Chapter 9
In Vitro Models and Molecular Markers for Assessing Nano-Based Systems Inflammatory Potential

Renata Lima, Vitoria Aparecida Nobrega Antunes, Tais Germano da Costa, and Mariana Guilger Casagrande

Abstract When nanotechnology proved to be a promising science with applications in several areas, there was a need for studies regarding the toxicity of nanomaterials. In vitro evaluation is a tool of potential interest among different study models since it can provide early signals of the possible behavior of the nanomaterial quickly and often accurately. In vitro studies allow the evaluation of both toxicological potential and nanomaterial activity. For confidence in these tests to reduce experiments using animal models, evaluative markers began to be studied and refined, along with different cell culture models, to ensure compatibility with in vivo exposure. Thus, two strands should be developed and used together for the application of in vitro models. One of them regards cell seeding and exposure techniques, and the other is the study of valuable markers to detect possible cellular alterations and their consequences. Although there are well-established techniques to evaluate cell viability and genotoxicity, these are not always appropriate for assessing cells exposed to nanocomposites due to the unique characteristics of these new materials. In this way, it is still necessary to verify the actual efficiency of the existing techniques when evaluating nanomaterials and envision possible changes and adjustments.

Keywords Cell culture · Nanoparticles · Organ-on-a-chip

R. Lima (✉) · V. A. N. Antunes · T. G. da Costa
Sorocaba University, Sorocaba, SP, Brazil
e-mail: renata.lima@prof.uniso.br

M. G. Casagrande
Sorocaba University, Sorocaba, SP, Brazil

Institute of Science and Technology, São Paulo State University–UNESP, Sorocaba, Brazil

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9.1 Introduction

Almost a century ago, most of the innovations and technologies that emerged in the research fields were directly or indirectly related to cell culture. Most cancer-related studies are directly related to Henrietta Lacks (HeLa) cells.

Since the discovery and culture of HeLa cells in 1952, innovations such as the polio vaccine in 1954 and sequencing of the human genome in 2000 have significant social and economic relevance showing that new technologies and discoveries play an essential role in the future of science (Freshney 2016; Masters 2002; Skloot 2010).

Over the years, it is possible to observe an increasing interest in developing protocols for in vitro cell culture. In 1885, when the embryologist Wilhelm Roux succeeded in conserving an embryonic tissue of chicken in a warm saline solution, a constant search for better culture conditions has begun. Harrison (1907) demonstrated the development of frog nerve fibers in a coagulated blood suspension, Carrel (1912) observed the importance of the nutrient content in the culture media, and in 1952, George Gey propagated the HeLa cell line from a cervical tumor tissue which has been used to this day (Freshney 2016; Gruber and Jayme 1994; Verma et al. 2020).

The emergence of nanomaterials triggered the need for new study models, mainly regarding in vitro evaluation since the assessment of newly developed nanomaterials aims to establish rules for their application and manipulation. As these are materials with totally differentiated characteristics, a new view concerning the applied tests must be considered, since the biological impacts on health and the environment are the primary concern of researchers to avoid future risks (Srivastava et al. 2015; Savage et al. 2019).

One of the possibilities presented using cell cultures is the rapid screening of these materials. However, the use of in vitro tests for the evaluation of nanomaterials goes far beyond screening. Nanomaterials act at the molecular level so that in vivo investigation studies will not keep up with the advances in nanotechnology, requiring a connection between in vivo and in vitro studies (Romeo et al. 2020). Therefore, tests should not be replaced but used in combination. The association between in vitro and in vivo tests can give accurate answers about nanomaterials, reduce animal experimentation, and introduce optimized tests. In vivo tests can track the routes of biodistribution and bioaccumulation of nanomaterials. However, previous evaluations using in vitro tests can identify highly dangerous nanocomposites (Hartung 2009, 2010; Berg et al. 2011).

Most studies involving the assessment of nanomaterials follow a sequence of in vitro tests with subsequent in vivo evaluations. However, it is predicted that this context will change within a few years. Both types of tests will be performed in an interconnected way, mainly regarding the evaluation of nanomaterials. Different cell culture models and markers are being developed, which nowadays enhance the assessments and will have even more impact in the future (Fig. 9.1).

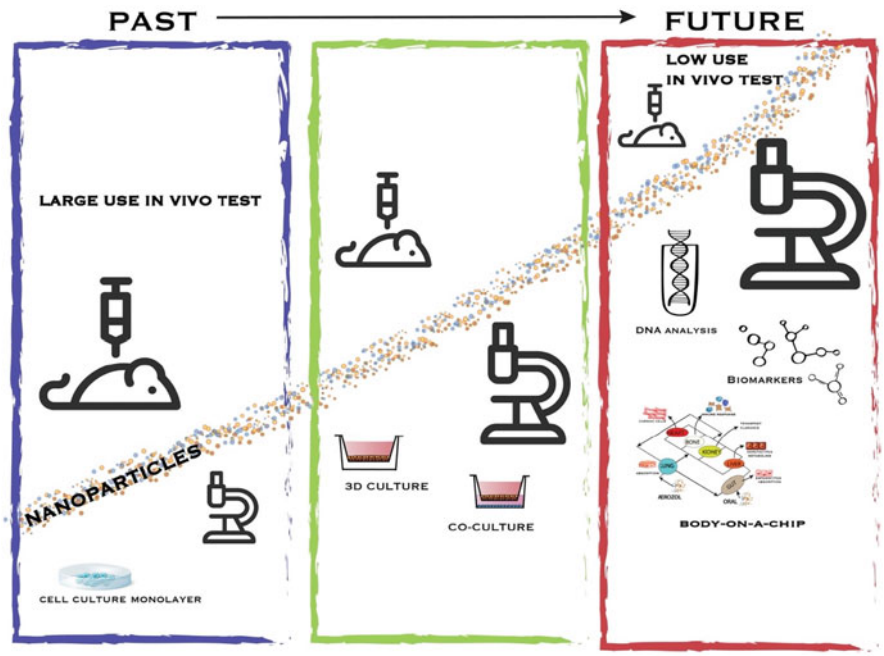

Fig. 9.1 Trends in nanomaterial research over time

Studies for improving some aspects of in vitro evaluation enable the development of models that employ different structures and materials and simulate a living organism (body-on-a-chip). It means that in the future, more elaborated in vitro evaluations could have a more significant contribution than the simple determination of risk potential, toxic dose, or as precursors to in vivo tests. In addition, it will be possible to reduce the use of animal tests, which will be performed just when the material is entirely safe (Frey et al. 2014; Romeo et al. 2020; Chen et al. 2021).

9.2 Evolution of Cell Culture Models

9.2.1 Cells

Multicellular organisms exist because cells can adhere to each other. This adhesion occurs through physical and biochemical mechanisms that happen in the extracellular matrix. In addition to enabling cell–cell adhesion, the extracellular matrix also promotes cell–substrate adhesion. The loss of cell adhesion can occur due to genetic mutations that cause alterations in the extracellular matrix proteins and, consequently, destabilize the tissue and alter the transduction of signals from the external

environment. Thus, it is a dynamic process that continuously moves and responds to changes in the microenvironment (Armingol et al. 2020; Windisch et al. 2019).

When using cell cultures as a strategy for performing in vitro tests, there is a need to provide a microenvironment-like in vivo systems, guaranteeing its homeostasis. One of the main limiting factors for this strategy is cell adhesion and cell–matrix–cell interaction (Oliveira et al. 2019; Zhou et al. 2018; Bich et al. 2019), which often are not maintained. The extracellular matrix comprises metabolites, receptors, ions, and multifunctional proteins such as growth factors, hormones, cytokines, chemokines, and neurotransmitters (Armingol et al. 2020). Cell adhesion molecules (CAMs) play a fundamental role, both physical and regarding cell signaling, influencing cell migration, mesenchymal remodeling, and contributing to critical processes such as embryogenesis, organ development, and wound healing (Canel et al. 2013; Windisch et al. 2019; Thiery et al. 2009; Epifano and Perez-Moreno 2012).

Therefore, over time and with the evolution of cell models, much has come to be questioned regarding the maintenance of the cell culture microenvironment, such as, for example, interactions mediated by cell adhesion molecules (Daley et al. 2008). These structures are widely distributed in the plasma membranes or clusters near the cellular junctions, which are responsible for maintaining the rigidity and strength of the tissues and epithelial barrier, transmission of information between intracellular and extracellular compartments, and the movement of molecules and ions from the cytoplasm of a cell into the cytoplasm of the adjacent cell (Saraiva et al. 2016; Nzou et al. 2019; Bergmann et al. 2018; Gloushankova et al. 2017). Cadherins, integrins, selectins, and immunoglobulins are examples of CAMs (Honig and Shapiro 2020; Mui et al. 2016; Aplin 2003; Juliano 2002).

9.2.2 Cell Cultures

Cell cultures have been used for material evaluation since the late nineteenth century when cells began to be isolated and cultured in the laboratory (Curtis et al. 1983; White 1946; Eagle 1955). An overview from 1907 to the present day (Fig. 9.2) shows that the techniques have evolved a lot concerning the employed technology, with several problems being solved over time, from cell adhesion in culture plates (1980) to the solution of issues related to the current three-dimensional cultures (Pardo et al. 2005; Sharrer 2006; Andrysiak et al. 2021; Hennies and Poumay 2021).

Depending on the type of culture, cells show different morphological properties and changes in gene expression, proliferation potential, cell interaction, and signal transduction (Fang and Eglen 2017; Riedl et al. 2017). An example was the work carried out by Ma et al. (2018), who compared the genome of glioblastoma multiforme cells in 3D cultures (polylactic acid scaffolds) and 2D cultures and found that cells cultured in a 3D system showed positive regulation of 8117 genes and negative regulation of 3060 genes in comparison with 2D cultures.

In vivo, the cellular response to external factors depends on the adhesion between cells and proteins of the extracellular matrix, mediated by the transmembrane

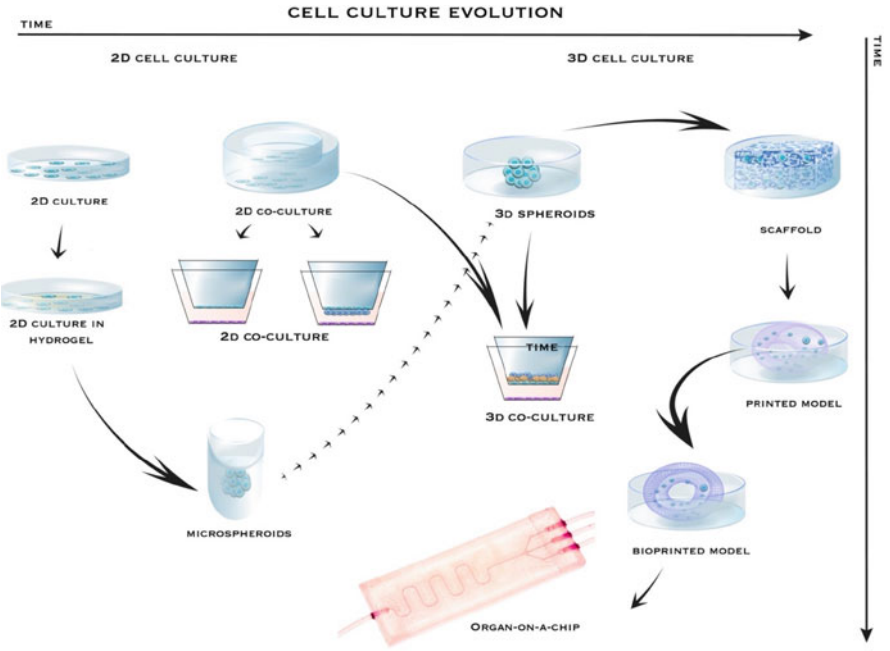

Fig. 9.2 Cell culture models over time. Cells grown in monolayers, in general, have a flat shape, not corresponding to the actual morphology. Coculture systems can mimic cellular interaction. 3D cultures are inserted in microenvironments like in vivo, being more representative

receptor system (Bachmann et al. 2019). In 2D culture, cell surface receptors have a structure and spatial arrangement different from in vivo organization. This change influences the way drugs and other substances bind to the cell, triggering varied responses (Edmondson et al. 2014). An example is a study by Loessner et al. (2010) in which ovarian cancer cells showed a viability decrease between 40% and 60% in 3D culture and 80% in 2D culture when exposed to paclitaxel. In this study, an increase in the expression of surface receptor integrins a3/a5/b1 and MMP9 protease was observed in 3D culture compared to the 2D culture model.

Analyzing the metabolic profile, Russell et al. (2017) found that in 2D culture, due to the monolayer arrangement, all the cells die when exposed to a cytotoxic drug, while in the 3D model, the cells form a protective barrier so that only those in the edge die. Soares et al. (2012) compared cardiac cells in 2D and 3D cultures and observed several differences, among them structural differences. The 3D culture showed a higher number of intercellular junctions, organized myofibrils, and preserved mitochondria and desmosomes, making the connection of neighboring cells and more significant deposition of extracellular matrix. A higher frequency of spontaneous contractions and an increase in the expression of the cardiac differentiation markers cadherin, sarcomeric alpha-actin, and desmin were also observed in the cells of the 3D model.

Liu et al. (2021) investigated the genomic architecture of mouse hepatocytes (AML12) in 2D and 3D cultures and observed differences in cellular organization, cell shape, and nucleus shape. They also observed differences in genomic interactions and a higher expression of genes involved in physiological processes in 3D culture. Chen et al. (2017) observed differences in the face of genomic regions related to structural changes in human fibroblasts grown in 3D and 2D models. More than 3000 genes showed altered expression.

Cells in 2D culture grow in monolayers attached to a plastic surface. Due to this arrangement, they present different morphology, physiology, interaction, and communication than the cells that compose living organisms (Edmondson et al. 2014). Thus, cells in 2D culture may be more sensitive when exposed to some substances (Chen et al. 2017; Lv et al. 2017); moreover, in this arrangement, all the cells receive the same amount of nutrients and growth factors, different from cells in natural conditions (Huang et al. 2013). In general, even though it is a low-cost and widely used practice compared to in vivo tests, 2D cultures present some limitations, mainly due to the impossibility of mimicking tissue architecture and the cellular microenvironment (Hartung 2013; Kieninger et al. 2018).

Since the presumption that the culture of monolayer cells limited cell–cell interaction and altered cell signaling, consequently causing discrepancies in the results of tests with cell cultures and organisms, new models of cell culture began to be studied and evaluated (Langhans 2018; Sieber et al. 2018; Chou et al. 2020; Turnbull et al. 2018). Then, the absence of a third dimension and a concentration gradient in the cell population in 2D models and the demand for more accurate models have triggered further studies.

Although there are gaps between the different types of cell models, the use of cell culture for the evaluation of new materials has been established, with varying attempts at combinations to obtain tremendous success (Fig. 9.2). With this exhaustive search for better in vitro evaluation parameters, it is possible to observe an increase in the number of studies that bring more effective and differentiated tests (Langhans 2018). All the advancement in this technology aims the search for study environments that resemble the in vivo cellular environment since many clinical trials fail in phase II and III due to safety and efficacy problems (Arrowsmith and Miller 2013).

3D cell culture models have advantages underrepresented in 2D cultures since they provide a complex cellular microenvironment closer to the in vivo environment, composed of proteins and extracellular matrix glycoproteins. Moreover, depending on the cellular composition, it is possible to simulate the signaling from other tissues (Vinci et al. 2012; Jedrzejczak-Silicka 2017; Chaicharoenaudomrung et al. 2019). In summary, the critical characteristic of 3D culture is the maintenance of the natural shape of the cell, which allows heterogeneous exposure to the medium, cellular communication, and better development (Chen et al. 2017; Lv et al. 2017). This system can be obtained using structures produced with biocompatible material denominated scaffolds or through the development of spheroids (Maia-Pinto et al. 2021; Saydé et al. 2021; Wang et al. 2020; Sokolova et al. 2020). In addition, it is

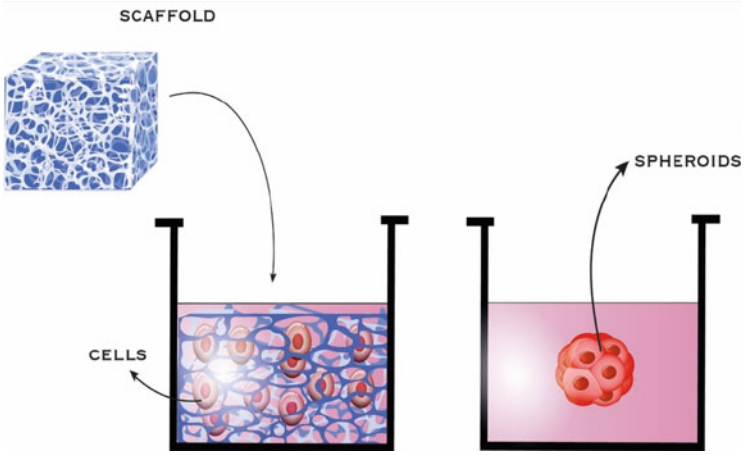

Fig. 9.3 Schematic representation of the two most used 3D cell culture models. The material used for the production of the scaffold may vary according to the needs of the study

worth mentioning that 3D models enable better exploration of space dimensions, providing greater cell–cell and cell–environment interactions (Fig. 9.3).

In the 3D cell culture model, the cellular organization is heterogeneous; that is, each cell is at a stage, with proliferating cells in the edges and cells in necrosis or quiescent within the system (Langhans 2018; Bonnans et al. 2014). Due to the cell–cell and cell–extracellular matrix interactions similar to in vivo experiments, the 3D cell culture model has become one of the most used methods for studying drugs and new materials (Jensen and Teng 2020).

Studies of scaffolds were introduced in the last decades, and, initially, these structures were composed of animal biomaterials such as collagen, gelatin, and chitosan. However, new biomaterials based on plants started to be studied and applied over time, including pectin and cellulose derivatives. Some studies showed that these scaffolds have favorable characteristics for developing cell cultures and contribute to the control of contamination and the improvement of cell–matrix interaction (Ravi et al. 2015; Campuzano and Pelling 2019; Mizoguchi et al. 2017).

Cellular interactions and communication play an essential role in several cellular functions, such as differentiation and proliferation, vitality, gene expression, response to stimuli, and metabolism, and are greatly influenced by the cell culture model (Kapałczynska et al. 2018). In addition to affecting cell–cell communication, the culture model also influences the extracellular matrix organization and its interaction with cells (Jensen and Teng 2020). The extracellular matrix biomolecules such as proteins, glycoproteins, and growth factors regulate cell proliferation, migration, differentiation, adhesion, and survival (Bonnans et al. 2014; Langhans 2018). Alterations in this organization, common in 2D cell cultures, give rise to inaccurate evaluations (Jensen and Teng 2020).

9.2.3 Coculture Models

Coculture models enable the study of two or more cell populations of different lines, the interactions between cell populations, exchange of substances, cell signaling or prediction of some events, as well as the development of methods for the creation of artificial tissues (Moraes et al. 2012; Costa and Ahluwalia 2019).

Another promising application of the coculture model, especially for nanotechnology, is tracking the transport of nanoparticles and other substances. These models enable the evaluation of materials permeation through biological membranes (Costa and Ahluwalia 2019). It is possible to assemble different models, from confluent monolayers and bilayers to 3D cell cultures, simulating different pathways such as pulmonary, cutaneous, and digestive, and then evaluate the permeability, translocation, and toxicity of substances and nanomaterials (Fig. 9.4).

These new evaluation methods employing cell cultures are more realistic. They provide essential strategies for the advancement of tissue engineering studies, discovering new drugs, organogenesis studies, and the modeling of diseases. In addition, they follow the 3Rs principle (reduction, replacement, and refinement), which boosts activities related to in vitro evaluation (Ravi et al. 2015; Jaroch et al. 2018). Another critical factor is that the development of 3D printing using biomaterials enabled new study models such as tridimensional organs (Ma et al. 2021).

One of the models of great importance for the evaluation of compounds using cell culture is the 3D coculture for the ocular surface. It is composed of rabbit conjunctival epithelium and lacrimal gland spheroid cells. According to the cell organization, Lu et al. (2017) tested different models for optical surface studies, which they named top, bottom, and membrane. The results proved that coculture introduced a beneficial effect on secretory function, mimicking the healthy ocular surface. This study provided a new platform for pathophysiological studies of the ocular surface.

Nanomaterials require molecular evaluation due to their unique characteristics, and the use of cellular cultures comes out as a great combination. Numerous studies involving different cell culture models have been developed for this purpose, which is increasingly well-elaborated, aiming not only for the previous assessment of nanomaterials but also for more robust analyses (Table 9.1).

Even in the face of different strategies of cocultures and 3D cultures, to obtain even more effective models, it is necessary to integrate different areas such as materials, molecular biology, and computational modeling, among others (Kamm et al. 2018). Moreover, although already used for a long time, in vitro analyses still need improvements mainly concerning current tests involving human biometric pathophysiology, which have a gap (Franzen et al. 2019; Ma et al. 2021). In the future, this gap may be completed using organ-on-a-chip, which enables the reproduction of organs or tissues in vitro, mimicking the architecture and functionality of in vivo systems, as an attempt to replace in vivo tests.

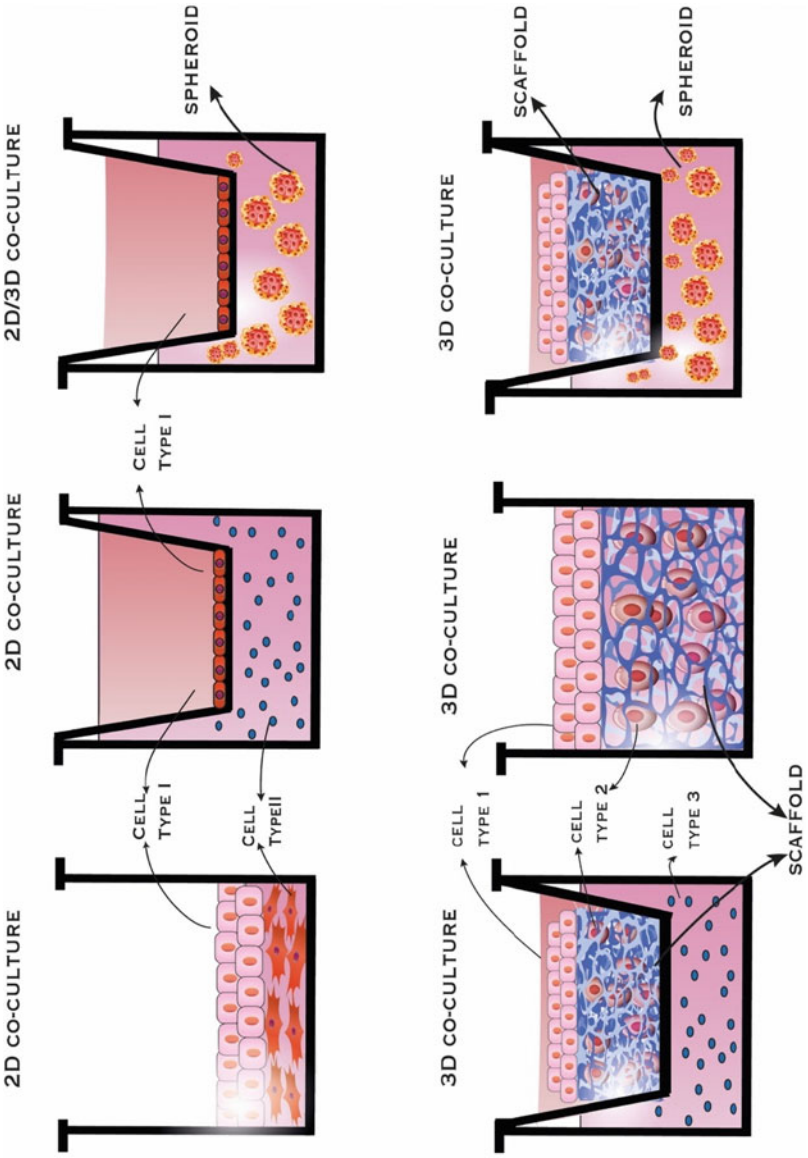

Fig. 9.4 Schematic representation of some coculture models

Table 9.1 Studies using cell culture for the evaluation of nanoparticles

Nanoparticle	Objective	Culture/cell type	In vitro tests	References
Magnetic nanoparticle-loaded human adipose-derived mesenchymal cells spheroids	Levitation of cell culture, maintenance of properties, and improvement of performance	Primary 2D and 3D cultures Human primary adipose-derived mesenchymal cells	• Cell viability: MTT • Cell migration • Assessment of osteogenesis, chondrogenesis, and adipogenesis	Labusca et al. (2021)
Silver nanoparticles synthesized with flavonoids from *Reinwardtia indica* leaves	Antioxidant, antimicrobial, and cytotoxic potential	Monolayer (2D) Human cervical tumor cells (SiHa)	• Cell viability: MTT • Enzymatic assays	Upadhyay et al. (2019)
Zinc oxide nanoparticles	Non-apoptotic cell death	Monolayer (2D) Murine microglial cells (BV-2)	• Cell viability: MTT • Trypan blue	Sruthi et al. (2020)
Cellulose nanofibers/hydroxyapatite/silver nanoparticles	Efficiency as a scaffold for tissue engineering applications	Monolayer (2D) Chicken embryo fibroblasts (CEFs)	• Cell viability: MTT • Cell fixation/microscopy	Sofi et al. (2021)
Silver and titanium dioxide nanoparticles	Toxicity	Monolayer (2D) Porcine ovarian granulosa cells	• RT-PCR • ELISA test	Sirotkin et al. (2021)
Silver nanoparticles with different surface modifications	Digestion on gastrointestinal fate and uptake	Monolayer (2D) and coculture Human colorectal adenocarcinoma cells (Caco-2) Human mucus-secreting adenocarcinoma cells (HT29-MTX)	• Cell viability: WST-1 • Integrity assessment: transepithelial electrical resistance • Cellular uptake/association and transport	Abdelkhaliq et al. (2020)
Natural organic matter coated silver nanoparticle	Kinetics of transport across the cell membrane	Monolayer (2D) Human lung carcinoma epithelial cells (A549)	• Cell viability • Uptake kinetics • Relationship intracellular/suspension nanoparticles • Cellular uptake pathway • ROS level	Zhong et al. (2021)

ROS-scavenger nanoceria encapsulated within mesoporous silica nanoparticles (Ce@MSNs)	Osteoporosis treatment/in stressed and normal conditions	Monolayer (2D) coculture Murine primary macrophage (RAW264.7) Pre-osteoblast cells (MC3T3-E1)	• Cell viability: MTT • Uptake • Antioxidant activity • Osteoblast differentiation • Therapeutic activity	Pinna et al. (2021)
Cytarabine-loaded poly(ε-caprolactone) nanoparticles	Sustained-release/anticancer activity	Monolayer (2D) Acute myeloid leukemia cells (KG-1) Breast cancer cells (MCF-7)	• Cell viability: MTT • Apoptosis quantification (flow cytometry) • Western blot	Jan et al. (2021)
Polyvinylpyrrolidone (PVP)-coated silver nanoparticles	Investigation of molecular mechanisms underlying AgNP induced lung cellular senescence	Monolayer (2D) Human fetal lung fibroblast (MRC5)	• SA-β-galactosidase staining • Western blotting • RNA-sequencing and data analysis • Immunofluorescence • PGE2 immunoassay • Apoptosis and senescence • Cell cycle	Chen et al. (2020)
Chitosan nanoparticles	Test and overcome the unfavorable influences of aggregated chitosan nanoparticles	Monolayer (2D) Human pancreatic adenocarcinoma (CFPAC-1)	• Cell viability: MTT • Uptake	Ozturk et al. (2020)
Cancer-specific prodrug nanoparticle (doxorubicin) with Bcl-2 anti-apoptotic inhibitor (Navitoclax)	Overcome acquired drug resistance during chemotherapy using nanoparticles	Monolayer (2D) Human breast adenocarcinoma (MDA-MB231) Human dermal fibroblast (HDF) Rat BDIX heart myoblast (H9C2)	• Uptake • Analysis of Bcl-2 expression (western blot) • Cytotoxicity assays (flow cytometry)	Kim et al. (2021)
Engineered silica nanoparticles	Investigate if the nanoparticles are biologically safe to deliver drugs or genes to liver cells	Monolayer (2D) Epithelial-like human hepatoblastoma cell (HuH-7) Liver sinusoidal endothelial cells (SK-HEP-1)	• Cell viability: MTT and Sulforhodamine B • Colocalization analysis with lysosomes and mitochondria • Flow cytometry • Genotoxicity/micronuclei • Hemolysis assay • Clonogenic assay	Tüncel et al. (2021)

(continued)

Table 9.1 (continued)

Nanoparticle	Objective	Culture/cell type	In vitro tests	References
Calcitonin-loaded octamaleimic acid-silsesquioxane nanoparticles in a hydrogel scaffold	Enrich the hydrogel scaffold with hydroxyapatite and platelet-rich plasma for bone tissue engineering	Monolayer (2D) Human osteosarcoma cells (MG-63)	• Cell viability: MTT and trypan blue • Alizarin red staining • Enzymatic assays • Osteogenic gene expression (qRT-PCR)	Ahmadipour et al. (2021)
Hyperthermic Ag and Au Fe_3O_4 nanoparticles	Anticancer activity	Monolayer (2D) Human embryonic kidney cells (HEK293) Human colorectal carcinoma cell (HCT116) Mouse mammary carcinoma cells (4T1) Epithelial-like human hepatoblastoma cell (HUH7)	• Cell viability: MTT • Hyperthermia • qRT-PCR	Katifelis et al. (2020)
Polydopamine (PDA)-coated magnetite nanoparticles (NPs) and spheres (sMAG) with PAMAM dendrimers and functionalized with NHS–PEG–Mal (N-hydroxysuccinimide–polyethylene glycol–maleimide) linker	Chemo- and photothermal therapy Generation of reactive oxygen species	Monolayer (2D) Hepatocellular carcinoma cells (HepG2) Human liver epithelial cells (THLE-2)	• Viability: WST-1 • Oxidative stress quantification assay	Jędrzak et al. (2020)
Cadmium oxide nanoparticles	Cytotoxicity and genotoxicity	Monolayer (2D) Human lymphoblastoid cells (TK6) Hepatocellular carcinoma cells (HepG2) Mouse lymphoma cell (L5178Y/Tk+/−3.7.2C)	• Cellular uptake (transmission electron microscopy) • Viability: MTS and ATP • Lactate dehydrogenase (LDH) activity assay • Micronucleus (flow cytometry) • Comet assay • Mouse lymphoma thymidine kinase assay (MLA)	Demir et al. (2020)

Multifunctional gelatin nanoparticles modified by NIR-emitting gold/silver alloy nanoclusters and loaded with ovalbumin (OVA) as a model antigen	Functionalized multifunctional nanovaccine for targeting dendritic cells and modulation of immune response	Monolayer (2D) and coculture murine bone marrow-derived dendritic cells (BMDCs) T cells derived from OT-I and OT-II mice spleens	• Viability, uptake, and cell activation • Analysis of chemokines• And cytokines	El-Sayed et al. (2021)
Copper oxide nanoparticles	Toxicity on intestinal barrier	Monolayer and 3D Human colorectal adenocarcinoma cells (Caco-2)	• Barrier integrity assay (transepithelial electrical resistance evaluation) • ELISA test	Bertero et al. (2020)

9.2.4 Organ-on-a-Chip

From the year 2010, with the construction of lung-on-a-chip (Huh et al. 2010), organ-on-a-chip systems started to be recognized with the development of several studies involving different tissues over the years (Si et al. 2020; Ma et al. 2016; Musah et al. 2017; Glieberman et al. 2019; Ugolini et al. 2018; Poceviciute and Ismagilov 2019; Koo et al. 2018; Bein et al. 2018). The use of chip systems for multi-cultures enables the control of interconnected independent cell cultures arranged to simulate tissue and organ physiology, which cannot be accomplished using only 2D or 3D cell cultures. This system also enables evaluating incompatible cultures in the same model simulating a specific microenvironment, leading to the discovery of new signaling mechanisms. The application of organ-on-a-chip models allows molecular and immunological analyses to promise future in vitro analyses (Ma et al. 2021; Chen et al. 2021).

Organ-on-a-chip systems enable the investigation of the toxicity of nanomaterials and other substances intermediating preclinical models such as 2D culture and animal models and population studies (Lu and Radisic 2021). A 3D culture system that involves fluid flow technology simulates living organisms' conditions with the continuous nutrient exchange, oxygenation, gas exchange, removal of residues and metabolites, shear stress, and other characteristics of in vivo systems. Among the advantages of organ-on-a-chip compared with static cultures such as 2D is that this system simulates cellular metabolism. An example is a study by Trapecar et al. (2020) in which the metabolism and inflammatory responses of CD4 T effector cells were observed in a multi-organ-on-a-chip model created with human hepatocytes and Kupfer cells, mimicking the liver, and ulcerative colitis epithelium, dendritic cells, and macrophages mimicking the gut.

Specifically, regarding the evaluation of the toxicity of nanomaterials, it is known that the dynamism of tissues has a significant influence on their behavior (Lu et al. 2020; Lu and Radisic 2021). In this way, different organ-on-a-chip systems are being developed, aiming at the investigation of nanomaterials effects. Huh et al. (2010) developed a biomimetic microsystem mimicking the alveolar-capillary interface of the human lung with human alveolar epithelial cells and microvascular endothelial cells to investigate the toxicity of silica nanoparticles. They observed high levels of intercellular adhesion molecule-1 (ICAM-1) expression in the underlying endothelium in the microvascular channel and an increase in reactive oxygen species production, which were intensified by mechanical stretching, suggesting that the toxic effects of nanomaterials may be induced by physiological breathing. Zhang et al. (2018) also developed a lung-on-a-chip system to investigate the effects of TiO_2 and ZnO nanoparticles. The system consisted of three parallel channels, with the culture of primary human lung epithelial cells (HPAEpiCs) on one side, a layer of 3D matrigel membrane with fluid flow in the center, simulating the human lung alveolar-capillary barrier, and vascular endothelial cells (HUVEC) on the opposite side. An increase in the system's permeability and the production of reactive oxygen

species were observed, especially in epithelial cells directly exposed to the nanoparticles, and apoptosis, with more significant effects of ZnO nanoparticles. Still focusing on the respiratory system, Chen et al. (2016) developed a human lung microtissue array using bronchial epithelial cells BEAS-2B to investigate the fibrogenic potential of multi-wall carbon nanotubes. After 72 h of exposure to carbon nanotubes, an increase in the microtissue contraction force and the fibrogenic marker miR-21 expression was observed, indicating the fibrogenic potential of the nanomaterial.

Directing the organ-on-a-chip model to investigate possible impacts of nanomaterials on the cardiovascular system, Ahn et al. (2018) evaluated the effects of TiO_2 and silver nanoparticles on the cardiac contraction tissue using a 3D mussel-inspired microphysiological model. The system consisted of bioadhesive polydopamine (PDA)/polycaprolactone (PCL) nanofibers introduced with neonatal rat ventricular myocytes, which developed into mature and functional cardiac tissue. The nanoparticles caused structural damage to the tissue architecture with disruption of the sarcomeric alignment and calcium signaling, decreasing the contractile function of the microphysiological system. Lu et al. (2020) also used a heart-on-a-chip system to evaluate the toxicity of air pollution CuO and SiO_2 nanoparticles. They developed a 3D vascularized microfluidic system that simulates cardiac tissue with cardiomyocytes derived from human pluripotent stem cells and human umbilical vein endothelial cells (HUVEC) into a bioscaffold. CuO nanoparticles showed high toxicity translocating from endothelium to cardiac tissue and causing electrical and contractile dysfunction, whereas SiO_2 nanoparticles did not translocate but induced the release of inflammatory cytokines.

Advancing even further, the inclusion of different organs in the organ-on-a-chip model to assess the effects of nanomaterials may present different results. Esch et al. (2014) developed a microfluidic body-on-a-chip system to evaluate the impacts of carboxylated polystyrene nanoparticles, combining in vitro models of the human intestinal epithelium with the coculture of enterocytes (Caco-2) and mucin-producing cells (HT29-MTX), and liver, with HepG2/C3A cells. When comparing the system which combined the intestinal tract and liver to a system that simulated a unique organ, the first one showed more significant toxic effects of the nanoparticles. Because of this, the authors suggest the greater effectiveness of multi-organ in vitro models for nanomaterials toxicity assessment.

Another essential point to be evaluated for the specific study of nanomaterials are the biomarkers, which can be safely employed to investigate how inert or potentially toxic a nanomaterial is. According to Salieri et al. (2020), there is a tendency to conduct more in vitro evaluations to replace in vivo tests in the future. However, new study strategies are necessary to use better data provided by in vitro analyses.

9.3 Inflammatory Effect Biomarkers of Exposure to Nanoparticles

Inflammatory effects occasioned by the exposure of cell cultures to nanomaterials are generally assessed by analyzing the release of soluble factors such as cytokines, chemokines, and growth factors by enzyme-linked immunosorbent assay (ELISA), with detection through flow cytometry or microplate reader (Drasler et al. 2017). Some studies have evaluated inflammatory responses using the ELISA assay with inflammatory markers, such as that performed by Huk et al. (2014), who investigated the inflammatory effects of silver nanoparticles (50, 80, and 200 nm) coated by polyvinylpyrrolidone (PVP) through the analysis of IL-8 and MCP-1 biomarkers in human lung carcinoma epithelial cells (A549). Greulich et al. (2011) quantified the release of the pro-inflammatory cytokines IL-6, IL-8, and TNF-α, the anti-inflammatory IL-1ra, and the IL-2 and IL-4 cytokines derived from T cells exposed to silver nanoparticles. Hackenberg et al. (2011) also quantified the release of the inflammatory cytokines IL-6 and IL-8 and the vascular endothelial growth factor (VEGF) in human mesenchymal stem cells silver nanoparticles exposed.

The evaluation of inflammatory proteins is widely used in studies of nanomaterials; however, it is still subject to interference from the evaluated nanomaterial, as it can interact with the culture medium or with the marker proteins. In addition, it is crucial to work with concentrations below the limit of cytotoxicity since a cytotoxic nanomaterial reduces cell viability and consequently reduces the release of cytokines, causing false-negative results (Drasler et al. 2017).

Due to the previously addressed problem, some authors prefer to use gene expression analyses, such as those performed by Shannahan et al. (2015), who evaluated the expression of the inflammatory marker TNF-α in mouse macrophages (RAW264.7) exposed to silver nanoparticles with and without protein corona. The assay consisted of the exposure of macrophages to the nanoparticles for 6 h, followed by the extraction of total RNA, reverse transcription for cDNA, and real-time PCR to quantify TNF-α. Cheng et al. (2020) evaluated the expression of the pro-inflammatory cytokine genes IL-1β and IL-6 and the chemokines CXCL1, CXCL2, CXCL3, CCL20, and CXCL8 in keratinocytes differentiated from embryonic stem cells exposed to ultrafine carbon nanopowder.

The pro-inflammatory potential of Al_2O_3, SiO_2, and CeO_2 nanoparticles was evaluated using a mouse alveolar macrophage cell model. The evaluation of the pro-inflammatory markers TNF-α, IL-1β, and IL-6 expression was performed, as well as the quantification of IFN-γ, IL-12p70, IL-1β, IL-6, IL-10, TNF-α, and mouse keratinocyte chemoattractant (KC) in the cell culture supernatant, using the Mouse ProInflammatory 7-Plex Ultra-Sensitive kit (Flaherty et al. 2015). The quantification of TNF-α, IL-1β, IL-8, and IL-6 markers in a 3D reconstruct of human bronchial tissue was performed by Di Cristo et al. (2020) after repetitive exposures to graphene oxide nanomaterial. The model simulated prolonged and repetitive human occupational exposure to the nanomaterial by nebulization using an air–liquid interface culture for 30 days. In this way, biomarkers are widely used for the evaluation of

nanoparticles. Some examples of studies that used biomarkers are shown in Table 9.2.

9.4 Evaluation of Genic Mutations for Exposure to Nanoparticles—Genetic Markers

In addition to the detection of biological markers that indicate inflammation triggered by cell exposure to nanomaterials, it is also possible to identify mutations through genetic features. Genes such as Tk (thymidine kinase) and Hprt (hypoxanthine guanine phosphoribosyltransferase) may be used to evaluate genetic mutations occasioned by nanomaterials (Kazimirova et al. 2020; Du et al. 2019; Doak et al. 2012).

Mouse lymphoma cell line L5178Y/Tk+/− (MLA) is employed to evaluate mutagenicity using the TK gene. At a specific time after exposure, trifluorothymidine (TFT), an analog of thymidine, is added to the cell culture, and then, only the cells that have undergone TK mutation in the presence of the nanocomposite can form colonies (Chen et al. 2014a; Demir and Castranova 2016; Du et al. 2019).

The test for mutation evaluation with the Hprt gene is performed according to the standardization proposed by the OECD Guidelines for the Testing of Chemicals 476 (OECD 2016) using 6-thioguanine (6-TG), a toxic guanine analog (Huk et al. 2014). Kazimirova et al. (2020) investigated the mutagenic effects of titanium dioxide anatase/rutile nanoparticles on different dispersions of V79–4 cell lines through the mammalian heart gene mutation test. Huk et al. (2014) used the same technique to evaluate the effects of polyvinylpyrrolidone (PVP) coated silver nanoparticles with different sizes (50, 80, and 200 nm) on the V79–4 cell line. Table 9.3 shows some studies that used the genes hprt, tk, and other genetic markers to detect the mutagenicity of different nanomaterials on in vitro cell cultures.

9.5 Conclusion

The emergence of nanotechnology has led to greater attention to new in vitro culture techniques. In addition to studies focused on the impact of the environment and health, it has also been necessary to improve molecular studies for more excellent knowledge of this new material. Therefore, different in vitro assays have developed an increasingly more effective approach to in vivo systems, which has led to a reduction in animal experimentation.

Coculture, 2D, 3D models, and new organ-on-a-chip models, together with a greater understanding of biomarkers, place in vitro analysis as one of the main tests that can safely assess the effects of nanomaterials, as well as collaborate to evaluate their inflammatory potential.

Table 9.2 Inflammatory and oxidative stress biomarkers for evaluation of nanomaterials effects on cell cultures

Nanomaterial	Cell line	Inflammatory or oxidative stress biomarkers	References
PVP-coated silver nanoparticles	Human lung carcinoma epithelial cells (A549)	IL-8 and MCP-1	Huk et al. (2014)
Silver nanoparticles	Human peripheral blood mononuclear cells (monocytes and lymphocytes)	IL-6, IL-8, TNF-α, IL-1ra, IL-2, IL-4 Reactive oxygen species (ROS)	Greulich et al. (2011)
Silver nanoparticles	Human mesenchymal stem cells	IL-6 and IL-8, and VEGF	Hackenberg et al. (2011)
Silver nanoparticles with and without protein corona	Mouse macrophages (RAW264.7)	TNF-α	Shannahan et al. (2015)
Ultrafine carbon nanopowder	Human embryonic stem cell (hESC)-based differentiation system towards keratinocytes	IL-1β and IL-6, CXCL1, CXCL2, CXCL3, CCL20, and CXCL8	Cheng et al. (2020)
Al_2O_3, SiO_2, and CeO_2 nanoparticles	Mouse alveolar macrophages (ATCC® CRL-2019)	TNF-α, IL-1β, IL-6 IFN-γ, IL-12p70, IL-1β, IL-6, IL-10, and TNF-α ROS	Flaherty et al. (2015)
Graphene oxide nanoparticles	EpiAirway™ tissues (AIR-100, PE6–5), 3D reconstruct of human bronchial tissue	TNF-α, IL-1β, IL-8 and IL-6	Di Cristo et al. (2020)
Silver nanoparticles	HeLa cells in infection with *Toxoplasma gondii*	IL-1β, TNF-α, IL-12p70, IL-8, IL-6, and IL-10 Nitric oxide, ROS	Machado et al. (2020)
Zinc oxide nanoparticles	Isolated human eosinophils	IL-1β and IL-8, ROS	Silva and Girard (2016)
Titanium dioxide and silica nanoparticles	Rat alveolar macrophages (NR8383)	84 rat chemokines and cytokines	Schremmer et al. (2016)
Multi-walled carbon nanotubes	A549 cells and normal human bronchial epithelial cells (HBEpC)	8-nitroG formation (iNOS expression, endocytosis, HMGB1, RAGE, TLR-2, and TLR-4), nitric oxide, GSH	Hiraku et al. (2016)
Zinc oxide nanoparticles	Transfected cells derived from A549 with reporter genes for IL-8 (Luc, RFP, and GFP)	IL-8	Stoehr et al. (2015)

Zinc oxide nanoparticles	A549 cells	NF-B-mediated NLRP3 inflammasome activation, IL-1β, and IL-18, ROS	Liang et al. (2017)
Silver nanoparticles	Human liver-derived hepatoma cells (HepG2)	NLRP3 inflammasome activation, IL-1β	Mishra et al. (2016)
Silver nanospheres and wires coated with PVP	A549 reporter cells possessing luciferase reporter genes	IL-6, IL-8, TNF-α, and NF-kB	Stoehr et al. (2011)
Spherical and wire-shaped aluminum oxide nanoparticles	Primary mice splenocytes	IL-1β and IL-18 NLRP3 inflammasome activation	Manshian et al. (2018)
Silver and silver-lipoencapsulated nanoparticles	THP1 monocytes and THP1 differentiated macrophages (TDM)	IL-1 β, IL-6, IL-8, and TNF-α	Yusuf and Casey (2019)
Silver nanoparticles	Caco-2/THP-1 coculture mimicking the intestine in a healthy or inflamed state	IL-1β, IL-8, TNF-α MCP-1, MIP-1α, IFN-γ, IL-4, and IL-6	Kämpfer et al. (2020)
Silver and metal oxide nanoparticles (CuO, Fe_3O_4, ZnO, TiO_2, NiO, and CeO_2)	GFP-tagged mouse embryonic stem (mES) cells	Bscl2-GFP, Srxn1-GFP, and Btg2-GFP	Karlsson et al. (2014)
Vegetable carbon (E153) and TiO_2 (E171) nanoparticles	GFP-tagged mouse embryonic stem (mES) cells	Bscl2-GFP, Rtkn-GFP, Btg2-GFP, Srxn1-GFP, BlvrbGFP, and Ddit3-GFP	Brown et al. (2019)
Silver nanoparticles	Human embryonic stem cell (hESC)-derived neural stem/progenitor cells (NPCs)	ROS	Oh et al. (2016)

Table 9.3 Genic mutation biomarkers for evaluation of nanomaterials effects on in vitro cell cultures

Nanomaterial	Cell line	Genic mutation biomarker	References
TiO_2 nanoparticles	Human lymphoblastoid cells (WIL2-NS)	Hypoxanthine guanine phosphoribosyltransferase (*hprt*)	Wang et al. (2007)
Ni and NiO nanoparticles	Human bronchial epithelial cells (HBEC3-kt)	*Hprt*	Åkerlund et al. (2018)
Cd/Se semiconductor quantum dots	Human lymphoblastoid-B cells (TK6)	*Hprt*	Manshian et al. (2016)
TiO_2 nanoparticles	Chinese hamster lung fibroblasts (V79)	*Hprt*	Chen et al. (2014b)
TiO_2 nanoparticles	V79	*Hprt*	Kazimirova et al. (2020)
Multi-wall carbon nanotubes (NM401)	V79	*Hprt*	Rubio et al. (2016)
Ag nanoparticles coated with PVP	V79	*Hprt*	Huk et al. (2014)
TiO_2 nanoparticles	Chinese hamster ovary cells (CHO-K1)	*Hprt*	Wang et al. (2011)
TiO_2 nanoparticles	V79	*Hprt*	Jain et al. (2017)
Multi-wall carbon nanotubes	Chinese hamster lung cells (CHL/IU)	*Hgprt*	Asakura et al. (2010)
TiO_2 nanoparticles	Mouse lymphoma cells (L5178Y)	Thymidine kinase (*tk*)	Du et al. (2019)
Ag nanoparticles	L5178Y	*Tk*	Mei et al. (2012)
Ag nanoparticles	L5178Y	*Tk*	Kim et al. (2010)
Poly(anhydride) nanoparticles	L5178Y	*Tk*	Iglesias et al. (2017)
Tungsten carbide–cobalt (WC–Co) nanoparticles	L5178Y	*Tk*	Moche et al. (2014)
Multi-wall carbon nanotubes	Mouse embryonic stem cells (ES)	Adenine phosphoribosyltransferase (*aprt*)	Zhu et al. (2007)
Zinc oxide nanoparticles	Human-hamster hybrid cells (AL)	*CD59*	Wang et al. (2015)

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