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# Features of spheroidogenesis of L929 cell line during prolonged three-dimensional cultivation

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**Abstract.** Cellular 3D cultures, in particular spheroids, are widely used to study intercellular interactions and test biocompatibility and toxicity, as they more accurately reproduce physiological conditions compared to 2D cultures. The aim of the study was to investigate the features of spheroid formation in L929 cell line in static suspension culture during long-term cultivation. To obtain spheroids, a suspension with a concentration of $2 \times 10^6$ cells/ml was placed in a 24-well plate coated with a 2% agar solution. Cultivation was carried out for 34 days. Every three days, half of the medium volume was replaced. The average diameter and volume of the spheroids were determined. The presence of necrotic cells in the spheroids was assessed by double staining of the spheroids with fluorescein diacetate and ethidium bromide. It was found that maintaining L929 cell line in a static suspension culture allowed spheroids with an average diameter of 118 μm to be obtained without the presence of a necrotic nucleus. Spheroid formation was a multi-step process that included initial cell aggregation, followed by compaction of the spheroids, which were kept in a floating state for one month of cultivation. During the study, it was found that L929 cell line spheroids were formed in several stages, including aggregation, compaction, and stabilisation of cells. All spheroids retained their spherical shape and remained viable, showing no signs of necrotic changes even on the 34th day of cultivation. The results of the study can be used to improve methods for testing biocompatibility and cytotoxicity, particularly in the development of new drugs and biomaterials using 3D cultures of L929 cell line

**Keywords:** 3D cultivation; spheroids; fibroblast cell line; static suspension culture; morphological analysis

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## Introduction

Cell 2D and 3D cultures are widely used in modern medical and biological research. When analysing the results obtained in 2D culture, it should be borne in mind that they only partially reflect the physiology of natural tissues. Cells that are forced to grow in a single plane as a monolayer can change their phenotypic and functional properties due to cytoskeletal rearrangement and disruption of cell-cell and cell-extracellular matrix contacts (Jubelin *et al.*, 2022). For this reason, studies of the toxicity of various substances and materials on 2D cultures do not provide adequate data for predicting cellular responses in the body. 3D models, in which cells are in three-dimensional cellular interactions, are closer to reality. As a result, 3D cultures (spheroids, organoids) are widely used to study intercellular interactions and cell differentiation, assess the toxicity of substances and the effectiveness of potential drugs, as components of bioengineered structures, and in regenerative medicine (Cardoso *et al.*, 2023).

Researchers C. Jubelin *et al.* (2022) summarised current approaches to the use of three-dimensional *in vitro* models in cancer research, in particular spheroids and organoids. The authors showed that 3D cultures better reproduce the architecture of tumour tissue, intercellular interactions, and oxygen and nutrient gradients compared to 2D cultures. Their advantage for evaluating the effectiveness of antitumour drugs and studying the mechanisms of drug resistance was emphasised. In 3D cell culture, a number of methods are used to induce spheroid formation by limiting cell adhesion and enhancing intercellular interactions. Thus, N.E. Ryu *et al.* (2019) noted that the use of non-adhesive surfaces, micro-well systems, and dynamic culture conditions is an effective approach to stimulate the self-organisation of cells into three-dimensional spheroid structures. In turn, A.P.P. Guimaraes *et al.* (2024) emphasised the importance of controlled cell aggregation methods, in particular the "hanging drop" technology, which allows the size and morphology of spheroids to be regulated and ensures reproducibility of results in 3D cultures.

It should be noted that the mechanisms of spheroid formation differ depending on the culture conditions. Under scaffold-based technology, spheroids are formed thanks to an artificial extracellular matrix that provides physical support for cells to attach and undergo three-dimensional reorganisation. At the same time, virtually all cells, including highly differentiated ones, participate in the formation of spheroids. K. Unnikrishnan *et al.* (2021) emphasised that artificial matrices are not limited to reproducing the architectural and biomechanical characteristics of the extracellular matrix, but also play an active role in regulating cell behaviour, in particular the processes of cell proliferation, migration and differentiation, as well as in the formation of spatial gradients of nutrients and oxygen within three-dimensional cell structures.

Under scaffold-free conditions, spheroid formation occurs through cell self-assembly. As noted by K.C. Liu *et al.* (2024), under such conditions, active synthesis and secretion of endogenous extracellular matrix components are observed, which contributes to the formation of 3D structures that are as close as possible to *in vivo* conditions. D. Stern-Tal *et al.* (2022) emphasised that scaffold-free approaches reduce the influence of foreign materials on the cellular response and allow the study of natural mechanisms of cell aggregation and spatial organisation. Unlike scaffold technology, this allows for the selection of stem/progenitor cells capable of forming cell colonies. The scaffold-free method of obtaining spheroids is the most promising for isolating and maintaining progenitor cells from different organs. One of the scaffold-free methods for obtaining spheroids is the use of a surface with low adhesive properties. Y. Xie *et al.* (2024) showed that in such a static suspension culture, the principle

of reduced adhesion of the culture surface is used: the attachment of cells to the surface is suppressed, so they try to realise their ability to attach through interaction with other cells and spontaneously form spheroids. Agar, chitosan, hyaluronic acid, etc. are used to create a low-adhesion surface.

The process of spheroid formation involves changes in cell interaction and cytoskeletal organisation, as well as the synthesis of the extracellular matrix. It is important to note that the main stages of spheroid formation are standard but may have specific features depending on the source of the cells or the method used to obtain the spheroids. Epithelial cells often show delayed aggregation, reflecting their dependence on polarisation and the development of specialised intercellular contacts (tight and adhesive contacts, desmosomes) (Bharathan *et al.*, 2024). In comparison, mesenchymal-derived cells, including the L929 line, typically aggregate more rapidly and form spheroids. A. Moisieiev *et al.* (2024) found that L929 cells have the ability to form spheroids in static suspension culture on low-adhesive surfaces. G.A. Bozhok *et al.* (2019) studied such spheroids only in the initial period (7-8 days), while the dynamics of their further existence under 3D culture conditions remain unknown.

Thus, the scientific literature notes the importance of using 2D and 3D cell cultures in biomedical research, in particular for studying intercellular interactions, assessing the toxicity and efficacy of drugs, and developing bioengineered structures and regenerative medicine. Despite the wide range of applications of 3D cultures, the details of the dynamics of spheroid formation of L929 cell line under conditions of prolonged 3D cultivation remain unexplored, which is relevant for assessing biocompatibility and cytotoxicity over a long period of time. In this regard, the aim of the study was to investigate the stages of spheroid formation in L929 cell line culture on a low-adhesive surface during prolonged 3D cultivation.

## Materials and Methods

The study was conducted at the Department of General and Clinical Pathology of the Medical Faculty of V.N. Karazin Kharkiv National University in collaboration with the Department of Cryoendocrinology of the Institute of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine in 2021-2022. To study the stages of spheroid formation, the L929 cell line was selected, which is a transplantable adhesive cell line obtained from the subcutaneous connective tissue of C3H/An mice. Due to its high proliferative properties and genetic stability, the line is widely used in laboratory and screening analyses in accordance with the international standard ISO 10993-5:2022 (2022) for assessing the biocompatibility (Sharma *et al.*, 2016) and cytotoxicity of substances (Cannella *et al.*, 2019). In addition, as shown by researchers F. Ehrhart *et al.* (2009) and A. Moisieiev *et al.* (2024), it has the ability to grow in 3D culture with the formation of spheroids.

L929 cell line were cultured in DMEM/F12 medium (Biowest, France) with the addition of benzylpenicillin (200 IU/ml), streptomycin (200 µg/ml), amphotericin B (2.5 µg/ml) and 10% foetal calf serum (Biowest) in a $CO_2$ incubator at 37°C and 5% $CO_2$. Initially, the cells were cultured under standard conditions in polystyrene flasks (SPL Life Sciences, Korea). The seeding density was $4 \times 10^4$ cells per 1 $cm^2$ of surface area. After the formation of a confluent monolayer, the cells were detached from the surface using a 0.5% trypsin solution prepared in physiological saline (Novopharm-Biosynthesis, Ukraine) and Versen's solution (Biowest, France) in a 1:1 ratio. To obtain spheroids, a suspension with a concentration of $2 \times 10^6$ cells/ml was transferred to a 24-well plate coated with a 2% agar solution (Ferak, Germany). The agar solution was sterilised by autoclaving, then heated in a boiling water bath to a liquid state and coated the bottom of the wells (250 µl/well) under sterile conditions. After the agar layer had solidified, the cells were seeded. Cultivation was

carried out for 34 days. Every 3 days, ½ of the medium volume was replaced.

Microphotography was performed using an AmScope XYL-403 inverted microscope (PRC) with a digital camera. The AxioVision Rel. 4.6 programme (Carl Zeiss, Germany) was used for morphometric analysis of the spheroids. The programme tools were used to determine the average diameter of the spheroids, from which their average volume (V) was calculated using the formula:

$$V = \pi/6d^3, \quad (1)$$

where d – spheroid diameter.

The presence of necrotic cells was assessed by double staining of spheroids with fluorescein diacetate (FDA) and ethidium bromide (EB) using a Zeiss LSM 510 META confocal microscope (Carl Zeiss, Germany). Confocal images were obtained at an excitation wavelength of 488 nm for FDA and 543 nm for EB. Statistica 10 software (StatSoft, USA) was used for statistical analysis of the data. Normality of distribution was determined using the Shapiro-Wilk W-test. The data are presented as the mean ± standard deviation. The sample size (n) was 25 spheroids for each cultivation term.

## Results and Discussion

After 2 days of cultivation on a low-adhesive surface, single cells and small aggregates of 2-3 cells were observed (Fig. 1). During the first week of cultivation, the cells formed loose clusters, which gradually increased in size due to aggregation and, possibly, proliferation. The process of aggregation and proliferation continued for the next 3-4 days. On day 7, the shapeless cell aggregates became denser and acquired a more spherical shape. On day 9, it was noticeable that the spheroids were becoming denser. In addition, large clusters began to appear, which may be the product of spheroid fusion. Such conglomerates of spheroids were not used in further analysis.

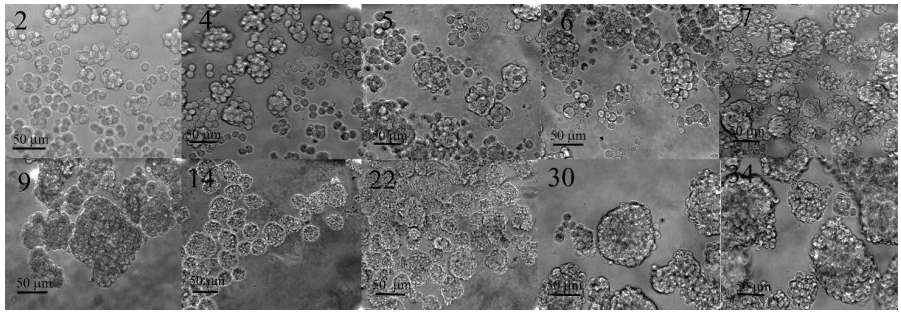


***Figure 1.*** *Stages of spheroid formation from L929 cell line during cultivation in low-adhesion conditions for 34 days*

**Note:** the number indicates the number of days of cultivation

**Source:** developed by the authors

In the next period (14-34 days), along with an increase in size, the spheroids became more spherical in shape, and individual cells became less distinct. However, not all cells merged, so single cells and small aggregates were observed in the culture along with the spheroids. This may be due to the dynamic process of proliferation and exfoliation of cells in spheroids. After 30 days of cultivation, the spheroids remained floating in the culture medium, while the process of their fusion was permanently observed, indicating the preservation of intercellular adhesion and the dynamic restructuring of three-dimensional cell aggregates (Fig. 2).

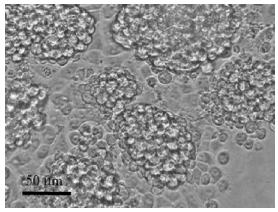


***Figure 2.*** *Attachment of spheroids formed during cultivation of L929 cell line in low-adhesion conditions for more than 30 days*
**Note:** around the spheroids, there are noticeable clusters of cells that have migrated and formed a monolayer
**Source:** developed by the authors

The fusion of spheroids was accompanied by an increase in their size and a change in morphology, which may be associated with prolonged maintenance of scaffold-free culture conditions and the absence of stable adhesion to the surface. After more than 30 days, gradual biodegradation of the agar coating was observed, accompanied by a loss of low-adhesion conditions. This led to the spheroids attaching to the bottom of the culture dish, from which cells began to migrate, forming a monolayer. These morphological changes emphasise the critical role of stable low-adhesion conditions in maintaining spheroid culture during long-term cultivation. The quantitative dynamics of spheroid sizes from L929 cells during 35 days of cultivation are shown in Figure 3.

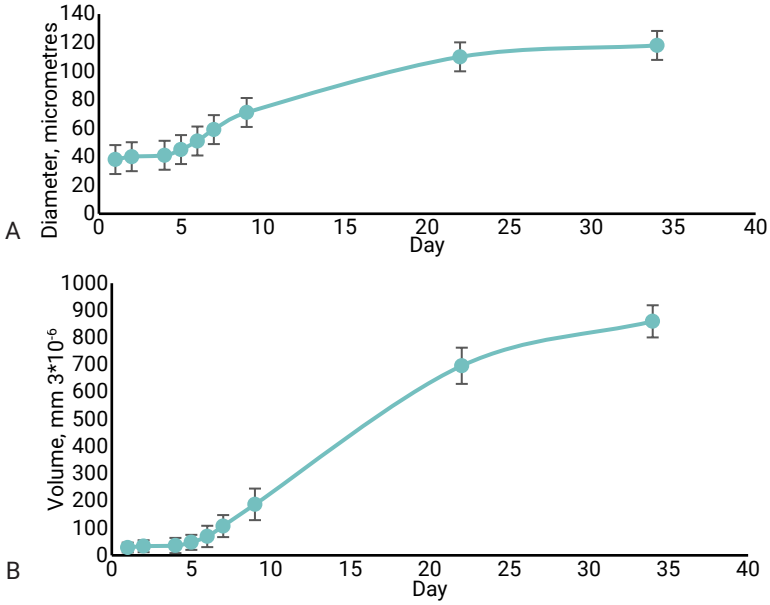


***Figure 3.*** *Dynamics of spheroid sizes from L929 cell line*
**Note:** A – average diameter and B – average volume of spheroids obtained over 34 days
**Source:** developed by the authors

The initial diameter of the cellular aggregates averaged 38 ± 3 μm, while their volume was 28 ± 18 × $10^{-6}$ $mm^3$, reflecting the formation of small primary cellular conglomerates. During the process of primary aggregation up to day 5, the linear dimensions did not increase, indicating the stability of the initial cellular aggregates and a limited number of cell divisions at this stage. Morphological analysis showed that by day 7 the aggregates acquired a spherical shape, reflecting the process of cellular self-organisation and optimisation of intercellular contacts. After this point, a sharp increase in both the diameter and the volume of the spheroids was observed. It is likely that from this stage the change in spheroid size was associated not only with

further aggregation but also with cellular proliferation within the three-dimensional structures. The increase in size was accompanied by a more compact and homogeneous spheroid morphology, indicating active intercellular adhesion and the organisation of extracellular matrix produced by the cells themselves. By day 34, the spheroids reached a mean diameter of 118 ± 36 μm and a mean volume of 859.85 ± 59.1 $mm^3$, demonstrating a substantial increase compared with the initial state and indicating the effectiveness of three-dimensional cultivation in maintaining the viability and functional activity of L929 cells over a prolonged period. Such data emphasise the phase-dependent nature of spheroid development: the initial formation and stabilisation of aggregates transitions into a phase of active growth, in which cellular proliferation and the strengthening of intercellular contacts predominate, ensuring the maintenance of structural integrity and functional organisation of the three-dimensional culture. At these average sizes, spheroids formed from the L929 cell line did not exhibit a necrotic core, as evidenced by the absence of red staining in the FDA and EB test (Fig. 4).

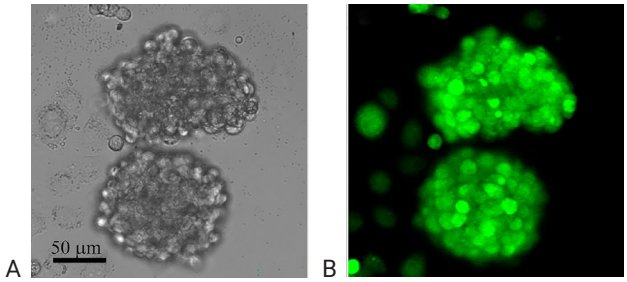


***Figure 4.*** *Viability of cells in spheroids according to FDA/EB staining*
**Note:** A – light microscopy; B – fluorescence microscopy
**Source:** developed by the authors

To assess cell viability based on plasma membrane integrity and enzymatic activity, a test using FDA and EB was used. FDA passively diffuses through the plasma membrane of viable cells. Inside the cell, under the action of intracellular esterases, FDA is hydrolysed to fluorescein, which is retained in the cytoplasm and emits green fluorescence, reflecting esterase activity and membrane integrity. EB enters only cells with impaired membrane integrity, binds to nucleic acids and emits an intense red fluorescence, indicating cell death or severe damage. The analysis showed high cell viability and sufficient diffusion of nutrients and oxygen to the centre of the spheroids with an average diameter of 118 ± 36 μm, which allows maintaining the functional activity of cells even in a three-dimensional structure. The results obtained emphasised the phase nature of spheroid development: the initial stabilisation of aggregates transitions into active growth with cell proliferation, the formation of spherical morphology and the maintenance of metabolic stability within the three-dimensional culture, which makes such aggregates relevant models for in vitro studies. Thus, as a result of the experiments, it was established that when using a low-adhesive agar coating, L929 cell line quickly organise into aggregates, after which, from the 7$^{th}$ day, in the process of compaction, they form spheroids that remain in a floating state for up to 34 days.

Continuing the analysis of the data obtained, it can be noted that at different stages of cultivation, changes in the structure of spheroids are observed, which is the result of active proliferation and organisation of cells. This opens up opportunities for a more detailed discussion of the mechanisms that regulate these processes. The results of the presented study showed that L929

cell line actively form spheroids when cultured on low-adhesive surfaces, which is consistent with the literature data on the mechanisms of spheroid formation. As noted by K. Białkowska *et al.* (2020), 3D spheroids are extremely important for research in biology because they provide an opportunity to recreate the natural conditions of the cellular environment, in particular cell-cell and cell-ECM interactions, which are limited in 2D cultures. They also emphasised that spheroid formation in 3D cultures depends on a number of factors, including culture conditions and cell type, which are critical for analysing biocompatibility and cytotoxicity *in vitro*.

Research by N.E. Ryu *et al*. (2019) showed that the first stage of spheroid formation is characterised by the initial aggregation of individual cells into loose clusters, which was also observed in the current study. This stage is mainly regulated by passive physical processes and early intercellular recognition events, as emphasised by Y. Zhou *et al.* (2017). Intravital observations of cell culture on non-adhesive surfaces allowed L. Adenis *et al*. (2020) to identify four phases characteristic of several cell lines during spheroid formation. The first phase is the random movement of individual cells. The second phase is the formation of aggregates of 5-10 cells. The third phase is the restructuring of aggregates, during which the cells inside are constantly moving and reorganising. During this phase, the process of cell proliferation begins. A few hours after formation, the aggregates condense and reorganise into a three-dimensional shape, which is the final phase of spheroid formation. Proliferation continues, constantly increasing the size of the spheroids. The cells inside the spheroids actively remodel their microenvironment by synthesising and depositing extracellular matrix components such as collagen, fibronectin and laminin. This endogenous extracellular matrix provides additional mechanical support to the spheroids and modulates their sensitivity to xenobiotics.

The rate of progression of the phases, density, and size of aggregates/spheroids may vary depending on both the culture conditions (static culture, surface properties, medium composition) and the characteristics of epithelial, mesenchymal, stem, or tumour cells, as demonstrated by L. Adenis *et al.* (2020). Moreover, not all cell lines form compact spheroids when cultured in static suspension culture. The key difference is that L929 spheroids derived from fibroblasts are less prone to early necrosis. In contrast, according to Y. Tan *et al*. (2020), more metabolically active or sensitive epithelial cancer cell lines are prone to earlier hypoxia and central necrosis. In the current study, L929 cells underwent all phases of spheroid formation, including initial aggregation, maturation and restructuring of aggregates, compaction and stabilisation due to extracellular matrix deposition.

During spheroid formation, cells organise themselves into a 3D structure, leading to the formation of gradients of metabolites and gases, such as oxygen, within the spheroid. As established by M. Acland *et al.* (2018), oxygen diffusion into the spheroid is limited to a cell layer of 150-200 μm. As the size of the spheroid increases, oxygen diffusion becomes more difficult, which can lead to the formation of necrotic zones in the centre (Barisam *et al*., 2018). Since the average size of the spheroids in the experiments did not exceed 120 μm, no necrotic zones were observed, confirming effective oxygen diffusion and maintenance of cell viability in all parts of the spheroid.

Thus, the experiments revealed that L929 cell line effectively form spheroids when cultured on low-adhesive surfaces, confirming their ability to self-organise and aggregate in three-dimensional conditions. The dynamics of spheroid formation includes several phases, starting from the initial aggregation of cells to their subsequent compaction and stabilisation due to the formation of an extracellular matrix. The observed changes in the size and morphology of spheroids are consistent with the literature data on the mechanisms of spheroid formation in other cell lines. Under cultivation conditions of up to 34 days, L929 spheroids do not show necrotic areas, confirming effective oxygen diffusion in three-dimensional

aggregates, preserving cell viability. These results highlight the importance of three-dimensional cultures for maintaining cell functional activity in long-term experiments, as well as for further application in biomedical research, such as biocompatibility and cytotoxicity testing.

## Conclusions

This article presents the results of a study of the stages of spheroid formation in L929 cells during prolonged three-dimensional cultivation on a low-adhesive surface. During the study, the morphological and quantitative dynamics of cell aggregate formation were monitored over 34 days, and their viability within three-dimensional structures was assessed. It was found that L929 cells in static suspension culture rapidly form primary aggregates, which from the 7$^{th}$ day become denser and acquire a spherical shape. Subsequently, the spheroids remain in a floating state, demonstrating active intercellular adhesion and the formation of an endogenous extracellular matrix, which maintains their structural integrity and functional activity throughout the entire cultivation period. With an average diameter of about 118 micrometres, no necrotic nuclei were found, indicating sufficient diffusion of nutrients and oxygen to the central zones of the spheroids. Thus, spheroid formation is a multistage process that includes initial cell aggregation, followed by compaction and stabilisation of three-dimensional structures over one month of cultivation.

Summarising the data obtained, it can be stated that the proposed approach using a low-adhesive agar coating allows obtaining stable three-dimensional models of L929 cell culture relevant for *in vitro* studies. Spheroids of this line are characterised by predictable growth dynamics, preservation of viability and absence of early necrosis, which makes them suitable for assessing biocompatibility, cytotoxicity and modelling cell responses in a three-dimensional environment. Thus, the results are of methodological and practical importance for the improvement of 3D models in biomedical research. Promising areas for further research include studying the effect of various characteristics of low-adhesive surfaces on the formation rate and stability of spheroids, detailed assessment of intracellular proliferation and apoptosis, analysis of the diffusion of nutrients and pharmacological agents into aggregates, as well as the application of the obtained three-dimensional models for testing cellular responses to external stimulants and therapeutic drugs.

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None.

## Conflict of Interest

None.

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## Особливості сфероїдогенезу клітин лінії L929 при тривалому тривимірному культивуванні

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**Анотація.** Клітинні 3D-культури, зокрема сфероїди, широко використовуються для вивчення міжклітинних взаємодій та тестування біосумісності та токсичності, оскільки вони точніше відтворюють фізіологічні умови в порівнянні з 2D-культурами. Метою дослідження було вивчити особливості сфероїдоутворення клітин лінії L929 у статичній суспензійній культурі протягом тривалого терміну культивування. Для отримання сфероїдів суспензію з концентрацією $2 \times 10^6$ клітин/мл приміщували у 24-лунковий планшет з покриттям 2 % розчином агару. Культивування проводили впродовж 34 діб. Кожні три доби здійснювалась заміна половини об'єму середовища. Визначався середній діаметр та об'єм сфероїдів. Наявність некротичних клітин у складі сфероїдів оцінювалась за допомогою подвійного забарвлення сфероїдів флуоресцеїндіацетатом і етидієм бромідом. Було встановлено, що підтримка клітин лінії L929 у статичній суспензійній культурі дозволяла отримувати сфероїди з середнім діаметром 118 мкм без наявності некротичного ядра. Формування сфероїдів було багатоступеневим процесом, що включало початкову агрегацію клітин, після якої відбувалась компактизація сфероїдів, які утримувались у флотуючому стані протягом одного місяця культивування. Протягом дослідження було виявлено, що сфероїди клітин лінії L929 утворювались через кілька етапів, включаючи агрегацію, ущільнення та стабілізацію клітин. Всі сфероїди зберігали свою сферичну форму та залишалися життєздатними, не демонструючи ознак некротичних змін навіть на 34-й день культивування. Результати дослідження можна використати для вдосконалення методів тестування біосумісності та цитотоксичності, зокрема при розробці нових лікарських засобів та біоматеріалів за допомогою 3D-культур клітин лінії L929

**Ключові слова:** 3D-культивування; сфероїди; клітинна лінія фібробластів; статична суспензійна культура