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# Exploring the Potential of Hydrogels for Alternative Cell Storage

# Alternatif Hücre Depolama Yöntemleri İçin Hidrojellerin Potansiyelinin İncelenmesi

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## ABSTRACT

Efficient long-term storage of mammalian cells is crucial for their applications in cell therapy and biologics production, but cryopreservation with additives such as DMSO and FBS can make it expensive, ethically challenging as well as raise potential complications further downstream. Desiccation, which involves removing all water from the cells, is a potential alternative to cryopreservation, but most cells require water for survival, and a supplement media/substrate may be necessary. Therefore, the presented study aimed to explore the feasibility of a number of hydrogels as protectants for mammalian cells during low-water, long-term storage, with a particular focus on gelatin, GelMA, agar, and a nanofibrillar cellulose-based commercial hydrogel. Material characterisations with dynamic mechanical analysis (DMA), scanning electron microscopy (SEM), Fourier-transform infrared spectroscopy (FTIR), and swelling degree ratio analyses were performed on these materials. C6 glial cells and L929 fibroblasts were desiccated for 6, 24, 48, and 72 h and assessed for their metabolic activity changes upon rehydration. The results demonstrated that desiccation temperature has a greater influence on cellular activity-based survival compared to the type of hydrogel that they are incorporated within. This study shows that entrapping cells in a suitable substrate can extend survival of cells in low-water conditions; gelatin- and nanofibrillar cellulose-based hydrogel materials could be promising candidates for long-term storage and delivery of mammalian cells for therapeutic applications.

**Key Words**

Hydrogel protectants, desiccation, long-term storage, nanofibrillar cellulose.

## ÖZ

Memeli hücrelerinin etkin bir şekilde uzun vadeli depolanması, hücre terapisi ve biyolojik ürünlerin üretimi gibi uygulamaları için önemlidir, ancak DMSO gibi toksik katkı maddeleri ile kriyoprezervasyon maliyetli ve zorlayıcı olabilir. Tüm suyun hücrelerden uzaklaştırılmasını içeren kurutma işlemi, kriyoprezervasyon kullanımının alternatifi olabilir, ancak çoğu hücre hayatta kalmak için suya ihtiyaç duyar ve bir tamamlayıcı ortam / substrat gerekli olabilir. Bu sunulan çalışma, özellikle jelatin, GelMA, agar ve nanofibrillar selüloza dayalı ticari bir hidrojel olmak üzere bir dizi hidrojelin memeli hücreleri için düşük su ve uzun süreli depolama sırasında koruyucu olarak kullanılabilirliğini keşfetmeyi amaçlamaktadır. Bu, malzemelerin ve karışımlarının dinamik mekanik analizi (DMA), taramalı elektron mikroskobu (SEM), Fourier Dönüşüm İnfrared (FTIR) ve şişme derecesi oran analizleri gibi malzeme karakterizasyonları gerçekleştirildi. C6 gliyal hücreleri ve L929 fibroblastları 6, 24, 48 ve 72 saat kurutuldu ve metabolik aktivite değişimleri üzerinden hücre aktivitesi değerlendirildi. Sonuçlar, kuruma sıcaklığının, hücrenin kültürlendiği hidrojel tipine kıyasla hücresel aktiviteye dayalı hücre sağkalımı üzerinde daha fazla etkiye sahip olduğunu gösterdi. Bu çalışma, hücreleri uygun bir substrata hapsederken, hücrelerin düşük su koşullarında yaşamlarını uzatabileceğini göstermektedir; jelatin ve nanofibriller selüloza dayalı hidrojel malzemeler, memeli hücrelerin uzun süreli depolanması ve terapötik uygulamalar için teslimat için umut vadeden adaylar olabilir.

**Key Words**

Hidrojel koruyucular, kuruma, uzun süreli depolama, nanofibriler selüloz.

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## INTRODUCTION

Three-dimensional networks of hydrophilic polymer chains, hydrogels, have gained significant attention in biomedical research and regenerative medicine thanks to their unique properties and potential applications. Hydrogels have been extensively explored for their ability to mimic the extracellular matrix (ECM), providing an optimal environment for cell growth which has long been considered for applications in tissue engineering, drug delivery, cell transport thanks to their biocompatibility and tailorable properties [1-3]. One promising area of research is the use of hydrogels for long-term human cell storage, which could revolutionize various fields, including cell-based therapies, tissue engineering, and drug development.

Efficient long-term storage of mammalian cells is crucial for their applications in cell therapy and biologics production, but cryopreservation suffers from high costs, dependence on significant infrastructure/logistical organisation as well as the use of undesirable additives (i.e. DMSO or animal serum). Desiccation, which involves the complete removal of water, is a potential alternative as it offers low maintenance and process compatibility, however most cells require water for survival [4].

The use of a hydrogel as a conduit for the storage of cells may be based on its absolute shielding and protective nature, or as a consequence of its innate high-water content. As such, the feasibility of using such hydrogels relies on their renowned properties of biocompatibility and biodegradability to ensure that it does not induce adverse effects or elicit immune responses when in contact with human cells. Moreover, they should offer a suitable microenvironment that supports cell survival, proliferation, and functionality [3,5,6]. Lastly, the hydrogel should allow for the retrieval of stored cells without compromising their viability or altering their biological properties.

One of the main advantages of hydrogels is their tuneable physical and chemical properties, which can be tailored to meet the specific requirements of cell storage. These properties include mechanical strength, porosity, water content, and the ability to encapsulate various types of cells. Hydrogels can be engineered to possess the appropriate stiffness and elasticity, resembling the native tissue microenvironment and providing mechanical support to the encapsulated cells [7,8]. The high-water content of hydrogels allows for the transport of nutrients, oxygen, and waste products, ensuring cell survival and metabolic activity. Moreover, the porous structure of hydrogels facilitates the diffusion of signalling molecules and growth factors, promoting cell growth and differentiation [8].

Despite these promising features, challenges and limitations remain in the field of hydrogel-based cell storage. Issues such as cell aggregation, limited diffusion of nutrients and oxygen, and difficulties in cell retrieval still need to be addressed. Furthermore, long-term stability, scalability, and cost-effectiveness of hydrogel-based storage systems need to be thoroughly investigated to ensure their practical implementation. This study aims to address the exploitation of hydrogels as protectants for mammalian cells during a low-water, long-term (>one year), storage environment induced by desiccation at the temperature of 4°C and 37°C.

## MATERIALS and METHODS

### Preparing Hydrogels, Culturing Cells and Desiccation Studies

Throughout this study, four different main materials were used and included gelatin (Sigma-Porcine skin 300 bloom, Type A, UK), GelMA (in-house synthesised), nanofibrillar cellulose (NFC) (UPM Biomedical, UK), and agar (Sigma-Aldrich, UK).

### Gelatin Preparation

Gelatin sourced from Porcine skin was dissolved in deionised water at a concentration of 10% w/v by slightly heating and sterilised using a 0.22 µm syringe filter (PES, Gamma-irradiated, Merck, UK).

Gelatin has not been chemically or enzymatically gelated but physically by temperature decrease below 25°C.

### NFC Preparation

NFC was prepared according to producers' directions (Figure 1). A total of 2 mL of 1% hydrogel mix was prepared with NFC (1334 µL) in PBS (466 µL) and cell suspension in media (200 µL).

### Agar Preparation

Agar was dissolved in deionised water at a concentration of 2% w/v. For sterilisation, the agar was autoclaved at 121°C for 15 mins. For casting, the agar was poured into culture and/or the test vessel whilst keeping the temperature above 35°C to prevent solidification.

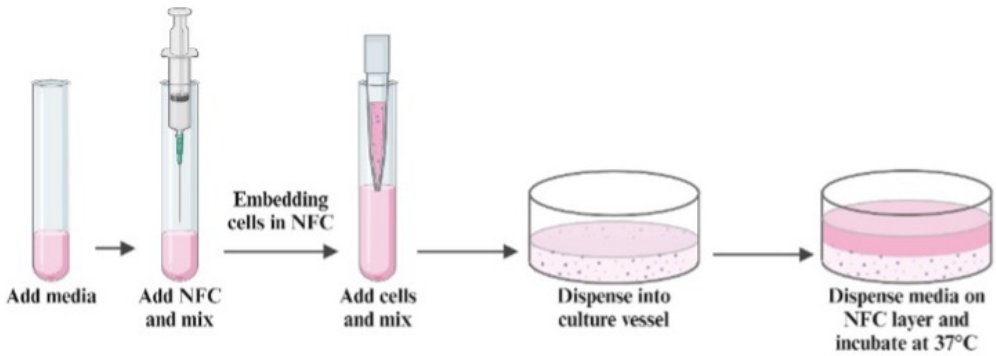


**Figure 1.** Preparation of NFC for 3D cell culture in hydrogels (Figure is created on Biorender.com by adapting from manufacturer's diagram).

**GelMA Preparation**

GelMA was synthesised as previously described in [7] with an over 80% degree of methacrylation. GelMA hydrogel was prepared by dissolving the freeze-dried GelMA in PBS (10% w/v), before photo-initiator of LAP (Lithium phenyl 2,4,6-trimethylbenzoylphosphinate, 900889, Sigma-Aldrich, UK) (0.1% w/v) was added to initiate UV crosslinking. For all characterisation and cell culture analyses, the GelMA was crosslinked in a UV chamber for 60 s (XYZ Printing UV chamber - Model 3UD10, Taiwan, UV LED λ 375–405 nm, 16 W), particularly for cell culture experiments to allow 3D cell culture and complete cell embedding crosslinking to occur. Crosslinking was done after mixing the hydrogel solution with cells to enable 3D cell culture.

The hydrogels were cast as 100 uL volumes in 96-well plates, frozen at -20°C and lyophilised (Heto Dry Winner connected with Edwards RV5 pump, UK) at <50 mbar pressure, -100°C. This ensured that they were obtained as dried/foam like structures.

**Cell Culture**

L929 mouse fibroblasts cells and rat C6 glioma cell lines were used for in vitro cell culture experiments. L929 cells were cultured in Dulbecco's modified eagle medium (DMEM; Gibco™, Thermo-Fischer Scientific, UK) and C6 cells were cultured in F-12 K (ATCC®30-2004™, USA) supplemented with 10% fetal bovine serum (FBS) (for L929) or 10% horse serum (HS) plus 5% FBS (for C6), 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (PS) in a humidified incubator at 37°C in 5% $CO_2$. Morphological examinations of cells were observed under inverted light microscopy (Olympus, CK2, Phase contrast, bright field).

**Desiccation Studies**

Cells were seeded into gelatin (10% w/v), NFC, agar (2% w/v) and GelMA (10% w/v) at 1x105 cells/mL cell seeding density, followed by desiccation studies of 6, 24, 48 and 72 h at two different temperatures of 4- and 37°C. Metabolic activity of cells were analysed using the AlamarBlue assay (DAL1025, Thermo Fisher Scientific, UK), and a fluorescence plate reader (Labsystems, Helsinki, Finland, ex 530 nm / em 590 nm).

**Characterisation of Hydrogels**

Fourier Transform Infrared (FTIR) Spectroscopy

Chemical structures of dry-formed gelatin, GelMA, NFC and agar, were analysed by attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR, System 2000, PerkinElmer, Seer Green, UK) between the ranges of 4000–400 $cm^{-1}$ with a resolution of 4 $cm^{-1}$ and within the area of interest of 2000–600 $cm^{-1}$. The results were plotted in Origin Pro and relevant pictures were annotated on the plot (Figure 2).

**Swelling Degree Ratio**

All hydrogels (n=4) were prepared as previously described in section 2.1. For swelling degree studies, freeze-dried samples were used and the initial weight (Wdry) of each sample was recorded using a sensitive scale (Ohaus PA224 Pioneer Analytical Balance, UK). The samples were immersed in PBS (without Ca/Mg ions) at 37°C, and the wet weights of samples were recorded (Wwet) at 5, 10, 20, and 30 mins, as well as 1, 2, 3, 4, 5, 24, 48, 120 and 180 h. Excess water was drained using filter paper very briefly on the hydrogel surfaces for each sample and time point. The swelling degree was calculated using the following equation:

*Swelling Degree ratio* % = ((W_wet-W_dry ))/(W_wet×100)

**Scanning Electron Microscopy (SEM)**

Prior to SEM, samples were freeze dried (Heto Dry Winner connected with Edwards RV5 pump, UK) at <50 mbar pressure, -100°C, as described in section 2.1. Then all samples were sputter-coated with gold (Polaron E5000) for imaging scanning electron microscopy (SEM; Philips XL30 field emission SEM, Netherlands). Analyses were performed on conventional SEM images to make observations on formational differences on materials surfaces and dried structure.

**Dynamic Mechanical Analysis (DMA)**

The mechanical properties of materials excluding NFC, with a width of 6.5 mm and a thickness of 4.0 mm with flat surfaces were characterized using a dynamic mechanical analyser (DMA; Discovery DMA 850, TA Instruments, New Castle, USA). Uniaxial compression testing was conducted at room temperature using cyclic sinusoidal load mode to frequency oscillations, which varied from 0.05 to 20 Hz with 5 Hz intervals. Samples were pre-loaded to 0.001 N force and dynamically subjected at low deformation (0.1% strain) compression.

**Statistical Analysis**

Statistical analysis was performed on Prism 8 GraphPad (Two-way ANOVA with Bonferroni Test, Tukey Tests (*p<0.05, ** p<0.01, *** p<0.001, ****p<0.0001) and all the plots on figures 1, 4 and 5 were drawn on Origin Pro.

**RESULTS and DISCUSSION**

FTIR spectroscopy involves exposing a sample to infrared radiation and detecting the absorbed wavelengths within the infrared spectrum. Each compound exhibits a unique pattern of absorption bands in its infrared spectrum. Proteins and polypeptides, feature characteristic bands such as amide I and amide II in their infrared spectra. The absorption associated with the amide I band leads to stretching vibrations of the C=O bond of the amide, absorption associated with the amide II band leads primarily to bending vibrations of the N—H bond (Figure 2a, graph annotations on FTIR spectra) [9].

According to FTIR spectra as seen on Figure 2 primary and secondary amide bonds are present in gelatin and GelMA at around ~1530 $cm^{-1}$ and ~1630 $cm^{-1}$ wavelengths respectively for amide I and amide II bonds, while agar only presents a secondary amide peak NFC and agar have shown glycosidic –(C-O-C) shifts around 1050 $cm^{-1}$ and 1043 $cm^{-1}$. All materials, apart from GelMA, have glycosidic bonds in their chemical structure.

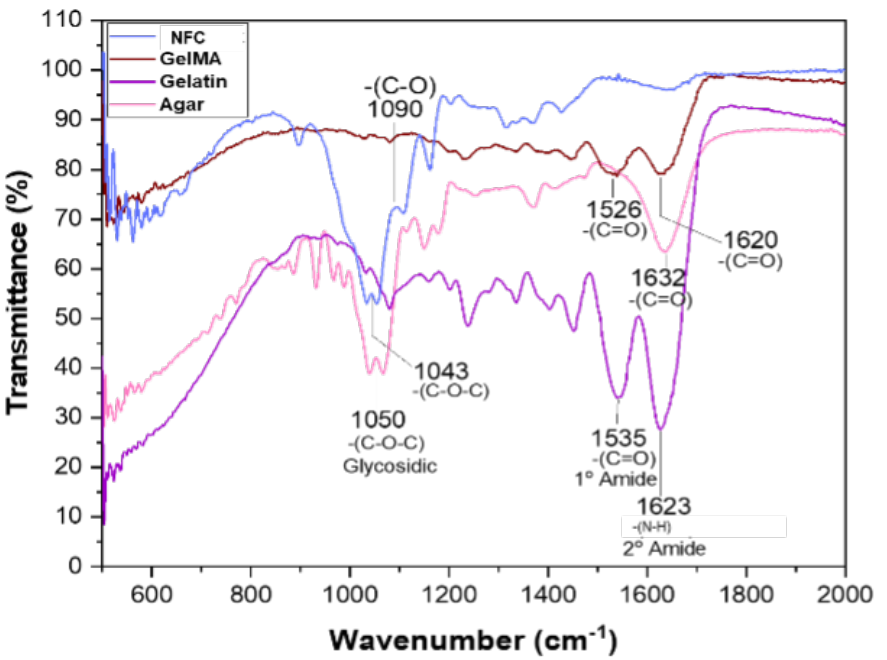


**Figure 2.** FTIR Spectra of hydrogels used in the study.

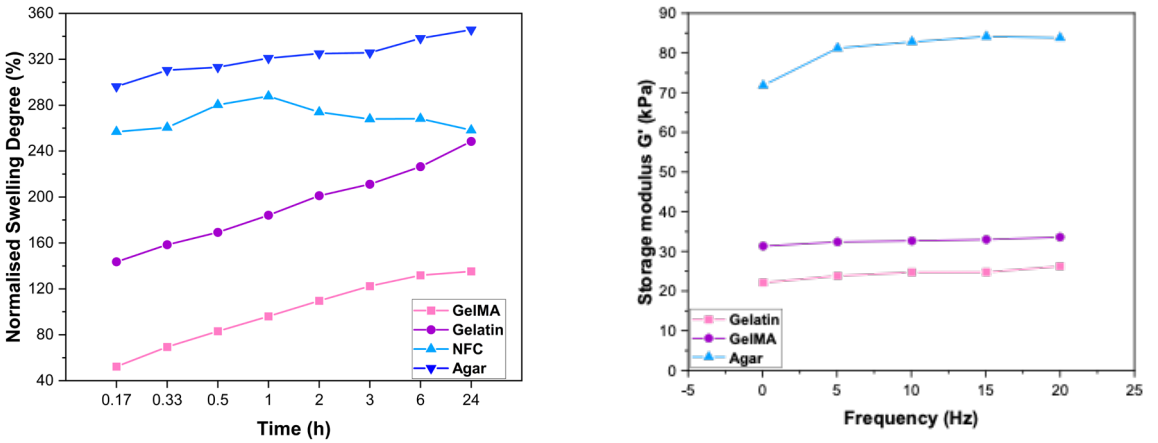


**Figure 3.** Swelling degree ratio and storage modulus profile of some hydrogels used.

The normalised swelling degree increased over time for all hydrogels Figure 3 except NFC which reached an optimum percentage and did not increase further. All hydrogels showed swelling behaviour greater than 100% due to their intrinsic hydrophilic nature. Among all the samples gelatin demonstrated the highest swelling rate, seen by the steep gradient, with GelMA having a similar initial gradient with gelatin which then stabilised after 6h. Hydrogels are known for their superior water intake capacity and hydrophilicity this property makes them serve as carriers for various water soluble bio-compounds and drugs as they can relatively easily be impregnated in the physical formation of hydrogel like materials as well as more advance loading techniques [10,11].

Storage modulus is an important property of viscoelastic materials like hydrogels which relates to the material's ability to store energy elastically [12,13]. As seen in Figure 3, agar had the largest storage modulus, ~50 kPa higher than GelMA at 10 Hz frequency. GelMA and gelatin demonstrated similar physical properties due to their shared gelatin component. However, since GelMA is modified with methacrylic groups and photocrosslinked, it has a higher storage modulus than gelatin. As the hydrogels were not crosslinked, NFC was not suitable for measuring mechanical properties via DMA due to its lack of stiffness. For future studies running DMA characterization with complex viscosity and tana values at 37°C would be more representative of physiological conditions for medical applications.

Figure 4 shows the conventional SEM images of the freeze-dried samples used and photographs in their hydrogel form (right). From the SEM images clear structural differences of the materials used could be seen in terms of their uniformity and interconnectivity of the holes and micropores that were created by the lyophilisation process. NFC appeared to have smaller holes that could be corresponding to the nanoporous structure of cellulose-based formulations [14,15].

Figure 5 shows images taken on an inverted light microscope, to elicit the morphology of the monolayer culture of the cell lines used in this work C6 glial cells (top) and L929 fibroblasts (bottom). Glial cells have a neuronal characteristic biradial morphology while L929 depicts standard fibroblastic morphology. L929 was chosen as it is a model cell line for ISO cytocompatibility analyses, and it is one of the most widely used cell types for general conventional cell analyses experiments [16]. C6 cells were used as a cancer-relevant cell line since they were isolated and immortalised from glioblastoma tissue.

Careful cell line selection should be implemented in future desiccation and cell preservation studies in hydrogels to ensure the most accurate physiological representation.

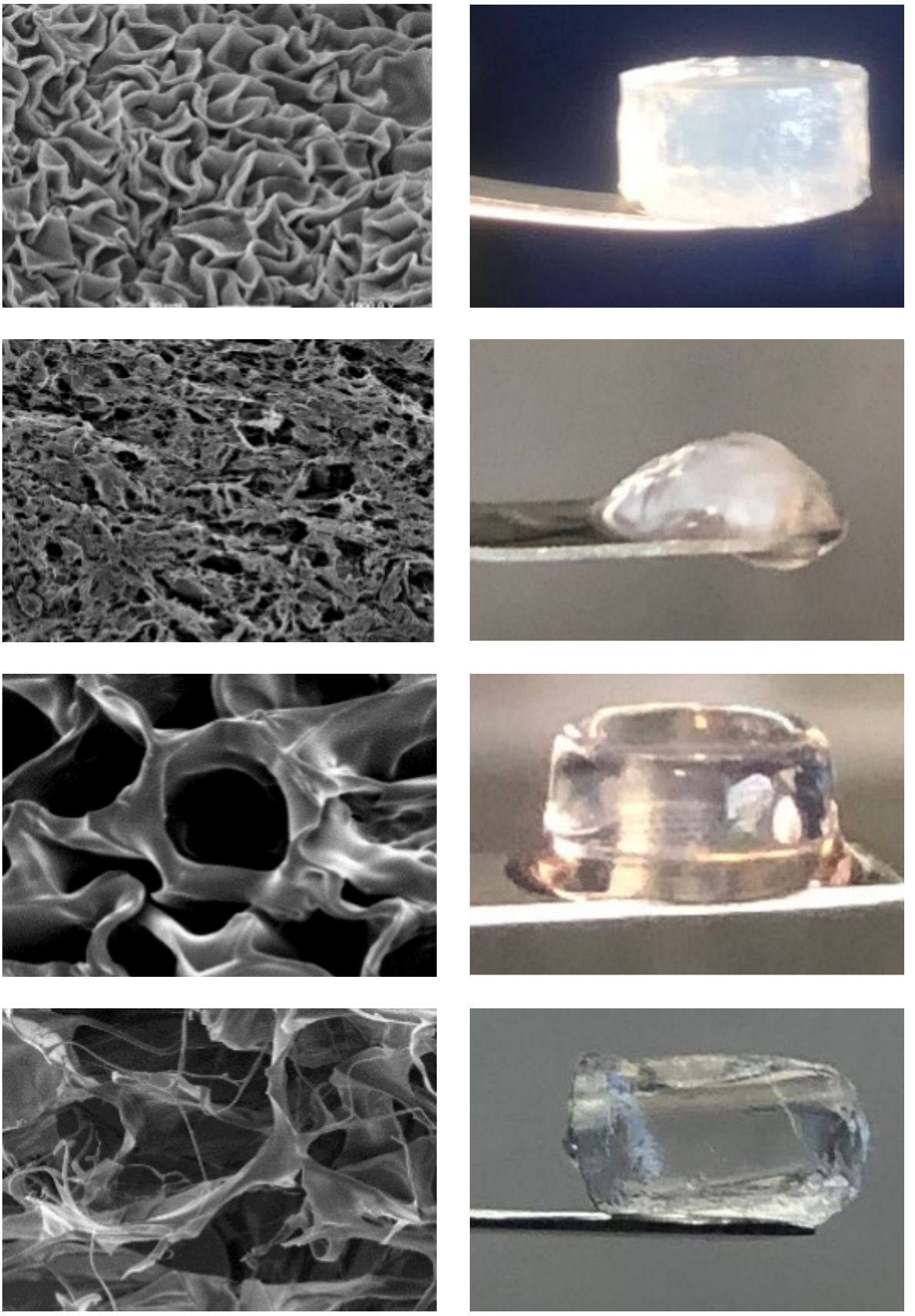

**Figure 4.** SEM images and macroscopic images of materials of Agar, NFC, GelMA, Gelatin respectively from top to bottom.

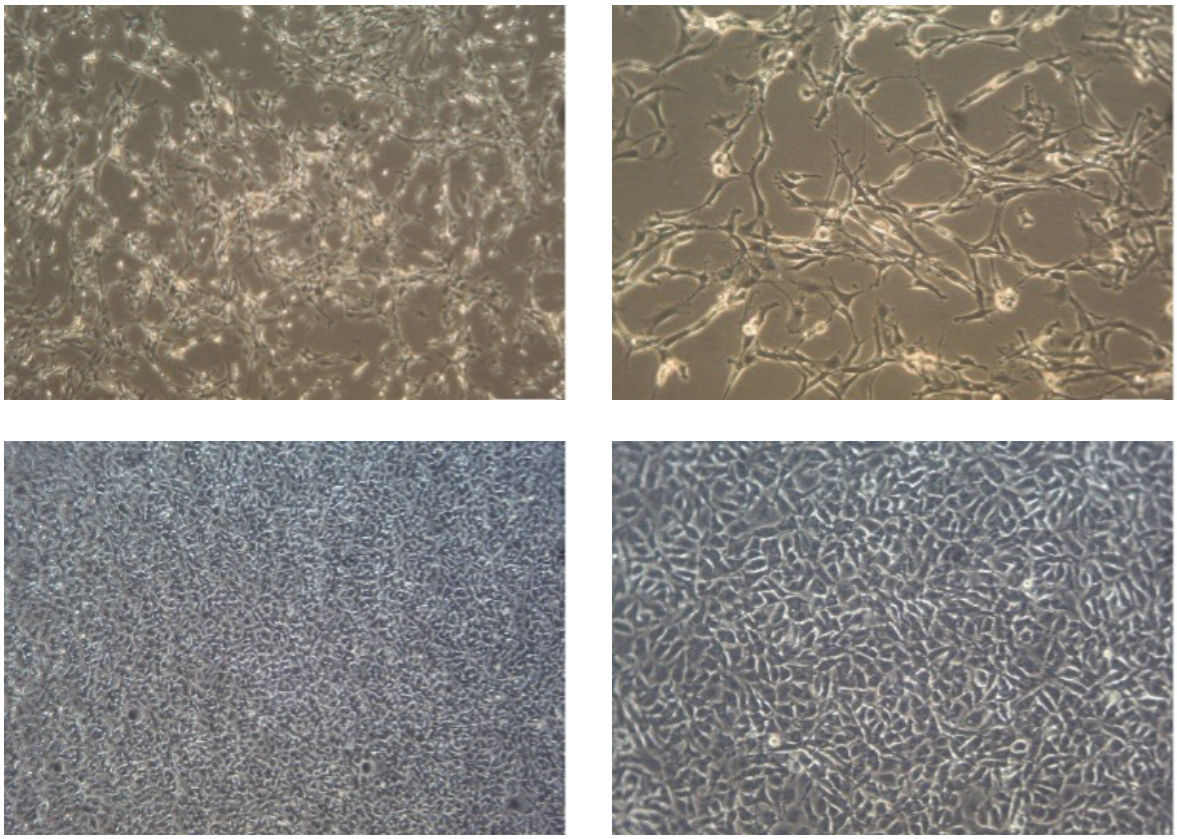

**Figure 5.** Culture images taken on inverted light microscope for C6 (top) and L929 (bottom) cells.

As seen in Figure 6, most cells were dead within 6 h in the absence of hydrogels. It was evident that the change in temperature from 37°C to 4°C had a greater influence on cell survival than the presence of media. The hydrogels appear to have increased metabolic activity and proliferation (Figure 7). NFC may enhance cell survival, however, the metabolic activity of agar and gelatin appeared lower than expected, this could be due to a lower culture temperature of 4°C being used. The lower metabolic activity may also be due to cells being removed with the media during the dehydration step, despite the positive impact of the hydrogels.

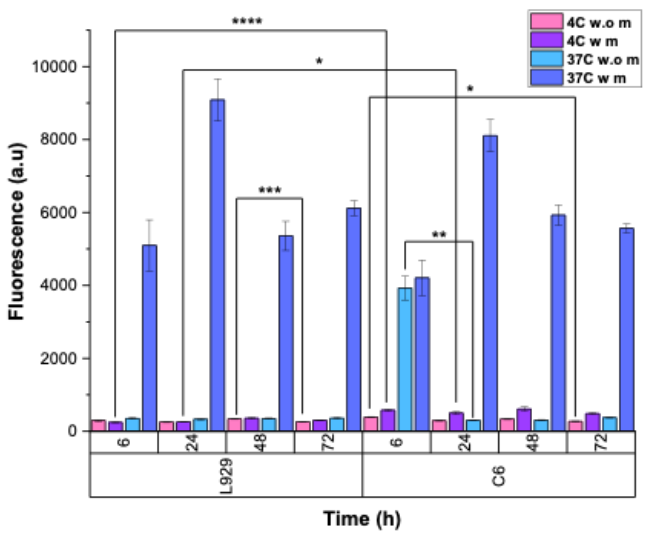


**Figure 6.** Metabolic activity of C6 and L929 cells during desiccation without hydrogels.

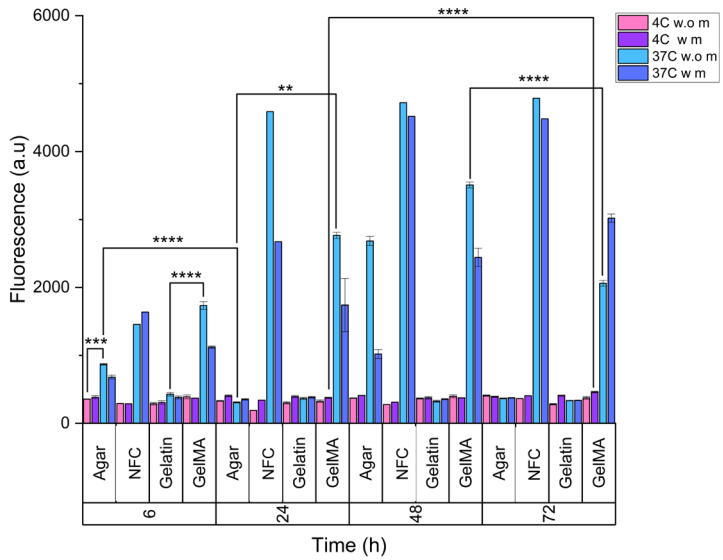


**Figure 7.** Metabolic activity of L929 cells seeded into hydrogels over 72 h of desiccation.

Gelatin is an attractive material as a scaffold element for tissue engineering and can be easily added into formulation of different natural and synthetic polymeric biomaterials [17], primarily to improve biocompatibility or adjust rheological properties and responsiveness to temperature it has been largely used in numerous studies in the field of biomaterials applications.

Agar is bioinert thermo-responsive algal originated material[18] that has been mostly used as coating material in cell culture applications to reduce cell attachment. It was chosen as a comparative inert material in this study.

Cellulose based biomaterials have long been investigated for tissue engineering applications, however for translational applications an aspect of biodegradability needs to be considered if the implant is not intended to be permanent. Moreover, cellulose-based materials can be considered for cell storage due to their durability, availability, and biocompatibility. These materials, such as NFC, can be prepared alone or combined with other hydrogels to improve cell storage and encapsulation. GelMA has gain significant attention in the field of biomaterials and cell and tissue engineering applications in the last decade thanks to its tuneable biophysical and mechanical properties [18]. As a semi-synthetic material GelMA offers both cellular compatibility motifs originating from gelatin in its structure and mechanical tunability and strength coming from methacrylate sites. Using these four hydrogel systems we have demonstrated the utility of controlled desiccation of materials for the storage of mammalian cells. The tuneable properties of hydrogels and their ability to provide a supportive matrix make them interesting materials for the development of cell storage techniques.

## Conclusion

Efficient long-term storage of cells is key due to their fragility, for example the risk of differentiation and biological deviation. Cryopreservation is the current gold standard for cell storage with extensive research proving its efficiency. However, it has several drawbacks such as high cost, difficulty transporting liquid nitrogen and the need for using unfavourable cryoprotectant additives such as DMSO. Along with the rise of cell banking platforms, there is a clear need for more cost effective and efficient methods for long-term cell and tissue preservation. This work aimed to demonstrate the potential utilisation of hydrogels as storage materials.

In conclusion, the use of hydrogels for long-term human cell storage represents an exciting avenue in regenerative medicine and biomedical research. These materials offer a versatile platform for maintaining cell viability,

preserving cell functionality, and enabling controlled release of bioactive molecules. Desiccation is a feasible and cost-effective alternative to cryopreservation. Hydrogels improved long-term survival of cells during desiccation at 37°C. Between the samples NFC appeared to have the largest impact on cell survival with increasing metabolic activity over time demonstrating cell proliferation. Further research and development are required to optimize the properties and design of hydrogels, incorporate exogenous factors to enhance longevity of cell survival, and pave the way for their widespread application in cell-based therapies, as well as tissue engineering and alternative cell storage technologies.

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