Chapter

Cytotoxicity and Cell Viability Assessment of Biomaterials

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Abstract

Biocompatibility testing is essential for medical devices and pharmaceutical agents, regardless of their mechanical, physical, and chemical properties. These tests assess cytotoxic effects and acute systemic toxicity to ensure safety and effectiveness before clinical use. Cell viability, indicating the number of healthy cells in a sample, is determined through various assays that measure live-to-dead cell ratios. Cytotoxicity measures a substance's potential for cell damage or death, and is evaluated through numerous assay methods based on different cell functions. Ensuring biocompatibility is crucial for the successful integration of medical devices and pharmaceuticals into clinical practice. As part of the evaluation process, researchers utilize a range of cell viability assays and cytotoxicity tests to assess the potential impact of these products on living cells. The results of these tests inform the optimization of cell culture conditions and drug candidates, as well as guide the development of safer, more effective medical devices. By thoroughly examining the interactions between devices, drugs, and biological systems, researchers aim to minimize the risk of adverse reactions and improve patient outcomes.

Keywords: cell viability, cytotoxicity, biocompatibility, trypan blue dye exclusion assay, ATP assay, MTT assay, DNA synthesis cell proliferation assays, Raman micro-spectroscopy, MTT assay

1. Introduction

Biocompatibility testing is a crucial aspect of the development and evaluation of medical devices and pharmaceutical agents. Ensuring that these products are safe and effective for human use is of paramount importance, as they can directly interact with the body's tissues and cells. The primary goal of biocompatibility testing is to assess the safety and effectiveness of medical devices and pharmaceutical agents before they come into contact with the human body. These products may possess a variety of mechanical, physical, and chemical properties that can potentially impact their interactions with biological systems. Biocompatibility testing helps identify any potential risks associated with these interactions, allowing for the optimization of product design and formulation to minimize the likelihood of adverse effects. The components of biocompatibility or the tissue response of the clinically relevant performance of biomaterials are cytotoxicity, genotoxicity, mutagenicity, carcinogenicity, and immunogenicity. Medical devices

and pharmaceutical agents must undergo a series of tests to determine their cytotoxic effects and acute systemic toxicity. These tests are essential in ensuring that the products are safe for clinical use and do not pose any undue risks to patients.

1.1 Cell viability and cytotoxicity assessments

A key component of biocompatibility testing involves the evaluation of cell viability and cytotoxicity. Cell viability refers to the number of healthy, functioning cells in a sample, while cytotoxicity measures the potential of a substance to cause cell damage or death. By assessing these parameters, researchers can gain insights into how medical devices and pharmaceutical agents may affect the body at the cellular level [1]. Various assays can be employed to measure cell viability and cytotoxicity, including dye exclusion methods, metabolic activity-based methods, ATP assays, and DNA synthesis cell proliferation assays, among others. These tests can help determine whether a substance exhibits direct cytotoxic effects or impacts cell proliferation, providing valuable information on its safety profile.

1.2 Biocompatibility testing for medical devices

Medical devices, which encompass a wide range of products used in various clinical disciplines, must demonstrate good biocompatibility to be deemed safe for use. This is particularly important for devices that come into direct contact with the body's tissues and cells, such as implants, prosthetics, and surgical instruments. Biocompatibility testing for medical devices involves assessing the compatibility of these products with the biological systems they will encounter during use. This includes evaluating the interaction between the device and the living tissues and cells it will come into contact with, as well as examining the potential for adverse reactions, such as inflammation, infection, or rejection. By thoroughly evaluating the biocompatibility of medical devices, researchers can develop products that are more likely to integrate successfully with the body and promote positive patient outcomes.

2. Parameters used in cell-based assays

Cell-based toxicological assays are designed to evaluate the potential toxic effects of various substances, including drugs, chemicals, and environmental pollutants, on living cells. These assays measure a range of cellular parameters that can be affected by toxic agents. By examining these factors in cell-based toxicological assays, researchers can gain a comprehensive understanding of the effects of various substances on cells and identify potential therapeutic targets, mechanisms of action, and potential side effects [2]. This information is crucial for the development of safer and more effective drugs, chemicals, and other products. Here are some common parameters used in cell-based toxicological assays:

2.1 Cell viability

Cell viability is a measure of the number of living cells in a sample. Cell viability assays are crucial for determining the overall health and survival of cells in response to various treatments. These assays are based on cellular functions that are specific to living cells, such as metabolic activity or membrane integrity [3] A decrease in

viability indicates a toxic effect, whereas an increase may indicate a protective or stimulatory effect. Several assays, such as MTT, XTT, WST-1, Neutral Red, and Alamar Blue, can be used to assess cell viability based on metabolic activity, dye uptake, or ATP levels. Damage to the cell membrane can be identified by the presence of intracellular substances, like lactate dehydrogenase (LDH), in the suspension medium. This can be assessed when cells are in contact with materials or material extracts in cell cultures [4]. A decline in metabolic activity might signal cell death before the breakdown of the membrane occurs. The MTT assay, introduced by Mosmann, is a widely used technique for evaluating cell viability [5].

2.2 Cell proliferation

Cell proliferation refers to the rate at which cells divide and increase in number. Assessing cell proliferation provides insights into how toxic agents affect cell division and growth. Inhibition of cell proliferation can indicate the potential anti-cancer effects of a drug, while excessive inhibition may indicate general toxicity. Cell proliferation is a crucial parameter in evaluating cytotoxicity. The cytotoxic effects of a biomaterial can threaten cell viability by compromising its structural or metabolic integrity and affecting its regenerative capacity [6]. Toxic agents can inhibit cell proliferation, which can be assessed using assays such as BrdU (bromodeoxyuridine) incorporation, EdU (5-ethynyl-2′-deoxyuridine) incorporation, or Ki-67 staining. A simple approach to measure cell proliferation involves comparing cell counts in cultures exposed to test material extracts for varying durations with control cultures. This method typically requires trypsinizing the cell culture and counting individual cells with a microscope or electronic cell counter [7]. Evaluating the protein content of cell cultures is a practical test for assessing the toxicity of biomaterials [8]. Cell proliferation assays can be used to determine the optimal concentration of a drug or compound for further testing.

2.3 Cytotoxicity

Cytotoxicity is a measure of cell damage or death caused by toxic agents. Cytotoxicity assays focus on detecting the extent of cell damage or death caused by toxic agents. These assays provide information on the direct effects of a substance on cells, which can be useful for identifying potential therapeutic targets or understanding the mechanisms underlying toxicity. Common cytotoxicity assays include LDH (lactate dehydrogenase) release assay, which measures the release of LDH from damaged cells, and the trypan blue dye exclusion assay, which measures membrane integrity. Cytotoxicity assays can also be used to determine the selectivity of a drug or compound for different cell types.

2.4 Apoptosis and necrosis

Apoptosis (programmed cell death) and necrosis (uncontrolled cell death) are distinct mechanisms of cell death that can be triggered by toxic agents.: Understanding the mechanisms of cell death induced by toxic agents is essential for developing targeted therapies and identifying potential side effects. Apoptosis and necrosis are distinct types of cell death, and differentiating between them can provide insights into the mode of action of a toxic agent and its potential therapeutic value. Assays such as annexin V/propidium iodide staining, TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay, and caspase activity assays can be used to assess apoptosis and necrosis.

2.5 Oxidative stress

Toxic agents can cause oxidative stress by inducing the production of reactive oxygen species (ROS) and/or impairing cellular antioxidant defenses. Assays such as DCFDA (2′,7′-dichlorofluorescin diacetate) fluorescence, lipid peroxidation assays, and glutathione assays can be used to evaluate oxidative stress. Evaluating oxidative stress is crucial for understanding how toxic agents affect cellular redox balance, which plays a vital role in maintaining cellular homeostasis. Oxidative stress can lead to cellular damage, dysfunction, and eventually cell death. Identifying agents that induce or prevent oxidative stress can help in the development of novel therapeutic strategies.

2.6 Genotoxicity

Genotoxic agents can cause DNA damage, which may lead to mutations, chromosomal aberrations, or DNA strand breaks. Genotoxicity assays assess the potential of a substance to damage DNA, which can lead to mutations, chromosomal aberrations, or other genomic changes. These assays can help identify potential carcinogens, mutagens, or teratogens and provide insights into the mechanisms of DNA damage and repair. Genotoxicity can be assessed using assays such as the comet assay (single-cell gel electrophoresis), micronucleus assay, and γ-H2AX (phosphorylated histone H2AX) staining.

2.7 Cellular morphology

Toxic agents can induce changes in cellular morphology, such as cell shrinkage, membrane blebbing, or cytoplasmic vacuolization. Examining cellular morphology provides a visual assessment of the effects of toxic agents on cell structure and organization. Changes in cellular morphology can indicate alterations in cellular functions, such as cell adhesion, migration, or differentiation, which can help elucidate the mechanisms underlying toxicity. These morphological changes can be visualized using light microscopy, phase-contrast microscopy, or fluorescence microscopy.

Studies examining morphological changes caused by cell adhesion to compatible and incompatible material surfaces have shown that human fibroblasts proliferate extensively on glass, but experience inhibition on hydrophobic biomaterials. Furthermore, cell rounding, detachment, and decreased proliferation have been observed [9]. This incompatibility of the biomaterial surface is known as intrinsic toxicity. The rounding of cells and other morphological alterations often occur before the loss of cell viability, which is accompanied by the disconnection of cells from the substrate [10]. Another characteristic of cell morphological changes is the increased vacuolation of the cytoplasm, often involving the formation of autophagosomes. Cytoplasmic vacuolation has been recognized as a dependable indicator of toxicity [11].

2.8 Protein synthesis and enzyme activity

Toxic agents can affect protein synthesis or the activity of specific enzymes, which can be assessed using assays such as western blotting, enzyme-linked immunosorbent assay (ELISA), or enzymatic activity assays. Assessing protein synthesis and enzyme activity can reveal how toxic agents affect specific cellular processes or signaling pathways. Identifying the proteins or enzymes affected by a toxic agent can help in understanding its mode of action and potential therapeutic applications.

2.9 Calcium signaling

Calcium signaling plays a crucial role in various cellular processes, and alterations in intracellular calcium levels can be indicative of toxic effects. Calcium signaling is involved in various cellular processes, such as cell division, migration, and apoptosis. Disruptions in calcium signaling can result in cellular dysfunction or death. Evaluating calcium signaling can provide insights into the effects of toxic agents on cellular communication and function. Calcium signaling can be assessed using fluorescent calcium indicators such as Fluo-4 or Fura-2.

2.10 Mitochondrial function

Toxic agents can affect mitochondrial function, leading to changes in mitochondrial membrane potential, respiration, or biogenesis. Mitochondria play a central role in cellular energy production and metabolism, and disruptions in mitochondrial function can have significant consequences for cellular health. Assessing mitochondrial function can reveal the potential effects of toxic agents on cellular bioenergetics and provide insights into the mechanisms of mitochondrial dysfunction and related diseases. Assays such as JC-1 staining (for membrane potential) and Seahorse XF analysis can be used to evaluate mitochondrial function.

3. Cell viability assessment

Cell viability assessment is an indispensable aspect of biocompatibility testing for medical devices and pharmaceutical agents. It plays a critical role in evaluating the safety and effectiveness of these products, ensuring that they do not cause undue harm to living cells when they come into contact with the human body. Cell viability is a fundamental parameter in the evaluation of the biocompatibility of medical devices and pharmaceutical agents. It is defined as the number of healthy, functioning cells in a sample and serves as an essential indicator of the impact of a particular substance on cellular health. By assessing cell viability, researchers can gain insights into the potential toxic effects of medical devices and pharmaceuticals, enabling them to optimize their design and formulation to minimize the risk of adverse effects. Moreover, cell viability assessment is crucial in various other aspects of biomedical research, such as understanding the mechanisms of action of specific genes, proteins, and signaling pathways involved in cell survival or death. Furthermore, it plays a significant role in the development of novel therapeutic strategies, as researchers must ensure that new drugs or treatments do not cause unacceptable levels of cell damage or death.

3.1 Applications of cell viability assessment

Cell viability assessment has a wide range of applications in biomedical research and drug development. Some key applications include:

- Evaluating the effect of drug candidates on cells: Cell viability assays can be used to determine the cytotoxic potential of new drug candidates, providing valuable information on their safety profile and aiding in the selection of promising candidates for further development.

- Optimizing cell culture conditions: Cell viability assessment can help researchers optimize the culture conditions for various cell types, ensuring that cells remain healthy and functional during in vitro experiments.

- Investigating the mechanisms of cell death: Cell viability assays can be employed to study the molecular mechanisms underlying cell death, providing insights into the biological processes involved in various diseases and enabling the development of targeted therapies.

- Screening for potential therapeutic agents: High-throughput cell viability assays can be used to screen large libraries of compounds for their potential to enhance cell survival or promote cell death, facilitating the identification of novel therapeutic agents.

3.2 Methods for determining cell viability

There are numerous methods available for determining cell viability, each with its advantages and limitations. Some of the most commonly used methods include:

Dilution: A simple technique in which the number of viable cells is estimated based on their ability to proliferate in a diluted environment. The dilution method involves serially diluting a cell suspension and then assessing cell growth in each dilution. Viable cells will continue to grow and divide, whereas non-viable cells will not proliferate. By comparing cell growth in each dilution, researchers can estimate the percentage of viable cells in the original sample. Although this method is simple and relatively easy to perform, it may not be suitable for all cell types or experimental conditions and may be less sensitive than other methods.

Surface viable count: This method involves counting the number of viable cells on a solid surface, such as a culture dish, after exposure to the test substance. The surface viable count method is based on the ability of viable cells to grow and form colonies on a solid surface, such as a culture dish or agar plate. After incubating the cells with the test substance, the viable cells are plated onto the surface and allowed to grow for a specified period. Researchers then count the number of colonies formed, which is proportional to the number of viable cells in the sample. This method is relatively simple and can provide accurate results, but it may not be suitable for non-adherent cell types or slow-growing cells.

Roll tube: A technique in which viable cells are embedded in a semi-solid agar medium and incubated for a specified period, allowing for the observation of cell growth and viability. In the roll tube method, viable cells are mixed with a semi-solid agar medium and poured into a glass tube, which is then rolled to create a thin layer of agar containing the cells. The tube is incubated, allowing viable cells to grow and form visible colonies within the agar. By counting the colonies, researchers can determine the number of viable cells in the sample. This method is useful for detecting slow-growing or fastidious cells but can be more labor-intensive and time-consuming than other methods.

Nalidixic acid: This method uses nalidixic acid to selectively inhibit the growth of nonviable cells, enabling the determination of viable cell counts. The nalidixic acid method involves selectively inhibiting the growth of nonviable cells by incorporating the antibiotic nalidixic acid into the culture medium. Viable cells will continue to grow in the presence of the antibiotic, whereas nonviable cells will not. By comparing cell growth in the presence and absence of nalidixic acid, researchers

can estimate the percentage of viable cells in the sample. This method can be highly specific but may not be suitable for all cell types, as some cells may be resistant or sensitive to nalidixic acid.

Fluorogenic dye: Fluorescent dyes, such as calcein-AM or propidium iodide, can be used to stain live or dead cells, respectively, allowing for the quantification of viable cells using fluorescence microscopy or flow cytometry. Fluorogenic dyes are molecules that emit fluorescence when bound to specific cellular structures or molecules. For cell viability assessment, researchers often use two different dyes: one that selectively stains live cells and another that selectively stains dead cells. By measuring the fluorescence intensity of each dye in the sample, researchers can determine the proportion of live and dead cells. This method is highly sensitive and can provide rapid results but may be affected by factors such as dye penetration, cell autofluorescence, and photobleaching.

Trypan Blue cell viability assay: A widely used method that involves the use of the Trypan Blue dye, which selectively stains dead cells, allowing for the estimation of viable cell counts using a hemocytometer or automated cell counter. The Trypan Blue cell viability assay is a widely used and straightforward method for determining cell viability. The Trypan Blue dye selectively stains dead cells with compromised membrane integrity, while live cells with intact membranes remain unstained. After incubating the cells with the dye, researchers count the number of stained (dead) and unstained (viable) cells using a hemocytometer or automated cell counter. This method is relatively simple and quick, but it may not provide accurate results for certain cell types or experimental conditions, such as when cell membrane integrity is temporarily altered or when cell autofluorescence interferes with the detection of the dye.

The diverse methods available for cell viability assessment offer a range of options to evaluate the impact of various substances on cellular health. Each method has its advantages and limitations, and the choice of the most suitable method depends on factors such as the cell type, experimental conditions, and desired level of sensitivity and specificity.

4. Cytotoxicity assessment

Cytotoxicity assessment is an essential component of the evaluation process for pharmaceutical agents, medical devices, and other substances that may come into contact with living cells or tissues. It involves the study of the potential harmful effects of these substances on cells, including cell damage and cell death. Cytotoxicity is the degree to which a substance can cause damage to cells. Assessing cytotoxicity is crucial for ensuring the safety and effectiveness of medical devices and pharmaceutical agents, as well as other substances that may come into contact with living cells or tissues. Understanding the cytotoxic effects of these substances helps researchers identify potential hazards and optimize the design and formulation of products to minimize the risk of adverse effects on the human body.

Moreover, cytotoxicity assessment is a valuable tool in various other aspects of biomedical research, such as the investigation of the mechanisms of cell death and the identification of novel therapeutic targets. By studying the cytotoxic effects of specific compounds or treatments, researchers can gain insights into the biological processes involved in cell damage and death, which may contribute to the development of new therapeutic strategies for a wide range of diseases and conditions.

4.1 Applications of cytotoxicity assessment

Cytotoxicity assessment has a wide range of applications in biomedical research and drug development. Some key applications include:

Evaluating the safety of medical devices and pharmaceutical agents: Cytotoxicity assays can be used to determine the potential harmful effects of medical devices and pharmaceuticals on living cells, ensuring that these products do not cause unacceptable levels of cell damage or death.

Screening for potential therapeutic agents: High-throughput cytotoxicity assays can be used to screen large libraries of compounds for their ability to selectively kill cancer cells or other target cell populations, facilitating the identification of novel therapeutic agents.

Investigating the mechanisms of cell death: By studying the cytotoxic effects of specific compounds or treatments, researchers can gain insights into the biological processes and signaling pathways involved in cell damage and death. This knowledge can contribute to a deeper understanding of the mechanisms underlying various diseases and conditions, as well as the development of new therapeutic strategies.

Evaluating the efficacy of therapeutic interventions: Cytotoxicity assessment can be used to measure the effectiveness of therapeutic interventions, such as chemotherapy or radiation therapy, in inducing cell death in target cell populations. This information is crucial for optimizing treatment regimens and developing more effective therapeutic strategies.

Assessing the potential toxicity of environmental contaminants: Cytotoxicity assays can be employed to evaluate the potential harmful effects of environmental contaminants, such as pollutants, pesticides, and industrial chemicals, on living cells. This information is vital for understanding the risks associated with exposure to these substances and developing strategies to minimize their impact on human health and the environment.

Cytotoxicity assessment is an indispensable tool in the evaluation of medical devices, pharmaceutical agents, and other substances that may come into contact with living cells or tissues. The various methods available for measuring cytotoxicity offer researchers a range of options for assessing the potential harmful effects of these substances on cells and for investigating the mechanisms of cell damage and death.

4.2 Methods for measuring cytotoxicity

There are numerous methods available for measuring cytotoxicity, each with its advantages and limitations. Some of the most commonly used methods include:

1. *MTT assay:* The MTT assay is a colorimetric method that measures the reduction of a yellow tetrazolium salt (MTT) to a purple formazan product by metabolically active cells. The amount of formazan produced is proportional to the number of viable cells, providing an indirect measure of cytotoxicity.

2. *LDH release assay:* Lactate dehydrogenase (LDH) is a cytoplasmic enzyme that is released into the culture medium when cells undergo damage or death. The LDH release assay measures the activity of LDH in the culture medium as an indicator of cytotoxicity.

3. *ATP assay:* The ATP assay quantifies the amount of intracellular adenosine triphosphate (ATP) as a measure of cell viability. A decrease in ATP levels can indicate cytotoxicity, as damaged or dead cells typically have reduced ATP content.

4. *Annexin V/propidium iodide staining:* This method uses fluorescent dyes to stain cells undergoing apoptosis (annexin V) or with compromised membrane integrity (propidium iodide). By analyzing the fluorescence intensity of each dye, researchers can determine the proportion of apoptotic and necrotic cells, providing insights into the cytotoxic effects of a test substance.

5. *Colony formation assay:* The colony formation assay measures the ability of single cells to grow and form colonies in the presence of a test substance. A reduction in the number of colonies formed can indicate cytotoxicity.

4.3 MTT assay

The MTT assay, also known as the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, is a widely used colorimetric method for assessing cell viability and cytotoxicity [12]. This assay measures cellular metabolic changes using colorimetric shifts. It is based on the conversion of the purple tetrazolium dye MTT into insoluble formazan by the nicotinamide adenine dinucleotide phosphate (NADPH)-dependent cellular oxidoreductase enzymes. The reductive activity occurring in the mitochondria of living cells is employed to assess cell viability [6]. The MTT assay quantifies live cells by gauging mitochondrial activity, as it correlates with the number of formazan crystals [13]. Despite being the gold standard for cytotoxicity testing, the conversion to formazan crystals is influenced by various factors like metabolic rate and the number of mitochondria [14].

The MTT assay is based on the idea that proliferating cells exhibit a higher rate of MTT conversion, while nonviable or slow-growing cells have reduced metabolism and lower MTT reduction levels. After MTT application, formazan crystals are dissolved in a solution containing dimethyl sulfoxide or sodium dodecyl sulfate. Formazan concentrations can be measured using a spectrophotometer between 540 and 720 nm [15]. This method can provide an accurate dose-response curve for small cell numbers, test multiple parameters simultaneously, and is straightforward and highly replicable. The MTT assay is primarily used for in vitro testing of cytotoxic effects of various novel drugs at different concentrations and evaluating drug resistance in cell lines. It also assesses in vitro drug effects and their potential clinical applications. Due to its simplicity, the MTT assay is widely used to determine the toxicities of polymers, alloys, and ceramics [16]. However, the assay does not differentiate between cytostatic and cytotoxic effects, and results may be inaccurate if the cell population is low [17, 18]. Additionally, the MTT test is cell-specific and requires solubilization. Although highly sensitive, it works only with adherent cell targets. Since all cells must be killed during the protocol, this assay cannot be used for follow-up studies.

The MTT assay is relatively easy to perform, requiring only the addition of the MTT reagent to cell cultures, incubation, and subsequent solubilization and quantification of the formazan product. Non-destructive: As the MTT assay measures cell viability indirectly through the reduction of the MTT reagent, it does not require the destruction of cells or the use of invasive techniques.

4.4 AlamarBlue assay

The AlamarBlue assay offers a straightforward and dependable approach to measuring cell viability. It employs a fluorometric technique to detect cellular metabolic activity. Mitochondrial enzymes with diaphorase activity, such as NADPH dehydrogenase, reduce resazurin (oxidized form; 7-hydroxy-3H-phenoxazin-3-1-10-oxide) to resorufin (reduced form) [19]. The AlamarBlue assay has been utilized to examine trophoblast viability, migration, and invasion [20].

Cell viability can be assessed in 96-well plates after exposure to the biomaterial being studied. This test offers several advantages [19]. It is a straightforward method that uses a water-soluble substance, applicable to both suspended and attached cells, and features a fluorometric and colorimetric growth indicator [21]. Furthermore, the reagents are harmless to both cells and technicians. This test eliminates the necessity for washing and extraction steps, allowing for easy differentiation of endothelial cell viability and cell concentrations. It is a cost-effective test that enables continuous monitoring of endothelial cell metabolism and viability [22]. However, the reduction process may reverse with high cell numbers and extended culture times. One limitation of this assay is that it is not a direct cell counting technique. The assay relies on metabolic pathways that can be influenced by various factors, such as individual cell-reducing capacity and agents that affect mitochondrial activity or directly reduce resazurin [23].

4.5 LDH (lactate dehydrogenase) release assay

The LDH (lactate dehydrogenase) release assay is a widely used method for evaluating cell viability, cytotoxicity, and membrane integrity in various cell types. LDH is an intracellular enzyme that is released into the extracellular environment upon cell membrane damage or cell lysis. Lactate dehydrogenase release assay is a two-step rapid colorimetric test to assess the quantification of cell numbers in vitro [24]. The principle of the LDH release assay is based on the conversion of lactate to pyruvate by LDH in the presence of a cofactor, NAD+ (nicotinamide adenine dinucleotide). During this process, NAD+ is reduced to NADH, which then reacts with a specific tetrazolium salt, producing a colored formazan product. The absorbance of the formazan product can be measured using a spectrophotometer or microplate reader, with the intensity of the color directly proportional to the amount of LDH released and, consequently, the number of damaged or non-viable cells. Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme in every cell. Cytotoxicity is assessed by the activity of cytoplasmic enzymes released by damaged cells [25]. LDH is released into the cell culture when there is apoptosis, necrosis, or other cellular destruction in the membrane [26]. This test can detect the cytotoxic effects of various agents or environmental factors [27]. In the first stage, LDH catalyzes the conversion of lactate to pyruvate by reducing NAD+ to NADH. Following this, diasphorase enzymes reduce the tetrazolium salt to a red formazan in the presence of NADH.

Colorimetric lactate dehydrogenase (LDH) assay has also been used for the evaluation of antiviral activity against bovine viral diarrhea virus in vitro. Using the NADH produced during the conversion of lactate to pyruvate to reduce a second compound in a coupled reaction into a product with easily quantifiable properties makes it simple to quantify LDH activity. In this assay, the reduction of a yellow tetrazolium salt, INT, by NADH into a red, water-soluble dye of the formazan class is measured using absorbance at 492 nm. The amount of formazan is proportional to the amount

of LDH in the culture, which is proportional to the number of dead or damaged cells. The advantages are that LDH assay reflects the membrane integrity, and the reagent does not damage viable cells. The drawback is that it is not super sensitive. Despite these advantages, there are some limitations to the LDH release assay.

4.6 MTS assay

The MTS assay is utilized to evaluate cell proliferation, cell viability, and cytotoxicity. It can determine cell viability after exposure to various cytokines, growth hormones, cytotoxic drugs, and anticancer agents [28]. The MTS assay can also be used to assess the effects of chemical and physical treatments on the biocompatibility of human bone and tendon tissues for clinical applications [12]. The test's principle is that a colored formazan dye is generated when MTS tetrazolium molecules are reduced by live mammalian cells and other species' cells.

Living cells' mitochondrial reductase enzymes convert MTS to formazan crystals in the presence of phenazine methosulfate, an electron-coupling agent. These reduced formazan crystals are water-soluble, eliminating the need for an additional solution or washing step to dissolve them in the cell culture medium [29]. A spectrophotometer can measure these formazan crystals at 490–500 nm. The MTS reagent solution has better storage stability compared to MTT or XTT molecules. One advantage of tetrazolium assays that yield a water-soluble formazan is the ability to periodically measure absorbance from the test plates during the initial incubation stages. Multiple readings may be useful during assay development, but it is crucial to keep the plates in the incubator between readings to maintain a relatively constant environment [30].

4.7 XTT assay

The XTT (2,3-bis-(2- methoxy-4- nitro-5-sulfophenyl) -2H-tetrazolium –5-carboxanilide) assay is similar to the MTT assay but relies on the reduction of the XTT reagent to a soluble orange formazan product. This allows for a simpler and faster assay, as the formazan product can be directly quantified in the cell culture medium without the need for solubilization. The colorimetric change indicates cell viability, proliferation, and cytotoxicity through a nonradioactive test [31]. The biomaterial to be evaluated is placed in 96-well microplates and an adherent or suspension cell culture. Metabolically active cells reduce yellow tetrazolium salt (sodium 3′-[1- (phenylaminocarbonyl)- 3,4- tetrazolium]-bis (4-methoxy6-nitro) benzene sulfonic acid hydrate or XTT) into an orange formazan dye.

A scanning multiwell spectrophotometer (ELISA reader) measures formazan dye. XTT assay can assess cell proliferation when exposed to growth factors, cytokines, and nutrients. It can measure the increase in the overall activity of mitochondrial dehydrogenases that corresponds to the increase in the number of living cells and the amount of orange formazan formed. Cytotoxicity can also be measured using XTT assay by measuring the cytotoxic or growth-inhibiting agents such as inhibitory antibodies [32]. This assay is compatible with resorbable and nonresorbable guided tissue regeneration membranes in cultures of primary human periodontal ligament fibroblasts and human osteoblast-like cells [33]. Unlike MTT, where cells must be lysed to solubilize the formazan salt before absorbance measurement, XTT does not require cell lysis. This allows easier monitoring of the same samples at different time intervals [32].

4.8 WST-1 assay

The WST-1 assay (Water-Soluble Tetrazolium Salt-1) is a colorimetric method used to evaluate cell viability, cytotoxicity, and proliferation. It is based on the reduction of a tetrazolium salt, WST-1, to formazan by cellular dehydrogenase enzymes present in metabolically active cells. The formazan dye produced is directly proportional to the number of viable cells, and its absorbance can be measured using a spectrophotometer [34]. WST-1 assay is an improved version of the MTT assay and offers several advantages. Unlike the MTT assay, which requires solubilization of the formazan crystals in a separate step, the WST-1 assay generates a water-soluble formazan product. It is water-soluble, quick and sensitive [35]. This feature simplifies the experimental procedure and allows for the absorbance measurement without the need for additional steps or cell lysis. As a result, it is possible to measure cell viability more quickly and monitor the same cell samples at multiple time points.

The WST-1 assay is widely used in various applications, such as assessing the cytotoxic effects of drugs, chemicals, or nanoparticles on cell lines, screening for potential anticancer agents, and testing the biocompatibility of biomaterials. The assay is performed by adding the WST-1 reagent to cell cultures in a 96-well plate format, followed by incubation for a specific period, usually ranging from 1 to 4 hours. The absorbance of the formazan dye produced is then measured at a wavelength of around 450 nm using a microplate reader. One of the limitations of the WST-1 assay is its sensitivity to environmental factors and culture conditions, such as pH and serum concentration [35]. WST-1 assay is unsuitable for assessing the cell toxicity of Mn-containing materials in vitro [35]. It is essential to optimize the experimental conditions and maintain a consistent environment during the assay to obtain reliable and accurate results. Additionally, the WST-1 assay measures metabolic activity rather than directly counting the number of viable cells, so it may not always accurately reflect the true cell viability, especially in cases where metabolic activity is altered by the experimental treatments. Despite these limitations, the WST-1 assay remains a popular and convenient method for assessing cell viability, cytotoxicity, and proliferation due to its simplicity, speed, and compatibility with various cell types and experimental conditions.

4.9 ATP assay

The ATP assay is a sensitive and reliable method for evaluating cell viability, metabolic activity, and cytotoxicity in various fields of biomedical research. It is based on the quantification of adenosine triphosphate (ATP), the primary energy currency of living cells, which serves as an indicator of cell health and metabolic activity. The ATP assay is centered on the detection and quantification of intracellular ATP levels, which directly correlate with the number of viable, metabolically active cells in a given sample. The most common method of ATP quantification involves the use of a bioluminescent enzyme, luciferase, which catalyzes the oxidation of luciferin in the presence of ATP, producing light as a byproduct. The emitted light is then measured using a luminometer, with the intensity of the luminescent signal being proportional to the ATP concentration and, consequently, the number of viable cells [36].

The ATP assay is highly sensitive, capable of detecting even small changes in ATP levels and cell viability, making it suitable for assessing the effects of various substances on cellular metabolism and health. The ATP assay provides rapid results, often within minutes, allowing researchers to quickly assess cell viability and metabolic activity in response to various experimental conditions. The ATP assay is a powerful tool for

assessing cell viability and metabolic activity in a wide range of applications, from drug discovery and toxicology studies to basic cell biology research. Its sensitivity, speed, and adaptability make it a popular choice among researchers in various fields of biomedical science. However, the limitations of the ATP assay, such as assay interference and extracellular ATP contamination, should be carefully considered when interpreting results and selecting the most appropriate method for a specific research question or experimental condition. The evaluation of biomaterials' cytocompatibility can be performed through a variety of cytotoxicity tests, as demonstrated in **Table 1**.

Assay	Merits	Demerits
MTT Assay	Considered as the gold standard for cytotoxicity testing	The conversion to formazan crystals depends on metabolic rate and number of mitochondria resulting in many known interferences
AlamarBlue Assay	This test eliminates the need for washing and extraction steps. It is a simple method based on a water-soluble substance. It can be used for both suspended and attached cells.	This is not a direct cell counting technique. It relies on metabolic pathways that can be affected by various factors.
LDH release Assay	LDH assay reflects the membrane integrity, and the reagent does not damage viable cells.	This assay lacks sensitivity.
MTS Assay	Superior storage stability of MTS reagent solution. Water-soluble reduced formazan crystals eliminate the need for the washing step.	The testing environment should be kept relatively constant for consistent results
XTT Assay	Measure the increase in the overall activity of mitochondrial dehydrogenases that corresponds to the increase in the number of living cells. Does not require cell lysis.	Time-consuming and not very sensitive
WST-1 Assay	It is water-soluble, quick, and sensitive	Particles such as carbon nanotubes and magnesium particles can interfere with the results
ATP Assay	Highly sensitive, as it measures the adenosine triphosphate (ATP) content in cells, which correlates well with the number of metabolically active and viable cells	ATP assay is vulnerable to interference from certain factors, such as the presence of extracellular ATP, ATP-degrading enzymes, or substances that affect the bioluminescent reaction.
Sulforhodamine B Assay	Simple, rapid, and cost-effective method for measuring cell viability and cytotoxicity based on the cellular protein content.	It measures cellular protein content rather than directly assessing cell viability or metabolic activity and the changes in protein content due to factors other than cytotoxicity could affect the assay results.
Neutral Red Assay	Cheaper and more sensitive than many other cytotoxicity tests. Does not require unstable reagents like in the case of tests using tetrazolium salts.	Once started, it must be completed in less than 3 hours. The accuracy of the absorbance readings of this assay is affected by the visible needle-like crystals precipitates of the neutral red dye.
Trypan blue dye exclusion Assay	It is a simple and rapid technique dye exclusion test	The viability is indirectly accessed based on the cell membrane integrity. Very time-consuming.

Assay	Merits	Demerits
GSH Assay	The assay's sensitivity is increased by the enzyme glutathione reductase, which causes enzymatic recycling of GSH.	High polarity and limited stability
Protease viability marker Assay	Highly specific for measuring cell viability, as they detect the activity of specific proteases that are released from cells when they undergo necrosis or apoptosis.	Have a limited dynamic range, which means that they may not be able to detect subtle changes in cell viability.
Clonogenic cell survival Assay	Highly sensitive, as they can detect small changes in cell survival and growth.	Time-consuming, as they require cells to grow and form colonies over a period of several days or weeks.
DNA synthesis cell proliferation assays (e.g., BrdU or EdU)	Highly specific for measuring cell proliferation, as they directly measure DNA replication, which is a key component of cell division.	DNA synthesis cell proliferation assays can lack precision, as they only measure one aspect of cell proliferation.
Agar Diffusion Assay	Cost-effective and straightforward cytotoxicity screening test	It cannot be used for biomaterials that do not dissolve through agar.
Raman Micro-Spectroscopy	High sensitivity; Raman micro-spectroscopy can provide quantitative data at a molecular level.	Raman micro-spectroscopy can be a complex technique to perform and analyze, requiring specialized equipment and expertise.

Table 1.
Cytotoxicity assays as part of the cytocompatibility assessment of biomaterials.

4.10 Sulforhodamine B assay

The Sulforhodamine B (SRB) assay is a colorimetric method used for measuring cell viability, proliferation, and cytotoxicity. It has been widely employed to evaluate the efficacy of anticancer agents and to determine the cytotoxic effects of various substances on cell cultures [37]. The SRB assay is based on the ability of the protein-binding dye, sulforhodamine B, to interact with the basic amino acid residues of cellular proteins under mild acidic conditions. Upon fixation, the dye binds to the proteins in the cells, and the amount of bound dye is proportional to the cell mass or protein content [38]. One of the advantages of the SRB assay is its sensitivity and accuracy, as well as its low cost compared to other cell viability assays. Additionally, the SRB assay is relatively simple and can be performed in a high-throughput manner, making it suitable for large-scale screening studies. Furthermore, the SRB assay is compatible with various cell types, including adherent and suspension cells, and does not require cell lysis or radioactive reagents [39].

However, the SRB assay has some limitations. For instance, the assay may not be suitable for measuring cell viability in certain situations, such as when cells produce high amounts of extracellular matrix, which can interfere with the dye binding to intracellular proteins. Additionally, the SRB assay is not a direct measure of cell number, and it relies on the assumption that protein content is proportional to the number of viable cells. Therefore, factors that affect protein synthesis or degradation may influence the results of the assay [39].

4.11 Neutral red assay

The neutral red dye easily penetrates nonionic cell membranes and accumulates in lysosomes. The structural integrity of these lysosomes serves as an indicator of cell viability, which is the foundation of the neutral red uptake assay. This method can quantitatively measure live cells [40]. The neutral red assay technique is based on the degree of absorption and binding of the dye by living cells [41]. This cell viability assay aids in the in vitro evaluation of biomaterials. The underlying principle is that dying cells, due to altered membrane properties, can no longer take up neutral red. As a result, living cells can be distinguished from dead or dying cells based on differences in neutral red uptake. After being exposed to the dye for 3 hours, the cells are briefly rinsed with a phosphate buffer solution. Cells are seeded on a 96-well plate and then exposed to test material or control substances in a nutritive cell culture medium for 24 hours. Live cells take up neutral red into their lysosomes after 24 hours of exposure, but as cells begin to die, their ability to incorporate neutral red decreases. The cells are subsequently treated with an acidified ethanol solution to release the incorporated dye. Neutral red that has been released is measured at 540 nm and correlated to the number of viable cells. The viability of unexposed cells measured at 540 nm is set at 100%. The lysosomal capacity for dye incorporation, the foundation of the neutral red dye assay, can be employed to differentiate between living, injured, and dead cells. Viability curves can be generated based on absorption data to determine the concentration of the test chemical needed to inhibit neutral red dye uptake by 50%. Lysosomal swelling agents have been demonstrated to cause an increase in neutral red uptake, potentially leading to an underestimation of cytotoxicity [42]. However, the neutral red assay can produce false-positive or false-negative results. The neutral red assay is more affordable and sensitive than many other cytotoxicity tests [43]. However, being a sensitive test, it must be completed immediately once initiated, usually within 3 hours after the cells have been treated with the dye [41]. This assay does not require unstable reagents like tests using tetrazolium salts. Another limitation is that the absorbance readings' accuracy is affected by the visible needle-like crystal precipitates of the neutral red dye [44].

4.12 Trypan blue dye exclusion assay

The trypan blue assay is a dye exclusion test that provides a straightforward and quick method for assessing cell viability. It is based on the principle that live cells possess intact cell membranes, which exclude the Trypan Blue dye, while dead or damaged cells take up the dye due to compromised membrane integrity. Viable cells have intact cell membranes, allowing them to exclude certain dyes (such as trypan blue, Eosin, and propidium), while dead cells cannot [45]. In this test, a cell suspension is mixed with trypan blue dye. The dye's absorption or exclusion is then visually examined, as viable cells will display clear cytoplasm, while nonviable cells will exhibit blue cytoplasm. A significant drawback of this technique is that it indirectly assesses viability based on cell membrane integrity. It is possible for a cell to be nonviable while still having an intact membrane. Conversely, cells with compromised membranes might recover and become fully viable. Another limitation is the subjective evaluation of dye uptake, which may cause small amounts of dye uptake to go undetected, potentially indicating cell damage.

One solution to this issue is to evaluate dye exclusion using a fluorescent dye and a fluorescence microscope instead of using trypan blue with a transmission microscope. However, determining dye uptake and cell viability using the cell's light scatter properties can be quite complex. A notable limitation of this method is that it is time-consuming, although some protocols show that the trypan blue exclusion assay can be performed in under 10 minutes [46].

4.13 GSH assay

In human cells, the majority of glutathione (90–95%) is present in its reduced form (GSH). It plays a role in numerous regulatory processes, such as signal transduction, gene expression, DNA and protein synthesis, proteolysis, cell growth and apoptosis, cytokine and immune responses, protein glutathionylation, and the maintenance of mitochondrial function and integrity [47]. The glutathione assay is a colorimetric test that identifies alterations in GSH and GSSG levels during oxidative stress [48] using the enzymatic recycling technique with glutathione reductase and Ellman's reagent. This assay can measure reduced glutathione (GSH), oxidized glutathione (GSSG), total glutathione (GSH + GSSG) concentrations, and their ratio in various samples, including blood, plasma, serum, cultured cells, and tissues.

The glutathione reductase enzyme converts GSSG to GSH, generating a yellow chromophore that can be detected spectroscopically at 415 nm. Consequently, the concentration in an unknown sample is determined by evaluating the absorbance at 415 nm and comparing it to the standard curve for GSSG. This curve is plotted each time glutathione quantification is performed. The assay's sensitivity is enhanced by the enzyme glutathione reductase, which facilitates the enzymatic recycling of GSH. However, some drawbacks of the glutathione assay include its high polarity, limited stability, and the aliphatic structure of the assay [49].

4.14 Protease viability marker assay

The Protease Viability Marker Assay is a fluorescence-based method employed for assessing cell viability, cytotoxicity, and proliferation. This assay takes advantage of the presence of intracellular proteases, which are released from cells upon loss of membrane integrity, as a marker for cell viability [50]. These proteases specifically cleave nonfluorescent substrates, such as the commercially available Calcein-AM or CellEvent Caspase-3/7 Green Detection Reagent, to generate a highly fluorescent product, which can be detected using a fluorescence plate reader or a fluorescence microscope [32, 51].

One of the main advantages of the Protease Viability Marker Assay is its high sensitivity and specificity, as the fluorescent signal is only generated when the substrate is cleaved by the intracellular proteases, ensuring minimal background fluorescence. Additionally, the assay is nontoxic to the cells, allowing for real-time monitoring of cell viability over time and facilitating the assessment of cellular responses to various treatments or conditions [52]. However, there are some limitations to the Protease Viability Marker Assay. The assay may not be suitable for all cell types or conditions, as the presence and activity of intracellular proteases can vary depending on the cell type, culture conditions, or experimental treatments. Moreover, the fluorescent signal generated by the cleaved substrate may not be directly proportional to the number of viable cells, as the protease activity can be affected by various factors, such as cell density, cell size, or cell cycle stage. The Protease Viability Marker Assay can be combined with other assays to obtain more comprehensive information about cell

viability, cytotoxicity, and the mechanisms underlying cellular responses to various stimuli. For example, the assay can be used alongside assays that measure apoptosis, necrosis, or autophagy to provide a more complete picture of the cellular response to a test substance. By integrating the Protease Viability Marker Assay with complementary methods, researchers can gain deeper insights into the complex biological processes that govern cell survival and death in response to various stimuli.

4.15 Clonogenic cell survival assay

The clonogenic cell survival assay, also known as colony formation assay, is a widely used method for evaluating the ability of cells to survive and proliferate following exposure to various stressors, such as radiation, chemotherapeutic agents, or other cytotoxic substances [53]. This assay is based on the principle that a single cell can give rise to a colony of cells, which can be counted and analyzed to determine the proportion of surviving cells with the ability to form colonies [53].

The assay involves seeding cells at a low density in culture dishes, followed by treatment with the agent of interest. After a suitable incubation period, typically 1–3 weeks, the cells are fixed, stained, and the number of colonies containing at least 50 cells is counted. The surviving fraction is calculated by comparing the colony formation efficiency of treated cells with that of untreated control cells. The clonogenic cell survival assay has several advantages. It provides a direct measure of the reproductive capacity of cells, allowing for the assessment of treatment-induced cytotoxicity at the level of individual cells. Additionally, the assay is highly sensitive and can detect changes in cell survival across a wide range of treatment doses [54]. There are also some limitations to the clonogenic cell survival assay. The assay can be time-consuming and labor-intensive, as it requires a long incubation period for colony formation and manual counting of the colonies. Additionally, the assay may not be suitable for all cell types, particularly non-adherent or slow-growing cells, which may not form distinct colonies under the experimental conditions [55].

4.16 DNA synthesis cell proliferation assays

DNA synthesis cell proliferation assays are a group of methods used to assess cell proliferation by measuring the incorporation of nucleotide analogs into newly synthesized DNA during the S phase of the cell cycle. These assays are valuable for studying the effects of various stimuli, such as growth factors and cytotoxic agents, on cell growth and division. One commonly used DNA synthesis cell proliferation assay is the 5-bromo-2′-deoxyuridine (BrdU) assay. BrdU is a thymidine analog that gets incorporated into newly synthesized DNA during the S phase of the cell cycle. After incorporation, the BrdU-containing DNA can be detected using specific antibodies, allowing for the quantification of proliferating cells. The BrdU assay has been used in various applications, including drug screening and evaluation of the cytotoxic effects of anticancer agents [56]. Another DNA synthesis cell proliferation assay is the 3H-thymidine incorporation assay. This assay involves the incorporation of radioactive tritiated thymidine (3H-thymidine) into newly synthesized DNA. The amount of 3H-thymidine incorporated into the DNA can be quantified using a scintillation counter, providing a measure of cell proliferation. The 3H-thymidine incorporation assay has been widely used in studies investigating cell proliferation in response to growth factors, cytokines, and other signaling molecules [57]. DNA synthesis cell proliferation assays offer several advantages. They provide a direct measurement of

DNA synthesis, reflecting cell proliferation rates, and they can be applied to a variety of cell types, including adherent and suspension cells. However, these assays also have some limitations, such as the potential for false-positive or false-negative results due to nonspecific incorporation of the nucleotide analogs and the need for proper controls to account for variations in DNA synthesis rates.

4.17 AGAR diffusion assay

The agar diffusion test is a cytotoxicity barrier testing method in which the test material is simply placed on an agar layer covering a monolayer cell culture that simulates the mucosal membrane [58]. In this technique, cells are incubated for 24 hours before evaluating cytotoxicity. They are stained with neutral red dye to identify viable cells, stressed, and lysed [59]. Toxicity is determined by the loss of viable cells around the test material, which appears as an unstained area under and around the material being tested. In cases of high concentration and cytotoxicity of the diffusing substance, a loss of dye within the cells may be observed as the leachable toxic substance causes cell lysis [60]. Agar diffusion assays can evaluate the nonspecific cytotoxicity of the tested material's leachable components.

This test has the advantage of being cost-effective and simple to perform as a cytotoxicity screening tool. However, one limitation of this test is that potentially cytotoxic leachates in a solid state may stick to the agar rather than spreading across the plate, resulting in cells being only partially exposed to the test substance. Additionally, this test can only be used for materials that diffuse through the agar covering the cell monolayer. If materials do not dissolve in or spread through the agar, they will not show toxicity by damaging cells. Nonetheless, such materials could still be cytotoxic in a clinical setting [58].

4.18 Raman micro-spectroscopy

Raman micro-spectroscopy is a nondestructive and label-free optical technique that combines Raman spectroscopy with microscopy to provide detailed information about the molecular composition, structure, and interactions within a sample. This technique relies on the inelastic scattering of light, also known as Raman scattering, which occurs when light interacts with molecular vibrations in a sample [61]. Raman micro-spectroscopy has been widely used in various fields, including materials science, biology, and medicine. In materials science, it is used to study the crystallographic structures, stress distributions, and phase transitions of materials [62]. In biology and medicine, Raman micro-spectroscopy has been applied to study cellular processes, molecular interactions, and disease diagnosis.

One of the significant advantages of Raman micro-spectroscopy is its ability to obtain high-resolution spatial information about the sample's molecular composition without the need for labeling or sample preparation. This allows for real-time, in situ analysis of living cells, tissues, and biomaterials. Additionally, the technique is sensitive to both chemical and structural information, enabling the identification and differentiation of various molecular species within the sample [63]. However, Raman micro-spectroscopy also has some limitations. The most significant challenge is its inherently weak signal, which often requires long acquisition times or high laser power, which can cause sample damage or photobleaching. Recent advancements in instrumentation, such as the development of near-infrared lasers and highly sensitive detectors, have significantly improved the signal-to-noise ratio and reduced the acquisition time.

5. Conclusions

Cytotoxicity screening assays provide a measure of cell death caused by biomaterials. The term cytotoxicity describes the cascade of molecular events that interfere with the macromolecular synthesis, causing specific cellular, functional, and structural damage. Different screening assays are available, and it is crucial to understand the advantages and limitations of the various available assays so that they can be selected for appropriateness and interpreted accurately.

Conflict of interest

The authors declare no conflict of interest.

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