-NPs was higher in the 2D cultured cells than those cultured in 3D. A higher concentration of NPs was required to exhibit the identical toxic effects in 3D spheroid cultures compared to the 2D cultures. Reproduced from Elje *et al.*[174] with permission from *Nanomaterials (Basel)*, copyright 2020. (D) Higher cytotoxicity of CdTe NPs to HepG2 cells was shown under 2D culture conditions than in 3D spheroid cultures. Reproduced from Lee *et al.*[175] with permission from *Small*, copyright 2009.

be influenced by three types of factors in the ECM: (1) collision with matrix fibres, (2) slow diffusion due to restricted thermal motion of water molecules near the fibres, and (3) electrostatic interactions between charged particles and ECM components.[158,159] The ECM can physically regulate the diffusion of nanoparticles because of its complex mesh-like structure. Particles larger than the pore size of the interstitial matrix can be trapped in the ECM network. On the other hand, for particles with a diameter smaller than the pore size of the ECM, molecular transport is dominantly influenced by the surface charge. Negatively charged components of the ECM, especially GAGs, can attract positively charged particles and prevent them from passing through the ECM.[160]

The effect of the ECM in the *in vitro* assessment of nanoparticle toxicity was demonstrated by Dubiak-Szepietowska *et al.*[161] (Fig. 5A). They cultured spheroids of HepG2 cells (human hepatoblastoma cell line) within a 3D collagen type 1 or Matrigel matrix and exposed them to three types of nanoparticles: ZnONPs, AgNPs, and SiO_2NPs. Cell viability was measured to assess the toxicity. The highest toxic effect was observed in the matrix-free monolayer culture system because of the high contact area with the nanoparticles. However, the toxic effects were observed to be different depending on the particle and matrix types. All tested particles showed the highest toxic effects in collagen-cultured spheroids. Spheroids cultured in Matrigel exhibited the highest cell viability when exposed to ZnONPs and SiO_2-NPs. Collagen type 1 has a large pore size (approximately 1–20 μm) and a neutral charge.[162,163] Conversely, Matrigel is negatively charged with high hydrophilicity, and has a smaller pore size (approximately 0.3 μm) than collagen.[72,164,165] This means that the penetration of nanoparticles into the tissue is also significantly affected by the surrounding matrix. The physiochemical properties of the ECM are determined by the type, composition, and density of ECM-composing proteins, which vary between tissues. These results indicate that the properties of the tissue-specific ECM must be considered in *in vitro* studies of nanoparticle mobility.

Additionally, the physical barrier between neighbouring cells influences the transport and toxicity of nanoparticles. Cell spheroids are an aggregated form of anchorage-dependent cells, which are available by promoting cell–cell interactions and suppress cell–substrate interactions. Usually, cell spheroids are formed using engineering techniques such as the hanging drop method, concave microwells, and low-attachment surface treatment.[166] Cell spheroid models have been widely utilised in drug and environmental toxicity testing because they can reflect various microenvironmental factors, including tight cell–cell interactions and spontaneous formation of concentration gradients from the shell to the core.[36] In addition, the availability of mass production allows high-throughput screening (HTS) of toxicity.[167]

In cell spheroids, the entry of nanoparticles into the deep core is hampered by tight cell–cell junctions, acting as a physical transport barrier. To visualise such a physical barrier effect in nanoparticle delivery, Albanese *et al.* embedded a tumour spheroid on a microfluidic device by immobilisation *via* geometrical confinement (Fig. 5B).[168] As the microfluidic device was transparent and the tumour spheroid was immobilised at a fixed position, the transport of nanoparticles could be monitored under a microscope. When the spheroids were exposed to nanoparticles with sizes of 40, 70, 110, and 150 nm, a significant decrease in intra-spheroidal delivery was observed in relatively larger particles, including those with diameters of 110 and 150 nm. These results indicate that the exposure to nanoparticles larger than the critical dimension might be limited to the surface of *in vivo* tissues. Therefore, unlimited access to nanoparticles during toxicity testing using conventional 2D culture dishes may exaggerate the real exposure scenario. Such limited transport into the spheroid core has also been reported using nanoparticles[167,169–171] and drugs.[172,173]

As a result of the limited transport of nanoparticles into the deep core, the toxicity of nanoparticles observed in 3D cell spheroids is usually lower than that observed in the 2D cell sheet model. For example, Elje *et al.* showed that the toxicity of silver (Ag) nanoparticles against HepG2 liver cells was higher in 2D culture (EC_{50} = 3.8 μg cm^{-2}) than in the 3D spheroid model (EC_{50} > 30 μg cm^{-2})[174] (Fig. 5C). Lee *et al.* directly visualised the reason for reduced toxicity in 3D spheroid models using scanning electron microscopy (SEM) images, showing the undamaged inner cell mass after partially detaching the dead outer shell[175] (Fig. 5D). Considering that the *in vivo* tissues have a dense structure, and thereby limited availability of exogenous materials, the effect of the physical transport barrier must be addressed in the testing of nanoparticle-induced toxicity.

4.5 Co-culture of multiple types of cells

Over the last few decades, single-cell type cell lines are usually cultured in a well plate or in a spheroid form when testing the cytotoxicity of particulate matter. However, growing evidence indicates that cell–cell interactions may induce or aggravate the toxicity of particulate matter towards cells.

In the case of brain tissue, the importance of cell–cell interaction upon exposure to diesel exhaust particles (DEP) was demonstrated in a co-culture experiment of neurons and microglia. Here, Block *et al.* showed that the DEP did not show notable toxicity when the dopaminergic neurons were cultured alone but exhibited toxicity when the dopaminergic neurons were cocultured with microglia[176] (Fig. 6A). In this study, they confirmed that the notable damage of neurons observed in the presence of microglia was due to the microglia-produced ROS. The effect of ROS on neurotoxicity was confirmed in DEP-microglia conditioned media experiments[177] (Fig. 6B) and NADPH oxidase-deficient ($PHOX^{-/-}$) mice experiments.[176] Such microglia-mediated toxicity of neurons was observed in silver nanoparticles, but

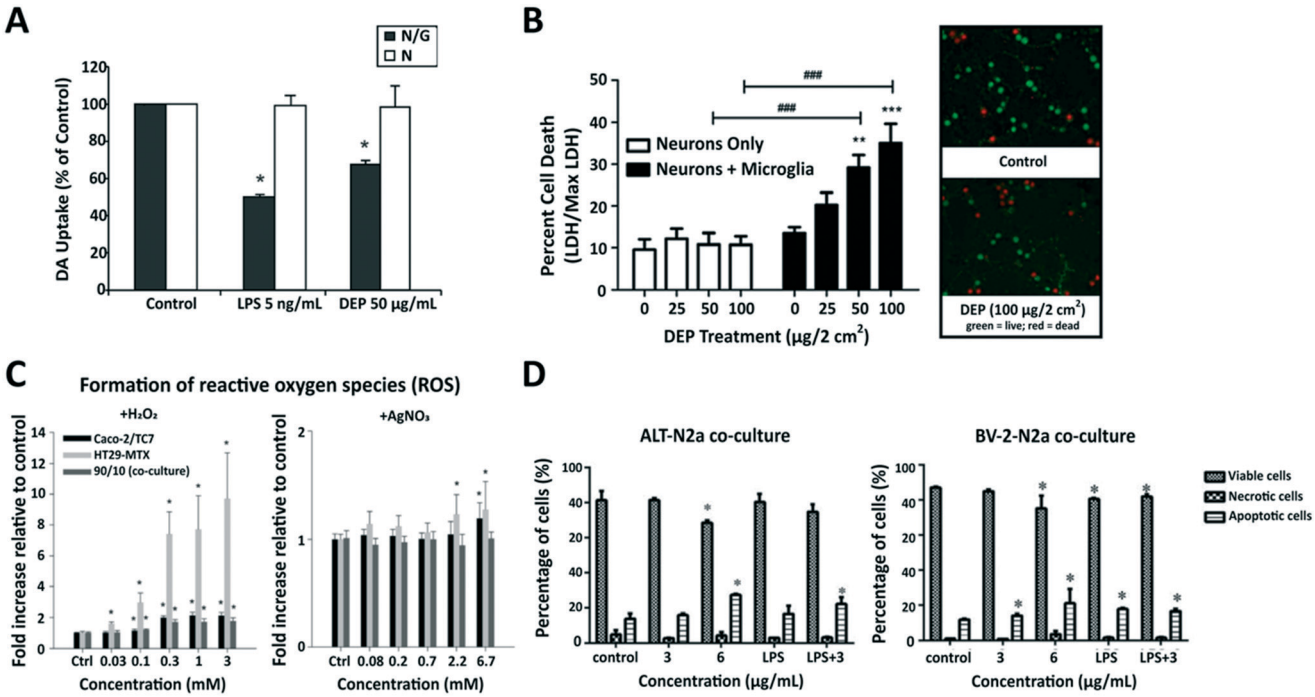

Fig. 6 The effects of the multicellular environment on toxicity, upon exposure to nanoparticles. (A) Dopaminergic (DA) neuron function was evaluated by measuring the cellular uptake of [^{3}H]DA. In the neuron–glia coculture case, lipopolysaccharides (LPS) and diesel exhaust particles (DEP) led to higher neurotoxicity compared with the neuron only case. Reproduced from Block *et al.*[176] with permission from *FASEB J*, copyright 2004. (B) DEP induced neuronal cell death only in the neuron–microglia coculture case while the neuron only group did not show any nanoparticle-induced toxicity. Reproduced from Roque *et al.*[177] with permission from *Neurotoxicology*, copyright 2016. (C) Exposure to H_2O_2 and $AgNO_3$ induced the production of reactive oxygen species (ROS) from the Caco-2/TC7 and HT29-MTX cells. Although 6.7 mM $AgNO_3$ elevated the production of ROS in mono-culture systems, there was no response in the coculture system. Reproduced from Georgantzopoulou *et al.*[181] with permission from *Part Fibre Toxicol*, copyright 2015. (D) The percentage of apoptotic N2a (neuroblastoma) cells was increased when they were co-cultured with ALT (astrocyte-like) or BV-2 (microglia-like) cells upon exposure to AgNPs. Indirect exposure to AgNPs under the coculture system (*i.e.* transfer of conditioned media) was more toxic to differentiated N2a cells rather than direct exposure of mono-cultured cells. Reproduced from Hsiao *et al.*[178] with permission from *Environ Toxicol*, copyright 2017.

the damage was mediated by H_2O_2 from astrocyte-like cells or NO from microglia[178] (Fig. 6D). Although microglia-mediated neuronal death is a known cause of nanoparticle-mediated brain damage, neuron-secreted factors also stimulate microglia to produce more neurotoxic factors. Cytokine- and soluble factor-mediated negative feedback between neurons and microglia resulting in the subsequent damage of neurons is known as reactive microgliosis.[179,180] Additionally, Georgantzopoulou *et al.* also demonstrated that intracellular ROS production decreased in the 90 : 10 co-culture system of Caco-2/TC7 and HT29-MTX cells when exposed to H_2O_2 and $AgNO_3$ (ref. 181) (Fig. 6C). These results clearly showed that the cytotoxicity test of particulate matter and nanoparticles in the culture of a single cell type may not be sufficient to estimate the mechanisms underlying cell damage.

From the previous examples, it can be hypothesised that cell-secreted cytokines may be responsible for nanoparticle-induced tissue damage. Along with ROS, altered levels of cytokines are widely observed in human and animal models exposed to particulate matter. For example, exposure to volatile organic compounds and fine particulate matter (PM2.5) is closely associated with nonsmoking chronic obstructive pulmonary disease (COPD), with a decrease in IL-5.[182] In a rat model, exposure to particulate matter during pregnancy increased the level of IL-4 cytokine in the foetal portion of the placenta.[183] In addition to these *in vivo* cases, several *in vitro* studies have also indicated the enhanced production of inflammatory cytokines when immune cells are exposed to particulate matter. Monn *et al.* reported 20 times higher proinflammatory cytokine levels of IL-6 and IL-8 upon exposure to fine (PM2.5) and coarse (PM2.5 to PM10) particulate matter in human monocytes.[184] Hetland *et al.* reported that alveolar macrophages produced higher levels of IL-6 and tumour necrosis factor-α (TNF-α) upon exposure to coarse particulate matter (2.5–10 μm) than to fine particulate matter (0.1–2.5. μm).[203] These studies indicate that immune cells residing in tissues produce large amounts of inflammatory cytokines, thereby causing secondary damage to nearby cells, or to the whole body, *via* circulation. Recent studies have showed that an indirect toxic effect was induced in a multicellular environment by the inflammatory cytokine or oxidative stress produced by cells exposed to air pollutants.[185–187] Therefore, consideration of the coculture environment, especially with immune cells, is necessary to estimate particulate matter-induced tissue damage and cytotoxicity.

The effect of the interaction between heterogeneous cell types is important in regulating fluidic condition-mediated cellular responses. Zhang *et al.* fabricated a 3D human lung-on-a-chip model by coculturing alveolar epithelial and endothelial cells and tested the toxicity of TiO_2 and ZnO nanoparticles.[188] They found that, in the case of monoculture of epithelial cells, there was no difference in permeability between static and perfusion conditions. However, in the coculture conditions of epithelial cells and ECs, the permeability under static conditions was higher than that under perfusion conditions, implying the synergistic effects of cell–cell interactions and fluidic conditions.

5. Perspective and conclusion

As the moral issue of animal models used in biomedical research, including efficacy or toxicity assays, has been debated, biomimetic technologies such as organ-on-a-chip and organoids have emerged as alternative platforms to accurately estimate *in vivo*-compatible results. In particular, organ-on-chips are regarded as innovative biotechnologies for toxicity evaluation because they can more accurately simulate the *in vivo* complexity and the local environment.[189] The most representative system would be the "lung-on-a-chip" and "heart-on-a-chip" integrated with an external source, such as a syringe or peristaltic pump, to induce mechanical stress. Many previous studies have shown that these miniaturised organ chips could provide closer physiological responses to organs than traditional 2D culture systems.[190,191] However, to accurately predict the cellular response and obtain more reliable preclinical data from these platforms, additional issues need to be considered.

First, cells that represent a human-specific phenotype must be introduced. Conventionally, immortalised cell lines have been widely used because of their ease of culture, cost efficiency, and batch-to-batch consistency. However, challenges remain to be solved for their use in drug screening and disease modeling.[192] These cell lines inevitably have several limitations, including limited tissue-specific function, low sensitivity to the cytotoxic environment, and low production of cytokines. Several studies have shown that immortalised cell lines are genetically and functionally different from primary cells.[193–196] As an alternative approach, primary tissue cells or induced pluripotent stem cell (iPSC)-derived tissue cells have drawn much attention in toxicology. These cells are known to present more *in vivo*-like responses in terms of cytokine secretion, mRNA expression, and recapitulation of tissue-specific cell functions. In particular, human-derived primary cells and iPSC-derived tissue cells can represent human-specific genetics and epigenetics, and are thus believed to be a reliable cell source that can mimic an individual's unique response to drugs or toxicants for toxicity testing in the future.[197,198]

Furthermore, the similarities in the physiological conditions under which the nanoparticles interact with tissue cells remain an unresolved issue. In this review, we presented five factors, including vascular geometries, fluidic conditions, cyclic stretching, physical barriers, and soluble factor-mediated interaction, as recently provided factors affecting cytotoxicity testing for mimicking the testing environment. The presence of these microenvironmental factors induced significant changes in cell viability, nanoparticle localisation and uptake, and soluble factor production. Consideration of the missing factors, such as electrical activity and pathological conditions, or the synergistic effect of microenvironmental factors described above may generate more *in vivo*-like results.

Additionally, the surface characteristics of nanoparticles are crucial factors that must be investigated. The surface characteristics of environmental nanocontaminants vary depending on the source and reaction mechanism.[2] In addition to the core materials, surface characteristics such as surface charge, hydrophilicity/hydrophobicity, and types of functional groups are known to significantly alter cellular uptake, binding with membrane-bound receptors, cytotoxicity, and clearance from the circulation system.[199] Although some studies have discussed the effect of these surface characteristics of nanoparticles, these studies are generally conducted in a 2D static environment. Considering that the interaction between nanoparticles and cells occurs in a 3D dynamic environment, which usually reduces the chance of interaction, the effect of surface characteristics of nanoparticles should be evaluated in a physiologically relevant *in vitro* microenvironment.

High-throughput screening (HTS) is also an important factor in toxicology testing using *in vitro* platforms.[200–202] In toxicology testing, the effects of exposure time, concentration, and cell type need to be considered. However, animal models cannot cover a wide range of exposure scenarios owing to ethical and cost issues. Although the 2D *in vitro* models could meet the needs of HTS, the dynamic or 3D *in vitro* models still pose limitations in establishing HTS. The development of a plate-compatible *in vitro* model is potentially beneficial for utilising readily available 2D well plate-based assay systems.

Finally, the similarity of the experimental results from *in vivo* and *in vitro* cases needs to be assessed. A pioneering study using the lung on a chip model showed increased cellular uptake and increased ROS production in the presence of cyclic stretching in both *in vivo* and *in vitro* cases.[25] However, the differences observed in static and dynamic conditions do not always support the superiority of the dynamic testing setup in cytotoxicity testing. A direct comparison with the *in vivo* results is needed to clearly demonstrate the benefits of the dynamic testing platform.

In conclusion, we have summarised the effects of physiological factors that must be considered in toxicology tests, including fluidic conditions, cyclic stretching, physical barriers, and soluble factor-mediated cell–cell interactions. The results consistently indicated that cell viability, cellular uptake of nanoparticles, and secretion of soluble factors are significantly affected by physiological factors, implying the

Table 1 Effect of microenvironmental factors on the toxicity of nanoparticles

Microenvironmental factors	Cell type	Nanoparticle type	Results	Ref.
Vascular geometry	*In vivo* zebrafish model	Carboxylate- coated polystyrene (200 nm)	Accumulation of NPs: higher in the branched and irregular areas.	31
	Acellular & synthetic microvascular model	Anti-P-selectin conjugated particles	Location of NPs: higher in the bifurcations of the vessel.	123
Fluidic conditions	BALB/3T3 mouse embryonic cell line	Quantum dot (core/shell; CdSe/ZnSe)	Cell viability: higher viability and low cell detachment under fluidic conditions	134
	H441 lung papillary adenocarcinoma cell line	Silica (AmSil30)	Cell viability: higher viability and proliferation of cells under fluidic conditions	135
	Human umbilical vein endothelial cell (HUVEC)	Quantum dots (CdTe) (2.7 nm and 4.7 nm) Silica (50 nm)	Cell viability: maximum viability in low shear stress of 0.05 Pa rather than 0.1 and 0.5 Pa Internalization of NPs: internalization of QDs and NPs only under fluidic conditions	136
	Human alveolar epithelial cells (EpiC) Human umbilicus vascular endothelial cells (EC)	TiO_2 ZnO	Permeability: higher in the coculture condition of EpiC and EC: higher in the static case than in the perfusion case	188
	Human endothelial cell	Mesoporous silica (less than 50 nm)	Cell viability: (unmodified mesoporous silica NPs) Decreased cell viability as the fluidic shear stress increases from 0 to 6.6 N m^{-2}: (highly organo-modified mesoporous silica NPs) No significant toxicity to EC even under flow conditions	149
	Human umbilical vein endothelial cell (HUVEC)	Carboxylate coated polystyrene (200 nm)	Internalization of NPs: higher intercellular uptake of NPs in the appropriate shear stress of 0.2 Pa: higher uptake of NPs in disturbed region	31
	Human umbilical vein endothelial cell (HUVEC)	Gold (100 nm)	Accumulation of NPs: lower in the high flow rate due to reduced residence time between particles and endothelium	131
Stretching	Human alveolar epithelial cells	Ultrafine silica (12 nm)	ROS production: increased ROS generation under 10% of cyclic stretching	25
	Human microvascular endothelial cells	Polystyrene (100 nm)	Internalization of NPs: increased uptake of polystyrene NPs under 10% cyclic stretching	
	Human umbilical vein endothelial cell (HUVEC)	Silica (30 nm and 70 nm)	Internalization of NPs: decreased uptake under stretching condition	153
	Bovine aortic endothelial cell (BAEC)	Carboxylate-modified fluorescent polystyrene (50, 100, and 200 nm)	Internalization of NPs: increased uptake as the strain increased from 0% to 15%	154
	Human epidermal keratinocyte (HEK)	Polyethylene glycol (PEG)-coated quantum dot (core/shell; CdSe/ZnS)	Internalization of NPs: increased cellular uptake of QD in 10% cyclic stretching Cell viability: decreased viability in the presence of 10% cyclic strain	155
	Primary rat alveolar epithelial cell	No particle (only cyclic stretching)	Permeability: increased by cell-produced ROS in the presence of cyclic stretching NF- κB activation ERK phosphorylation: induced by ROS produced by cells in cyclic stretching	150
Physical barrier (extracellular matrix)	HepG2 (liver spheroid)	ZnO Ag, SiO_2	Toxicity: the highest toxic effect in the collagen-cultured spheroid (all particles): the lowest toxic effect in the Matrigel-cultured spheroid (ZnO NPs and SiO_2 NPs)	161
Physical barrier (bicellular junction)	MDA-MB-435 (tumor spheroid)	Gold (15, 30, 70, and 100 nm)	Penetration and accumulation of NPs: decreased in the interstitial space of spheroid as the size of nanoparticle increases	168
	HepG2 (liver spheroid)	TiO_2 Ag ZnO	Toxicity: stronger in 2D cultures than in 3D spheroid cultures	174
	HepG2 (liver spheroid)	CdTe Gold	Penetration of NPs: protected inner cells in the spheroid models	175
Cell–cell interaction	Primary neurons (rat and mouse) Primary microglia (rat)	Diesel exhaust particles (DEP)	Neurotoxicity: higher in the presence of microglia: low neurotoxicity in NADPH oxidase deficient mouse	176
	Primary mouse cerebellar granule neuron (CGN) Primary mouse microglia	Diesel exhaust particles (DEP)	Neurotoxicity: neuronal cell death in the coculture condition: neuronal cell death by the microglia-conditioned medium (DEP treated)	177
	Murine neuroblastoma Neuro-2a (N2a) cell	Silver	Neurotoxicity: NO release from microglia: H_2O_2 from astrocyte-like cells	178

Table 1 *(continued)*

Microenvironmental factors	Cell type	Nanoparticle type	Results	Ref.
	Murine brain astrocyte-like ALT cell Murine microglial BV-2 cell			
	Human primary monocyte	PM_{10} $PM_{2.5}$ (collected)	Cytokine (IL-6 and 8): 20 times higher upon exposure to particulate matter	184
	Primary rat alveolar macrophage	PM_{10} $PM_{2.5}$ (collected)	Cytokine (IL-6): higher upon exposure to PM_{10} than $PM_{2.5}$	203
	Human alveolar epithelial cells (EpiC) Human umbilicus vascular endothelial cells (EC)	TiO_2 ZnO	Permeability: lower when EC was cultured with EpiC: lower under fluidic conditions	188
	Human umbilical vein endothelial cell (HUVEC) Human astrocyte (HA)	Indoor airborne particulate matter (collected)	Astrocyte activation: increased proliferation of HA as a result of INPM exposure oxidative stress: increased level of oxidative makers related to neurotoxicity	204
	Maternal decidual cells (DECs) Fetal chorion trophoblast cells (CTCs) Amniotic mesenchymal cells (AMCs)	Cadmium (Cd)	Cadmium transport: Cd propagation to the cells in neighboring chamber toxicity: cell death in the DEC chamber due to direct exposure of Cd: indirect toxic effect in the CTC chamber: no toxic effect in the AEC chamber due to barrier structure	185
	Amniotic epithelial cells (AECs)		Cytokine (IL-10, TNF-α): increased level of TNF-α: no change in IL-10 level	
	Human cerebral microvascular endothelial cell line (hCMEC/D3)	$PM_{2.5}$	Neurotoxicity: astrogliosis and microgliosis-mediated neurotoxicity when exposed to $PM_{2.5}$: pTau accumulation	186
	Human neural progenitor cells (ReNcell VM) Human adult microglia (SV40) cell line		Cytokine: increased production related to microglia recruitment (CCL1, CCL2) and activation (IL-1β, IFN-γ)	
	Human umbilical vein endothelial cells (HUVECs)	Au (control)	Cardiac toxicity: increased time to peak: lower amplitude of calcium transient	187
	Human cardiac microvascular endothelial cells (HCMVECs)	CuO	Permeability: increased permeability with disrupted endothelial junctions	
	Ventricular cardiomyocytes derived from hiPSCs	SiO_2	Increased production of ROS cytokine: increased production of proinflammatory cytokines (IL-6, MCP-1, IL-1β) from ECs	

possibility that the conventional 2D static testing conditions may over- or underestimate the cytotoxicity of nanoparticles. As the *in vivo* physiology is quite complex and diverse depending on the organs, a detailed investigation of the tissue-specific environment is needed. By understanding and mimicking organ-specific microenvironmental features, the toxicity of nanomaterials can be evaluated with high reliability. We expect that the utilisation of human-organ-physiology-mimicking platforms can help in the evaluation of human-relevant toxicity of environmental nanoparticles (Table 1).

Author contributions

Conceptualisation, S. S., K. L., and H. N. K.; investigation, S. S., J. E. L., and H. N. K.; writing – original draft, S. S. and H. N. K.; writing – review & editing, S. S., K. L., and H. N. K.; funding acquisition, J. E. L., K. K., and H. N. K.; supervision, K. L. and H. N. K.

Conflicts of interest

The authors declare no competing financial interests.

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