Gelacell™ 3D nanofibrous scaffold

Taking cell culture to the next dimension

Introduction

In the past decades, the two-dimensional culture approach has been the standard for research that involves the culture of eukaryotic adherent cells. This approach is extensively documented and well characterized; however, it suffers from major drawbacks. Mainly stemming from the adaptation that cells have to undergo a 2D culture approach. For cells to correctly adhere and grow on a surface, they tend to flatten and stretch into a monolayer. This not only creates a change in their shape, but also in their gene expression, limits their cell-to-cell interactions, and modifies their natural response to external stimuli. Thus, the biological relevance of research performed on a 2D monolayer is limited by its inability to reproduce the complex behavior found in native tissues. That is why in recent years there has been a marked effort towards the use of 3D culture systems that can better resemble living tissue conditions [1].

Whether it is for oncological studies, toxicity screening, tissue engineering for regenerative medicine, angiogenesis and complex systems modeling, or efficiency assessment of chemical compounds for novel therapeutics, performing experiments in environments that closely resemble the structure of a natural extracellular matrix, is an essential factor to improve the accuracy and relevance of the results.

Among the different technology platforms that support 3D cell culture, nanofibrous scaffolds offer several unique benefits that make them stand out as a superior alternative. Namely, the high surface-to-volume ratio, the variety of its possible polymeric components, the tunability of their mechanical and surface chemical properties, their high porosity that allows nutrients transfer and cell motility, and their ease for scalable standardized production [2]. All these elements make nanofibrous scaffolds an ideal candidate to act as an *in-vitro* ECM with exceptional performance.

Concurrently, and especially regarding tissue engineering applications, obtaining scaffolds that mimic the tissue native architecture at the nanoscale has always been a major challenge, specifically the reproduction of the interwoven nanostructure created by the natural ECM proteins, which supports and directs cell adhesion, proliferation, and bioactivity.

The use of nanofiber scaffolds can address this challenge by providing a porous and highly interconnected fibrous network that can act as an unparalleled substitute for the native microenvironment, providing an ideal tissue engineering and regenerative medicine building block.

Product description

Gelacell™ 3D nanofibrous scaffold, is a non-woven highly porous scaffold specially designed for in vitro 3D cell culture and tissue engineering. It is made from a crosslinked network of randomly oriented pharmaceutical-grade porcine-derived gelatin nanofibers. It is biodegradable, bioresorbable, non-toxic, compatible with many cell lines, and suitable for the culture of complex systems [3]. The scaffold has been optimized to provide chemical, thermal, and mechanical stability for 3D cell culture, as well as an adequate swelling capability and porosity to allow for nutrient diffusion and avoid cellular waste build-up.

The Gelacell™ scaffolds come with several advantages that are inherent to our patent-pending method itself, ultimately producing a 3D microarchitecture fundamentally different from the planar and stiff alignment surfaces of alternative nanofiber production methods like electrospinning. Gelacell™ scaffold's nanofibers, which range from 0.5-0.8 µm in diameter, form a unique porous network in a bundle-like arrangement which provides a 3D framework for an *in vitro* ECM analog suitable for the culture of many cell types such as fibroblasts, MSCs, liver cells, etc. In Figure 1, SEM pictures of the nanostructure of Gelacell™ under different magnifications can be appreciated.

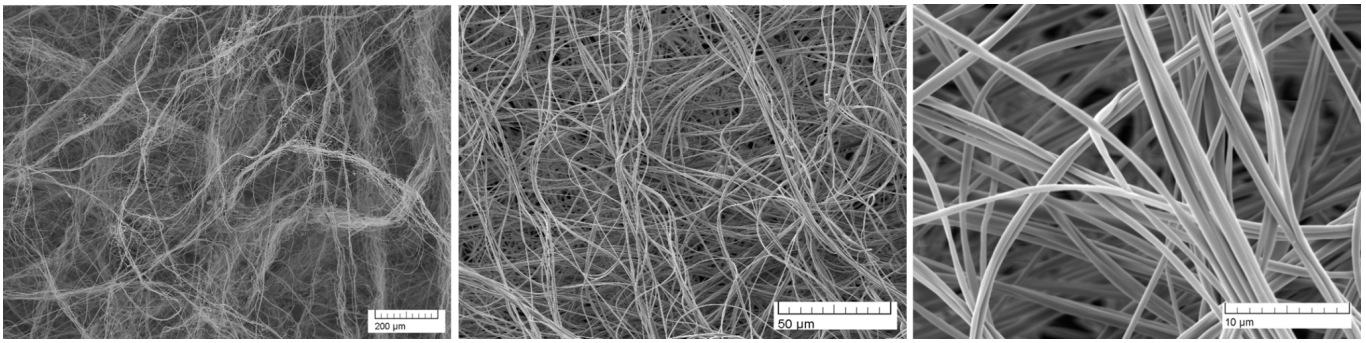

Figure 1: SEM images of Gelacell™ 3D nanofibrous scaffold nanofibers structure under different magnification.

The technology from which Gelacell™ 3D nanofibrous scaffold is made offers a standardized, easily scalable, high-throughput production process, with batch-to-batch reproducibility, at a lower cost when compared with electrospinning.

The scaffold remains viable for long periods of time when stored under dry conditions at room temperatures.

The scaffold can be sterilized with ease using radiation, dry heat, or ETO gas.

Product performance

The physical properties of Gelacell™, namely, thickness, swelling, degradation, tensile strength, and elongation were evaluated in-house and the results can be found in the Product Data Sheet (PDS).

Cell viability and cell behavior in a 3D culture were assessed by seeding eukaryotic cells (Baby Hamster Kidney, BHK-21) onto the Gelacell™ scaffolds, and analyzing cell adhesion and proliferation through morphology observations and MTS activity assays. The following section illustrates the cell culture studies carried out with Gelacell™ scaffolds.

Cell viability and microscopy after 24 hours

To evaluate the performance of the Gelacell™ scaffold in comparison with a standard nitrocellulose membrane, and a traditional 2D culture at the bottom of the well, cell viability assays, and confocal microscopy observations were performed 24 hours after seeding. Three different seeding concentrations were tested for the MTS assays, and one seeding density for the confocal microscopy. The detailed methodology of the tests can be found in the Methods section of this report.

Figure 2 depicts the results of the MTS cell activity assay after 24 hours with the three different platforms and the different seeding concentrations. It is evidenced that for all the different seeding concentrations, the Gelacell™ scaffold supports better cellular activity than the nitrocellulose membrane. This could be explained by a better cellular adherence profile of the Gelacell™ scaffold when compared to the nitrocellulose membrane.

Moreover, it is noticeable that at the highest seeding concentration tested, namely the 800k cells per well, there is a remarkable similarity in the cell viability between the Gelacell™ scaffold and the control cells in the bottom of the well.

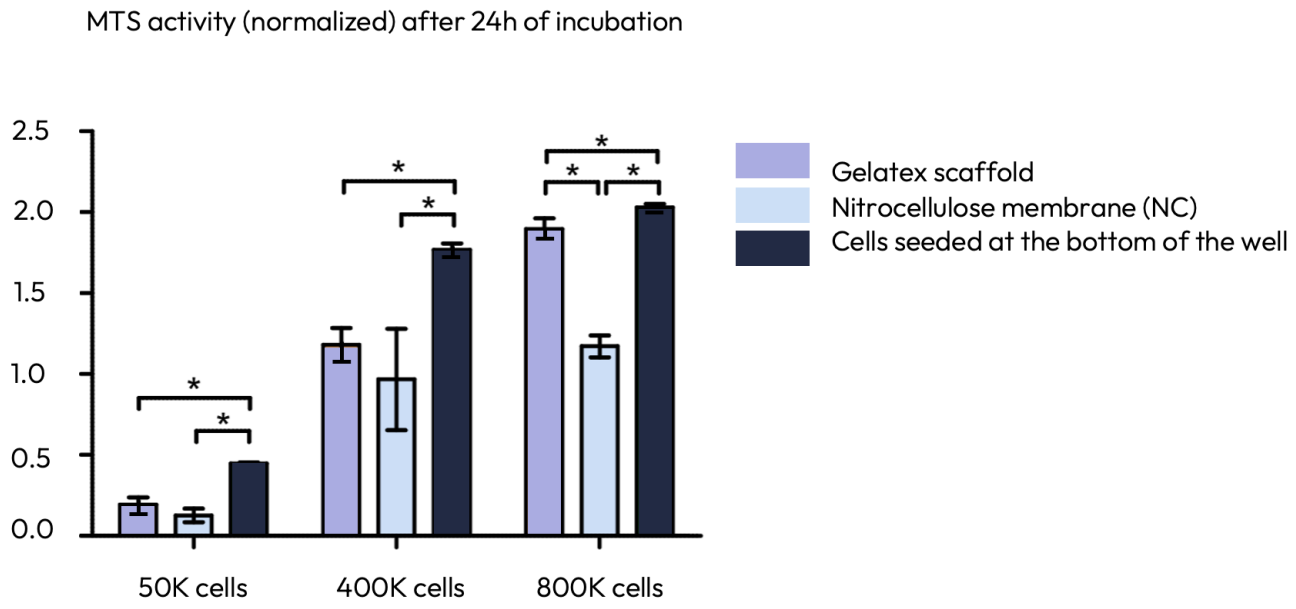

Figure 2: Viability of BHK 21 cells on Gelacell™ scaffold and a nitrocellulose membrane (NC) after 24 h of incubation using different cell seeding concentrations. Cells on the bottom of 24 well plates and nitrocellulose were used as controls.
* statistical significance (p<0,05). Results are shown as a mean of 3 biological replicates ± standard deviation.

For the confocal microscopy, the cells were stained with DAPI and Alexa 568 after 24 hours of incubation. The main objective of these observations was to determine the structural differences between the cells that had been incubated in the 3D environment of the Gelacell™ scaffold, and the cells that had grown into the traditional 2D monolayer at the bottom of the well. Examining the structural changes and growth patterns of the cells in these different conditions provides insights into the feasibility of using the Gelacell™ scaffold as a suitable growth substrate for eukaryotic cells.

Figure 3 depicts confocal images of viable cells in the Gelacell™ scaffold, attached to nanofibers, and compares them with the cells in the 2D control. Figure 4 shows the 3D render of a confocal image Z-stack shows cells at different depths of the Gelacell™ scaffold.

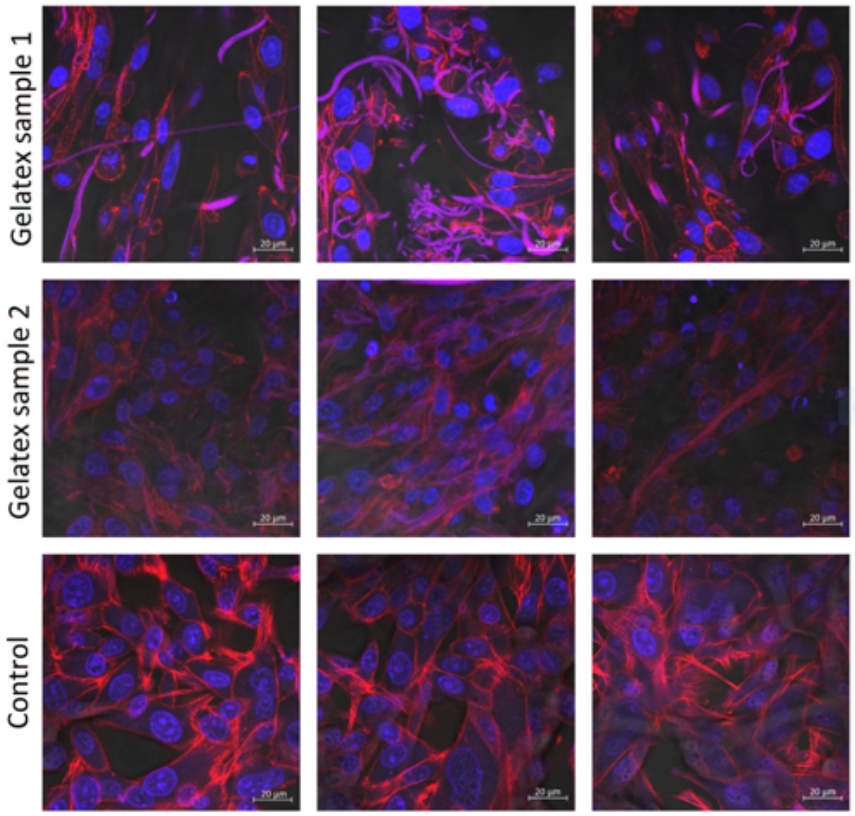

Figure 3: Confocal microscopy images. Cell structure after growing on the Gelacell™ scaffold for 24 h and using an initial seeding concentration of 400 000 cells per well. Images from different locations within the sample are depicted in the same row. Blue – DAPI stained cell nucleus. Red – Alexa 568 stained actin filaments. Images show that fibers also exhibit autofluorescence (stained pink-purple). The control is the cells grown for 24 hours on a 2D layer at the bottom of the well.

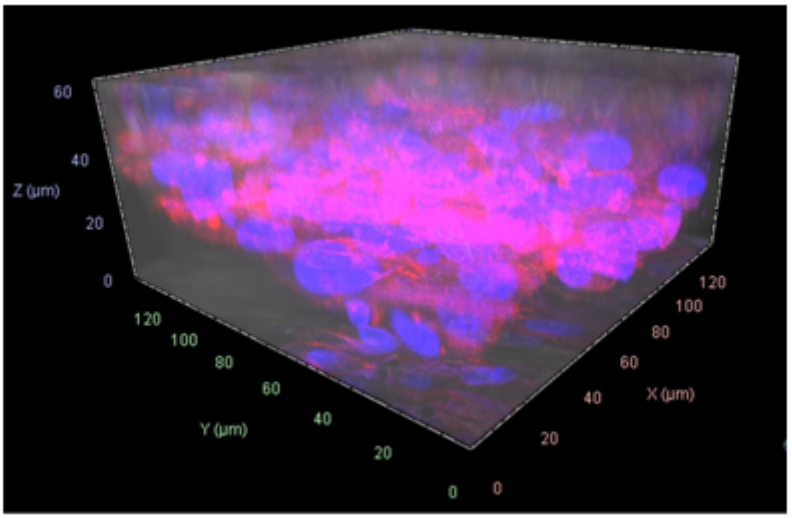

Figure 4: Z-stack 3D image showing cell locations in different planes within the Gelacell™ matrix (400 000 cells per well after 24 h).

Both Figure 3 and 4 illustrate that there was successful cell motility into deeper layers of the Gelacell™ scaffolds, demonstrating the cells' ability to navigate, migrate inwards, and distribute throughout the matrix. This is mainly believed to be due to the intrinsic highly porous and flexible 3D structure of the nanofibrous network inherent of the Gelacell™ scaffold.

When compared to the control set, the actin filaments prevalence of the cells cultured in the Gelacell™ scaffold is less noticeable, hinting that the cells do not need to undergo harsh morphological changes in order to improve their adherence to the surface. It is believed that, aside from a support network, the nanofibrous structure of the scaffold provides mechanosensory feedback for the cells that is more similar to that of a naturally occurring tissue. This idea is further supported by the observation of the flat cell morphologies of the control sample and comparing them with the more elongated shape of the cells grown on the Gelacell™ scaffolds.

Cell viability and microscopy after 7 days

To evaluate the performance of the Gelacell™ scaffold in comparison with a standard nitrocellulose membrane, and a traditional 2D culture at the bottom of the well, cell viability MTS assays and confocal microscopy observations were performed 1, 3, and 7 days after seeding. The seeding concentration for all samples was 150k cells per well. The detailed methodology of the tests can be found in the Methods section of this report.

Figure 5 shows the results of the cell viability MTS assays for all staples throughout the 7-day period. Although no statistically significant differences were detected between the different platforms at the measured times, it is important to note that cells presented a market growth in all substrates. In the specific case of the Gelacell™ scaffold, it was observed that after 7 days, it had the same cell viability measurement as the cells cultured on the bottom of the well. By this, it can be inferred that the 3D nanostructure of the Gelacell™ scaffold is not negatively impacting the cell growth and viability when compared to the traditional 2D well approach.

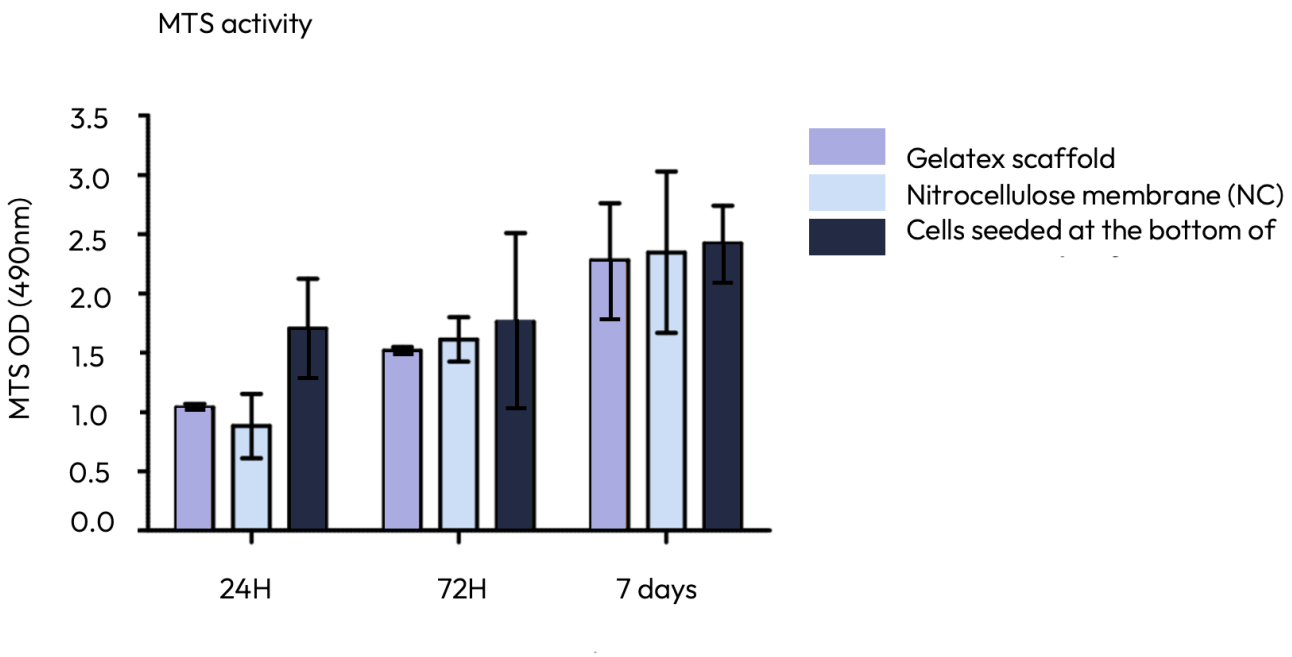

Figure 5: Viability of cells at different time-points (24 hrs, 72 hrs, and 7 days) after seeding of cells on Gelacell™ scaffolds, nitrocellulose (NC) membrane and at the bottom of the well-plate (n=3; error shows the standard deviation).

For the confocal microscopy, the cells were stained with DAPI and Alexa 568 after 1, 3, and 7 days of incubation. Again, the main objective of these observations was to determine the structural differences between the cells that had been incubated in the 3D environment of the Gelacell™ scaffold, and the cells that had grown into the traditional 2D approach at the bottom of the well after a week of culture.

A visual inspection of the wells at the 72-hour mark revealed that the cells seeded on the bottom of the well were reaching a high level of confluency and the first instances of multi-layering occurred. This phenomenon continued until the seventh day of the experiment as cells kept expanding as indicated by the MTS assay results.

The images resulting from the confocal microscopy observations for the 1, 3, and 7 days can be seen in Figure 6, Figure 7, and Figure 8 respectively.

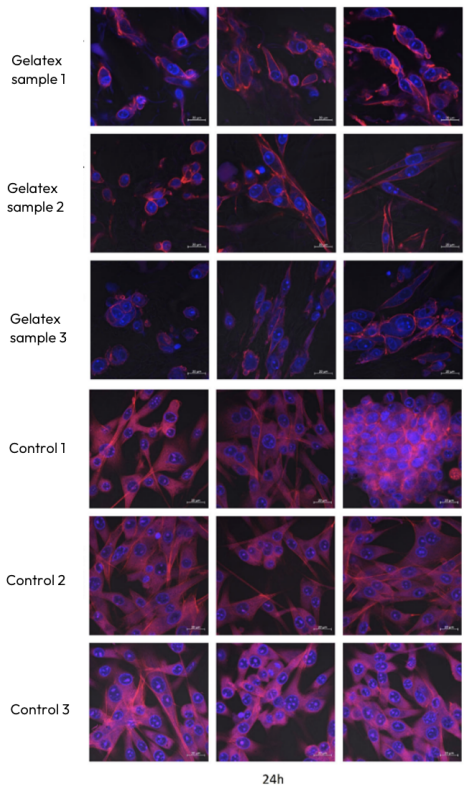

Figure 6: Results from confocal microscopy after 24 hours. Cell seeding concentration was 150 000 per well. Measurements from the same samples are shown in one row. Blue – DAPI stained nucleus of the cell. Red – Alexa 568 stained actin filaments.

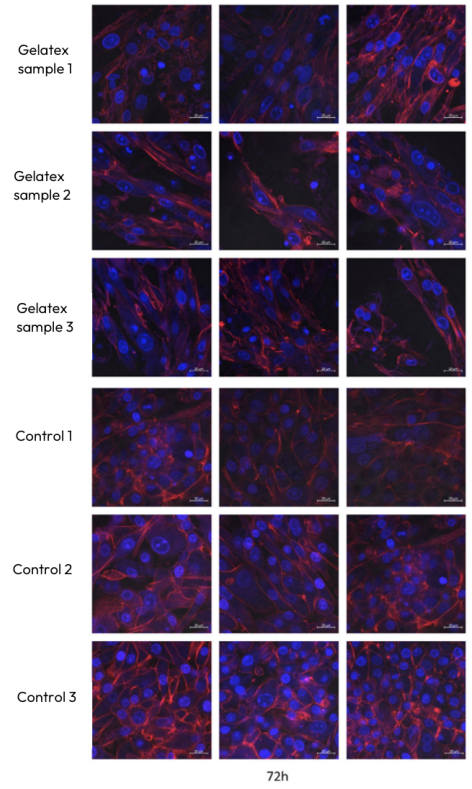

Figure 7: Results from confocal microscopy after 72 hours. Cell seeding concentration was 150 000 per well. Measurements from the same samples are shown in one row. Blue – DAPI stained nucleus of the cell. Red – Alexa 568 stained actin filaments.

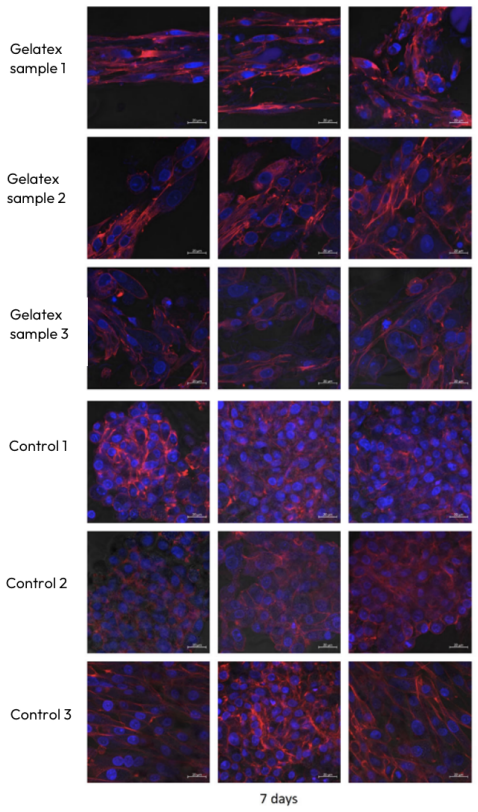

Figure 8: Results from confocal microscopy after 7 days. Cell seeding concentration was 150 000 per well. Measurements from the same samples are shown in one row. Blue – DAPI stained nucleus of the cell. Red – Alexa 568 stained actin filaments.

When comparing Figures 6 to 8, it can be noticed that the highest number of cells visualized in the Gelacell™ increased from the 24-hour mark to its maximum at 72 hours, coinciding with the time point where layering started to occur in the cells seeded at the bottom of the well. After 7 days, fewer cells were visually observed at the Gelacell™ scaffold superior layers. This is believed to be caused by the migration of cells inside the scaffold and the limitations of the visualization method to penetrate deeper into the matrix layers. This inference is supported by the increase of the OD measured on the MTS assay for the Gelacell™ scaffold during the experimental period.

Moreover, like the observations from the previous experiment, in these figures, it can be seen that the actin filaments are less activated in the Gelacell™ scaffold samples at all times measured when compared to the 2D approach. This strengthens the position that the nanofibrous environment of the scaffold provides a richer environment for cellular interaction and a support network in which the cells do not need to greatly modify their morphological confirmation to improve their adhesion and enable their motility.

Conclusion

In this paper, several experiments provided evidence that the Gelacell™ scaffolds are suitable for the 3D culture of eukaryotic cells. The scaffold is biodegradable, biocompatible, and does not present cytotoxicity nor negatively impact cell adhesion, proliferation, or metabolic activity.

It is evidenced that the internal network of interconnected gelatin nanofibers creates an adequate environment to facilitate cell migration and proliferation while maintaining lower levels of cellular stress,

demonstrated in less morphological deformation and actin activity when compared to a traditional 2D culture approach.

It can be then concluded that the Gelacell™ scaffolds mimic, through their composition and morphology, the natural collagenous arrangement of a native extracellular matrix. This in turns, promotes cell adhesion, cell motility, and provides an ECM-like support for 3D cell-to-cell, and cell-to-scaffold interactions. Therefore, it is expected that results from cell culture experiments that use the Gelacell™ 3D nanofibrous scaffold platform, will better reflect the processes found in natural tissues and might achieve improved biological relevance.

Materials

Gelacell™ scaffolds, Nitrocellulose membranes (pore size 0.45 μm), GMEM broth (Biowest), FBS (Corning), TPB (Corning), HEPES (Corning), Penicillin and Streptomycin mixture (Corning), Formaldehyde (3.7%) solution in PBS, Triton-X solution in PBS, Trypsin-EDTA, Phosphate buffer saline (PBS), MTS Proliferation Assay Reagent (Quick Cell Proliferation Colorimetric Assay Kit Plus, Biovision), DAPI and Alexa 568 (Confocal Microscope stain).

Methodology

Cell viability and microscopy after 24 hours

BHK-21 cells were cultured on a petri dish up to 90% confluency to be posteriorly seeded into the respective platforms in triplicate (n=3), namely the Gelacell™ scaffold and the nitrocellulose membrane. As an extra control, cells were grown on round microscopy glasses at the bottom of the well. Special 24-well crown inserts were used to stabilize both matrices inside the respective wells. The seeding densities were 400k, 500k, and 800k cells per well. The seeding volume was 500 μL. Posteriorly after seeding, 750 μL of the medium was added to each well as a nutrient source. The cell culture media used contained GMEM broth, FBS, TPB, HEPES, and a mixture of Penicillin and Streptomycin. The cells were incubated at standard conditions, 37°C and 5% CO2 for 24 hours.

After incubation the MTS viability assay was performed in all samples following the established assay protocols, adding the MTS proliferation assay reagent, incubating, and measuring OD at 490 nm. Between groups, single-way ANOVA statistical analysis was performed. If a statistical significance was found, a t.test was performed and corrected with the Holm-Bonferroni method. Results were considered statistically significant if $p<0.05$.

For the confocal microscopy, the samples seeded on the Gelacell™ scaffold were compared with the controls grown on round microscopy glasses at the bottom of the well. In both cases, the samples used were the ones with a seeding density of 400k cells per well. After 24 hours of incubation, the samples were fixed with a standard method using formaldehyde and stained with the mixture of DAPI and Alexa 568 dyes. The confocal microscope Zeiss LSM 710 was used for imaging and visualization.

Cell viability and microscopy after 7 days

BHK-21 cells were cultured on a petri dish up to 90% confluency to be posteriorly seeded into the respective platforms in triplicate (n=3), namely the Gelacell™ scaffold and the nitrocellulose membrane.

As an extra control, cells were grown on round microscopy glasses at the bottom of the well. Special 24-well crown inserts were used to stabilize both matrices inside the respective wells. The seeding density was 150k cells per well. The seeding volume was 500µL. Posteriorly after seeding, 750µL of the medium was added to each well as a nutrient source. The cell culture media used contained GMEM broth, FBS, TPB, HEPES, and a mixture of Penicillin and Streptomycin. The cells were incubated at standard conditions, 37°C and 5% CO2 for up to 7 days with a 500 L cell medium change every 48 hours.

MTS viability assay was performed in all samples at 1, 3, and 7 days, following the established assay protocols; adding the MTS proliferation assay reagent, incubating, and measuring OD at 490 nm. Between groups, single-way ANOVA statistical analysis was performed. If a statistical significance was found, a t.test was performed and corrected with the Holm Bonferroni method. Results were considered statistically significant if $p<0.05$.

For the confocal microscopy, the samples seeded on the Gelacell™ scaffold were compared with the controls grown on round microscopy glasses at the bottom of the well. In both cases, the samples used were the ones with a seeding density of 150k cells per well. Microscopy was performed 1, 3, and 7 days after incubation. The samples were fixed with a standard method using formaldehyde and stained with the mixture of DAPI and Alexa 568 dyes. The confocal microscope Zeiss LSM 710 was used for imaging and visualization.

Citation

[1] Ravi, Maddaly, et al. "3D cell culture systems: advantages and applications." *Journal of cellular physiology* 230.1 (2015): 16-26.

[2] Vasita, Rajesh, and Dhirendra S. Katti. "Nanofibers and their applications in tissue engineering." *International Journal of nanomedicine* 1.1 (2006): 15.

[3] Wang, Chia-Yu, et al. "Polymeric gelatin scaffolds affect mesenchymal stem cell differentiation and its diverse applications in tissue engineering." *International Journal of Molecular Sciences* 21.22 (2020): 8632.