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ORIGINAL ARTICLE

Bioprinting of a Liposomal Oxygen-Releasing Scaffold for Ovary Tissue Engineering

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This study addresses a critical challenge in bioprinting for regenerative medicine, specifically the issue of hypoxia compromising cell viability in engineered tissues. To overcome this hurdle, a novel approach using a microfluidic bioprinter is used to create a two-layer structure resembling the human ovary. This structure incorporates a liposomal oxygen-releasing system to enhance cell viability. The bioprinting technique enables the simultaneous extrusion of two distinct bioinks, namely, bioink A (comprising alginate 1% and 5 mg/mL PEGylated fibrinogen in a 20:1 molar ratio) and bioink B (containing alginate 0.5%). In addition, liposomal catalase and hydrogen peroxide (H_2O_2) are synthesized and incorporated into bioinks A and B, respectively. The liposomes are prepared using thin film hydration with a monodisperse size (140–160 nm) and high encapsulation efficiency. To assess construct functionality, isolated human ovarian cells are added to bioink A. The bioprinted constructs, with or without liposomal oxygen-releasing systems, are cultured under hypoxic and normoxic conditions for 3 days. Live/Dead assay results demonstrate that liposomal oxygen-releasing systems effectively preserve cell viability in hypoxic conditions, resembling viability under normoxic conditions without liposomes. PrestoBlue assay reveals significantly higher mitochondrial activity in constructs with liposomal oxygen delivery systems under both hypoxic and normoxic conditions. The evaluation of apoptosis status through annexin V immunostaining shows that liposomal oxygen-releasing scaffolds successfully protect cells from hypoxic stress, exhibiting a proportion of apoptotic cells similar to normoxic conditions. In contrast, constructs lacking liposomes in hypoxic conditions exhibit a higher incidence of cells in early-stage apoptosis. In conclusion, the study demonstrates the promising potential of bioprinted oxygen-releasing liposomal scaffolds to protect ovarian stromal cells in hypoxic environments. These innovative scaffolds not only offer protection but also recapitulate the mechanical differences between the medulla and the cortex in the normal ovary structure. This opens new avenues for advanced ovary tissue engineering and transplantation strategies.

Keywords: bioprinting, ovarian tissue engineering, hypoxia, PEGylated fibrinogen, alginate

Impact Statement

Ovary tissue engineering is a strategy to preserve fertility in young women and prepubertal girls diagnosed with cancer, who cannot benefit from transplantation of cryopreserved ovarian tissue. In this study, we used bioprinting technology as a pioneering approach to investigate the fabrication of human ovarian tissue, incorporating oxygen-releasing liposomes to mitigate the adverse effects of hypoxia on transplanted engineered ovarian tissue. Bioprinting offers an innovative platform capable of mimicking the intricate architecture of the human ovary, including its mechanical nuances, while facilitating the precise

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extrusion of functional materials, such as nanocarriers. Therefore, this research holds significant promise for advancing the field of ovary tissue engineering and providing a ray of hope to those in need of fertility preservation under challenging clinical circumstances.

Introduction

Preserving fertility in patients with cancer, especially in prepubertal girls and women requiring immediate treatment, stands as a paramount concern. Although ovarian tissue cryopreservation and transplantation have shown promising results, there are still significant challenges to overcome, especially for patients diagnosed with tumors carrying a high risk of ovarian involvement, such as blood-borne and metastatic cancer. These cases pose significant challenges as the transplantation of cryopreserved ovarian tissue is not allowed. These cases pose significant hurdles to the transplantation of cryopreserved ovarian tissue because of the potential reintroduction of malignant cells, thereby heightening the risk of disease recurrence.^{1–5} It is important to note that chemotherapy and radiotherapy, while crucial in treating cancer, can lead to primary ovarian insufficiency, early menopause, and infertility. Moreover, they are associated with higher risks of osteoporosis and cardiovascular mortality, posing significant concerns for female patients with cancer.⁶

In response to these challenges, the ongoing research on the development of an engineered ovary emerges as a promising avenue.^{7–13} This innovative approach aims to create bioengineered structures that mimic the function of the ovary, providing a means to restore fertility without the risk of reintroducing malignant cells. This transformative technology holds the potential to instill hope in patients with cancer facing fertility issues because of their treatment, presenting a safer alternative to traditional transplantation methods.

Cell survival after transplantation of tissue-engineered scaffolds remains an important challenge, primarily because of the limited oxygen supply that results from a lack of vascular network. Moreover, it is well established that oxygen dissolved in a liquid can only diffuse a maximum distance of 200 μm in a scaffold.^{14,15} Based on Fick's first law, the diffusion of oxygen in *in vitro* culture systems decreases as the medium volume increases. Although reducing the medium height in culture plates can mitigate the gradient decrease in oxygen, it introduces the risk of medium evaporation and cell dehydration.¹⁶

Several strategies have been devised to overcome the limitations of oxygen diffusion within engineered scaffolds, such as using oxygen-releasing particles,^{17–19} vascularization-inducing growth factors,^{20,21} and endothelial cells.²² Notably, hydrogen peroxide (H_2O_2) can be used as an oxygen-generating source, breaking down into water and oxygen in the presence of catalase.^{23–25} Abdi et al.²⁶ demonstrated that encapsulation of H_2O_2 offers precise control over its decomposition, thereby protecting cells from direct exposure of H_2O_2 and its potential harmful effects.

Another limitation of conventional tissue-engineered constructs is their inability to provide the complex architecture

found in natural tissues.²⁷ However, advanced technologies, such as bioprinting and microfluidics, can be used to some degree to recapitulate the heterogeneity observed in normal tissues. For instance, in an effort to mimic the diverse mechanical properties inherent to ovarian tissue's cortex and medulla, Choi et al.²⁸ encapsulated ovarian follicles in two-layered microcapsules using microfluidic technology. In this innovative approach, the outer layer exhibited greater rigidity, whereas the inner layer demonstrated a softer composition, thus effectively replicating the contrasting mechanical characteristics present in the ovarian tissue.

The bioprinting technology uses robotic additive manufacturing techniques that dispense biomaterials precisely in X, Y, and Z directions to control cell distribution for fabricating 3D complex tissue constructs that mimic natural tissues.^{29–31} Three distinct types of bioprinting approaches have been developed, which include vat photopolymerization-based (including stereolithography, digital light processing, and volumetric printing), jetting-based (including inkjet, microvalve, acoustic, and laser assisted), and extrusion-based bioprinting.^{32–35} Light-based vat-polymerization bioprinting creates 3D structures using photoactivatable bioresins in vats, initiated by patterned light doses for precise patterning in 2D and 3D. Inkjet bioprinters can be divided into two categories, namely, thermal and piezoelectric setups. The thermal printers generate bubbles from heated bioink and force the liquid to exit, whereas piezoelectric devices use mechanical stimulation to produce droplets.

The droplets created by both types of inkjet printers are deposited accurately. Laser-based bioprinters, as a nozzle-less method, use a laser beam to print bioink. The laser pulse is focused on the donor slide coated with an absorbing layer, such as titanium or gold, and induces a vapor bubble that ejects the bioink droplet onto the collection substrate. Extrusion-based bioprinters, in contrast, extrude bioink using pneumatic, piston, or screw dispensing systems to create 3D structures.^{30,36–39} Microfluidic extrusion represents an advanced methodology for generating printable hydrogels by simultaneously coextruding bioink and cross-linker through microchannels. This technology enables printing with multiple cell types while protecting them from shear stress during extrusion. The printhead has a microfluidic design that allows for effortless integration with various precise fluid and cell manipulation modules before fiber extrusion, ensuring versatility and efficacy.^{40–42}

The goal of this study was to address two critical aspects. First, we aimed to assess the capabilities of microfluidic bioprinting technology to fabricate a bioprinted scaffold for ovarian tissue engineering, which replicates the intricate mechanical properties observed in natural ovarian tissue. Second, our study sought to assess the impact of an oxygen-generating bioprinted scaffold, enriched with liposomal catalase and liposomal H_2O_2 , on the viability of ovarian stromal cells.

Materials and Methods

Ovarian tissue collection

For this study, ovaries from deceased multiorgan donors were collected following approval from the Université Catholique de Louvain's Institutional Review Board for the use of human ovaries on May 25, 2019 (IRB reference 2018/19DEC/475). After transferring ovaries to the laboratory and the removal of the medullar section, ovarian cortices were frozen based on our standard method.⁴³

Cell isolation and culture

The stromal cells were isolated after thawing cryopreserved ovarian fragments at 37°C, as previously described.⁴⁴ Briefly, tissues that were mechanically minced by a tissue chopper (Mickel Laboratory) were enzymatically digested using Liberase DH (05401054001; Sigma-Aldrich) and DNase I (10,104,159,001; Sigma-Aldrich) at 37°C for 75 min. Then, the activity of enzymes was stopped by adding an equal volume of Dulbecco's phosphate-buffered saline (DPBS; 14,190,144; Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS; 16,140–071; Gibco).

The final suspension was consecutively filtered through 80 (NY8002500; Millipore, Sigma-Aldrich) and 30 µm (NY3002500; Millipore) nylon net filters and centrifuged at 500 g for 10 min. Afterward, the cells were counted and cultured in a culture medium containing Dulbecco's modified Eagle's medium F-12 nutrient mixture (DMEM/F12; 21,041–025; Gibco), 10% FBS, and 1% antibiotic and antimycotic (Anti-Anti; A5955; Gibco) in a humidified incubator (37°C, 5% carbon dioxide [CO₂]). Cells were subcultured after they had reached confluence, and the culture medium was replaced every other day.

Liposomal catalase and liposomal H₂O₂ syntheses

Liposomal catalase and liposomal H₂O₂ were prepared by the thin-film hydration method and followed the optimized formulation described previously.⁴⁵ Briefly, DSPC (1,2-Distearoyl-sn-glycero-3-phosphocholine; P1138; Sigma-Aldrich), DOPE (2-Dioleoyl-sn-glycero-3-phosphoethanolamine; 76548; Sigma-Aldrich), and cholesterol (C8667; Sigma-Aldrich) at a molar ratio of 1:1:1 were dissolved in chloroform, and the solvent was then evaporated at 60°C using a rotary evaporator. The film was then dried in a vacuum desiccator overnight to get rid of any remaining solvent. After hydrating the film with a 1 mL solution of PBS containing 800 mg/L catalase or 1M H₂O₂, the mixture was vortexed, heated to 40°C for 15 min in an ultrasonic bath (Powersonic 405, 40 kHz), and then centrifuged at 20,000 g for 20 min at 4°C to separate the liposomes from the aqueous solution. The final samples were sterilized by filtering through a 0.22 µm sterile filter before being kept at 4°C.

Liposomal catalase and liposomal H₂O₂ characterization

Dynamic light scattering technique using the Zetasizer Nano ZS (Malvern Instruments Limited) was used for the evaluation of the size, polydispersity index, and zeta potential of liposomal catalase and liposomal H₂O₂. The ultraviolet spectrophotometer method was used to measure the catalase concentration (NanoDrop 2000/2000c, Thermo Fisher Scientific) and the fluorometric Amplex UltraRed/horseradish

peroxidase (HRP) was used for the quantitative assessment of H₂O₂.^{46,47} Briefly, a mixture of Amplex UltraRed (100 µM) and HRP (1U/mL) was added, and after a 15-min incubation, the fluorescent reaction products were quantified using a fluorometer (Multilabel reader, Victor X4). The total amount of catalase or H₂O₂ (WT) and their concentration in the supernatant following centrifugation (WF) were measured to compute the entrapment efficiency (EE %) using the following equation:^{48,49}

$$EE (\%) = (W_T - W_f / W_T) \times 100 \quad (1)$$

Bioink preparation

Alginate (9842; Aspect Biosystems) was dissolved in PBS at 4°C overnight to prepare a concentration of 1.18%. PEGylated fibrinogen (PF) was also prepared as described previously⁵⁰ with a molar ratio of PEG: Fib 20:1. Briefly, syringe-filtered human fibrinogen (F3879; Sigma-Aldrich) at a concentration of 85 mg/mL was incubated at 37°C before PEGylation. A total of 10 mg/mL of O,O'-Bis[2-(N-Succinimidyl-succinylamino)ethyl]polyethylene glycol (NHS-PEG-NHS; 2,000 Da; 713783; Sigma-Aldrich) in PBS was prepared, incubated at 37°C, and syringe filtered. Then, an equal volume of NHS-PEG-NHS was added to the fibrinogen solution and incubated at 37°C for 2 h.

Two types of bioinks (shell A and shell B) were prepared to print the oxygen-releasing scaffold. Shell A was composed of alginate, PF 20:1, ovarian stromal cells at passage 7, and liposomal catalase with a final concentration of 1%, 5 mg/mL, 1 × 10⁶ cells/mL, and 40 mg/L, respectively. Shell B contained alginate and liposomal H₂O₂ with a final concentration of 0.5% and 40 mM, respectively.

Bioprinting and in vitro culture of the scaffolds

The extrusion-based RX1 bioprinter (Aspect Biosystems) was used to print parallel filaments of shell A and shell B. This bioprinter is operated by air pressure and contains microfluidic printheads that allow the printing of different materials at the same time. The channel specified for the cross-linker initiates cross-linking of bioinks as well as minimizes shear stress on the cells during printing.

A rectangular print structure with a volume of 1 × 1 × 10 mm³ was designed in the bioprinter software (Aspect Studio). The CENTRA™ printhead was used to print the desired structure with optimized printing pressures of 105, 43, 200, and 150 mbar for shell A, shell B, cross-linker, and buffer, respectively. CAT-2™ cross-linker (0458; Aspect Biosystems) and saline buffer solution (flushing solution; 0460; Aspect Biosystems) were used to cross-link filaments during printing and flush the channels of the bioprinter printhead after printing, respectively. Moreover, 40 mM calcium chloride (CaCl₂; C5080; Sigma-Aldrich) and 5 IU/mL of human thrombin (T7009; Sigma-Aldrich), which was reconstituted and diluted in 40 mM CaCl₂, were used as postprinting cross-linkers. Three repetitions were bioprinted for each condition of hypoxia (1% oxygen [O₂], 5% CO₂) and normoxia (20% O₂, 5% CO₂) with or without liposomes and cultured for three days in humidified incubators at 37°C (Fig. 1).

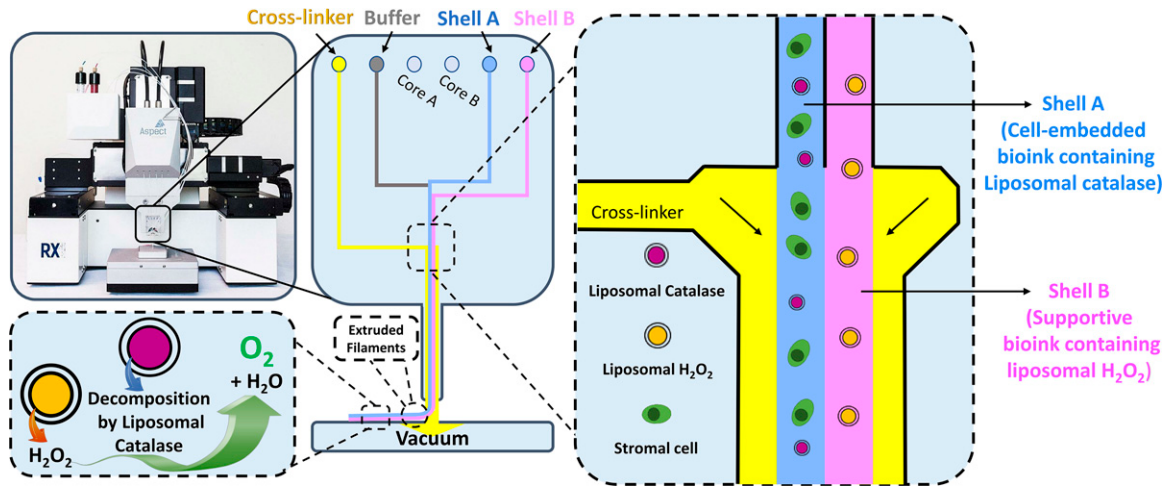


FIG. 1. A schematic of bioink compositions in a microfluidic printhead, RX1 bioprinter, and a representative bio-printed scaffold.

Live/Dead assay

The viability of cells was analyzed using a Live/Dead™ viability/cytotoxicity kit (L3224; Invitrogen), which is based on the membrane integrity and intracellular esterase activity. To perform this analysis, the medium of constructs was removed and washed with DPBS before adding the Live/Dead solution containing 0.5 μ L of calcein-AM and 2.5 μ L of ethidium homodimer-1 in 997 μ L DPBS. Then, the samples were incubated for 45 min at 37°C. The cell survival was characterized under a fluorescent microscope, in which green and red colors indicate live and dead cells, respectively. The viable and dead cells were counted on a surface of 200 $\times$ 200 μ m² using Image J software (<https://imagej.nih.gov/ij/>).

Mitochondrial activity assay

The mitochondrial activity of cells in each condition was assessed by using a resazurin-based solution of PrestoBlue™ HS (P50200; Invitrogen) following our published protocol.⁵⁰ PrestoBlue solution was prepared in the culture medium with a proportion of 1:10 PrestoBlue in the medium. Then, the PrestoBlue solution was added to each well after aspirating the medium of samples. After an incubation time of 3 h at 37°C, the medium in each well was pipetted a few times to be homogenized, and 100 μ L of the medium was transferred to a 96-well plate to measure the fluorescent values by fluorescence spectroscopy (Multilabel reader; Victor X4). The emission and excitation wavelengths were set at 620 nm and 560 nm. The average fluorescent value of hydrogels on day 0 and normoxic samples without liposomes on day 3 were used for normalization at 0% and 100% viability, respectively.

Immunofluorescence staining

After 3 days of *in vitro* culture of bioprinted samples, the samples were put in HistoGel (HG-4000-012; Thermo Fisher Scientific) and fixed with a combination of 75% of paraformaldehyde and 4% and 25% of CaCl₂ 200 mM for 1 h at room temperature. Then, the constructs were dehydrated and clarified before embedding in paraffin. The paraffin-embedded samples were cut, and the slides were prepared for an immunohistochemical study. Given that early apoptosis in cells can be

identified through the detection of membrane phospholipid phosphatidylserine (PS) exposed on the cell's outer membrane, we conducted annexin V immunostaining to detect cells in the early apoptosis state. Briefly, after deparaffinizing and rehydrating different sample sections on slides, the nonspecific antigens were blocked using 10% NGS in 1 $\times$ Tris-buffered saline (TBS) for 1 h at room temperature.

Afterward, the slides were incubated with annexin V polyclonal primary antibody (11060-1-AP; Proteintech), diluted 1:400 in normal goat serum (NGS) 1%, for 1.5 h at room temperature. After washing slides in 1 $\times$ TBS, they were incubated with Alexa Fluor 488 goat anti-rabbit secondary antibody, diluted 1:250 in NGS 1%, for 40 min at room temperature. Then, the nuclei were stained with 1:500 Hoechst 33342 (H3570; Thermo Fisher Scientific) for 5 min followed by washing in 1 $\times$ TBS and distilled water. Finally, the slides were mounted with a fluorescence mounting medium (S3023; Dako) and scanned by a fluorescent scanner (ZEISS Axio Scan.Z1). The pictures were analyzed by ZEN 3.5 (blue edition), and the annexin V-positive cells were counted in a square with dimensions of 160 $\times$ 160 μ m².

Statistical analysis

One-way analysis of variance was performed to statistically analyze the data, and a *p*-value of 0.05 was used to define statistical significance. The quantitative data were reported as mean $\pm$ standard deviation (SD), and error bars in graphs reflect one sample SD.

Results

Liposomal catalase and liposomal H₂O₂ characterization

The size, polydispersity, and zeta potential of liposomal catalase and liposomal H₂O₂ were measured using dynamic light scattering technique (Table 1). Results indicated that the sizes of both formulations are in the range of 140–160 nm with a polydispersity index of 0.2–0.4, which is classified as relatively monodisperse nanoparticles (Fig. 2a).⁵¹ In addition to being a crucial factor in the stability and biocompatibility of nanoparticles, zeta potential is an effective way for determining the strength of colloidal particle interactions.⁵² The

TABLE 1. CHARACTERIZATION OF LIPOSOMAL CATALASE AND LIPOSOMAL H₂O₂ AFTER PREPARATION

	Liposomal catalase	Liposomal H ₂ O ₂
Size (nm)	145.545 ± 3.235	152.750 ± 17.150
Polydispersity index	0.243 ± 0.056	0.377 ± 0.032
Zeta potential (mV)	-7.740 ± 2.220	-10.190 ± 0.960
Entrapment efficiency (%)	72.551 ± 5.699	78.214 ± 3.112

zeta potential of liposomal catalase and liposomal H₂O₂ was -7.74 ± 2.22 and -10.19 ± 0.96 , respectively, which demonstrate relative stability, which is important in ensuring that aggregation does not occur during the bioprinting process (Fig. 2, b).

Cell survival and mitochondrial activity of bioprinted scaffolds under hypoxic and normoxic conditions

The viability of cells bioprinted along with liposomes and cultured under hypoxic conditions exhibited no significant difference when compared with the viability of cells cultured in normoxia without liposomes ($78.2 \pm 5.3\%$ and $83.9 \pm 8.7\%$, respectively). Conversely, the viability of cells in hypoxia without liposomes ($74.7 \pm 8.4\%$) was significantly lower compared with the other experimental groups ($p < 0.05$).

In contrast, samples containing liposomal catalase and liposomal H₂O₂ and cultured in normoxia demonstrated the highest cell viability ($90.1 \pm 5.2\%$) compared with all other groups (vs. normoxia, $p < 0.05$; vs. hypoxia, $p < 0.0001$; vs. hypoxia contained liposomes, $p < 0.01$) (Fig. 3a–e). Moreover, mitochondrial activity was significantly higher in bioprinted oxygen-releasing constructs in both hypoxia and normoxia culture conditions when compared with bioprinted constructs without liposomes (normoxia–liposomes [Normoxia-LIP] vs. normoxia, $161.8 \pm 23.8\%$ vs. $100 \pm 6.2\%$, $p < 0.001$; hypoxia–liposomes [Hypoxia-LIP] vs. hypoxia, $110.1 \pm 7.9\%$ vs. $89.3 \pm 4.9\%$, $p < 0.05$). Notably, the group cultured in normoxia containing liposomes showed the highest mitochondrial activity compared with all other groups (vs. all groups, $p < 0.0001$) (Fig. 3f).

Immunofluorescent staining on bioprinted scaffolds

Cells cultured under both normoxic and hypoxic conditions were examined for early apoptosis using annexin V antibody. The results demonstrated that cells in hypoxia exhibited a higher degree of translocation of PS to the outer cell membrane

layer ($39.5 \pm 19.8\%$) compared with the bioprinted cells in normoxia (20.3 ± 15.7 , $p < 0.01$) and hypoxia with the incorporation of liposomal catalase and liposomal H₂O₂ ($17.9 \pm 11.4\%$, $p < 0.01$). Interestingly, the number of annexin V-positive cells in the normoxia group did not significantly differ from the number observed in bioprinted constructs containing liposomal catalase and liposomal H₂O₂ cultured in hypoxia (Fig. 4).

Discussion

Catalase is an antioxidant enzyme known for its ability to break down H₂O₂ into water and oxygen.^{53,54} In this study, liposomal catalase was used to produce oxygen from H₂O₂ molecules, which cross the lipid bilayer of liposomal H₂O₂ at a gradual rate.^{55,56}



The encapsulation of liposomal H₂O₂ was executed in a separate bioink, deliberately excluding cells to avoid any potential adverse effect during the printing process. Following the bioprinting process, liposomal encapsulation yielded a sustained release of H₂O₂, thereby protecting the surrounding cells from high concentrations of free H₂O₂ and minimizing potential cellular damage. Therefore, this approach serves as a dual barrier for the controlled release of H₂O₂ before it undergoes decomposition by catalase. Furthermore, liposomal catalase was incorporated into the cell-laden bioink.

Recent literature has demonstrated sustained activity of liposomal catalase over a 24-day period, whereas free catalase exhibited a loss of 90% of its original activity within the same time frame.⁵⁷ Encapsulating catalase within liposomes enhances the enzyme's short lifetime and provides a controlled, gradual release pattern.^{58–60} Notably, the usage of liposomal catalase and liposomal H₂O₂ has found application in enhancing cancer therapy. For instance, Song et al.⁵⁶ injected liposomal catalase and H₂O₂ into 4T1 tumor-bearing mice and reported effective oxygenation of tumors. In our study, we use catalase and H₂O₂-loaded liposomes for the oxygenation of scaffolds in a hypoxic environment.

Two distinct alginate-based bioinks were formulated: shell A (bioink A) and shell B (bioink B). Shell A contained a higher concentration of alginate (1%), PF, isolated human ovarian stromal cells from the cortex, and liposomal catalase. Shell B included liposomal H₂O₂ in a lower alginate concentration (0.5%). PF has been successfully used for encapsulation of either isolated human

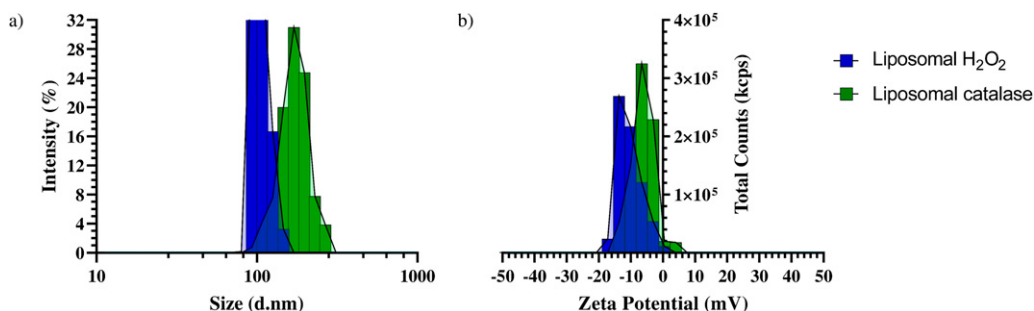


FIG. 2. Characterization of liposomal catalase and liposomal H₂O₂ after preparation: size distribution (a) and zeta potential (b).

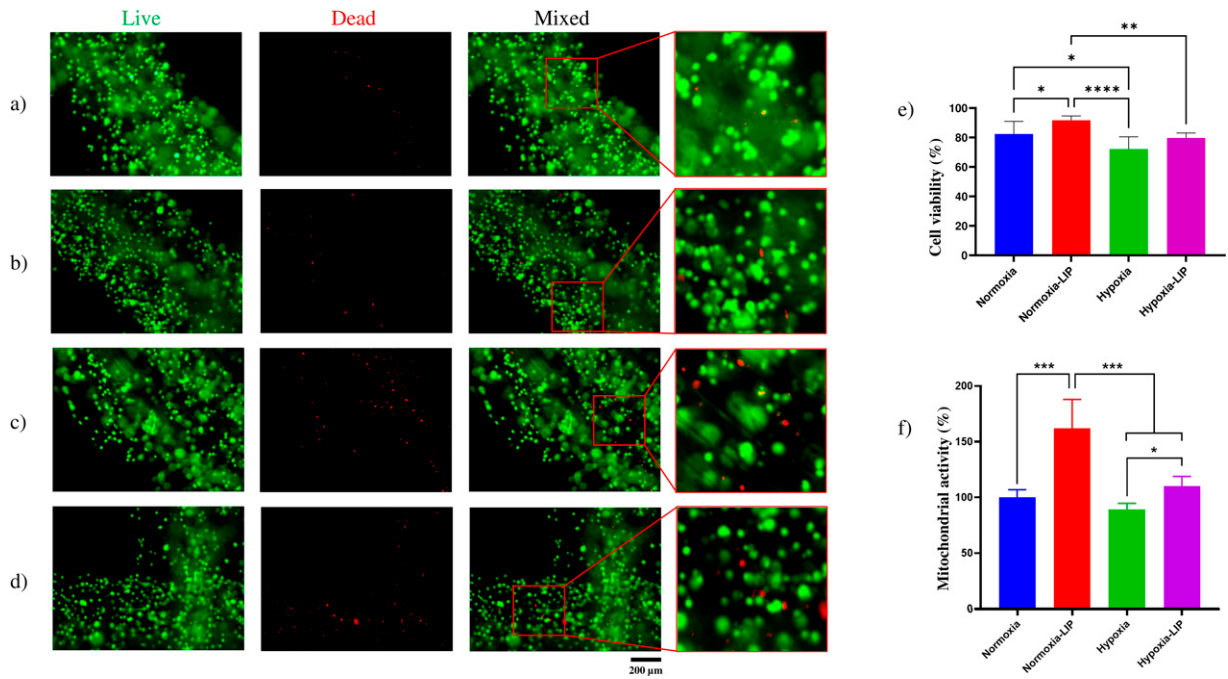


FIG. 3. Cell viability and mitochondrial activity of bioprinted cells under hypoxic and normoxic conditions. Representative Live/Dead cytotoxicity test pictures of samples in normoxia (a), normoxia-included liposomal catalase and liposomal H_2O_2 (Normoxia-LIP) (b), hypoxia (c), and hypoxia-included liposomal catalase and liposomal H_2O_2 (Hypoxia-LIP) (d); the scale bar indicates 200 μm . Graph showing quantification of Live/Dead assay (e); mean $\pm$ SD; $n = 9$. Graph showing the mitochondrial activity of samples in different groups (f); mean $\pm$ SD; $n = 6$. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. SD, standard deviation.

ovarian stromal cells⁵⁰ or follicles⁴⁴ and has been reported to have positive effects on preserving the survival of both ovarian stromal cells and follicles *in vitro*. Moreover, Dadashzadeh et al.⁵⁰ reported the positive impact of thrombin on ovarian cell proliferation.

In this study, the *in vitro* culture medium of bioprinted constructs was enriched by thrombin to polymerize the PF incorporated in bioink A. The selection of varying alginate concentrations aimed to replicate the mechanical differences between the ovarian cortex and the medulla. The mechanical

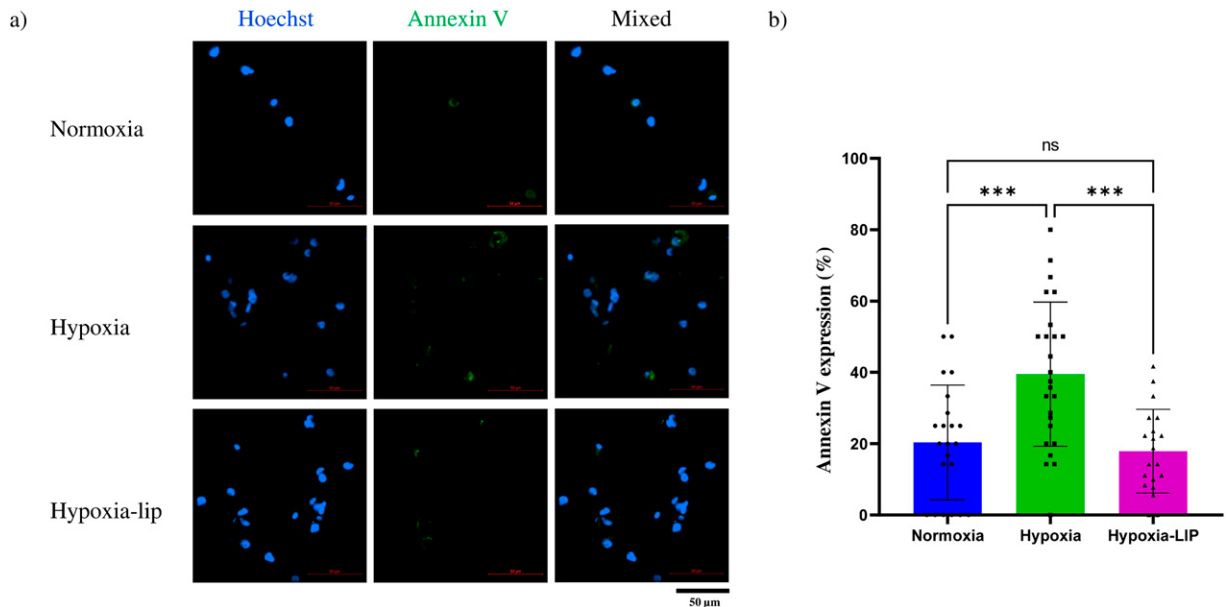


FIG. 4. Early apoptosis analysis of bioprinted ovarian stromal cells cultured under hypoxic and normoxic conditions for 3 days. Representative images of annexin V bound to translocated PS in different constructs and conditions (a); the scale bar indicates 50 μm . Graph showing quantification of annexin V-immunoassayed samples (b); mean $\pm$ SD; $n \geq 20$; *** $p < 0.001$. PS, phosphatidylserine; SD, standard deviation.

properties of alginate hydrogels depend on several factors, including the molecular structure of alginate, concentration of divalent cations, cross-linking time, and alginate concentration.^{61–65} Here, CaCl_2 and cross-linking time were kept constant, whereas alginate concentration was varied to form a stiffer alginate hydrogel in shell A (1%) than in shell B (0.5%). The microfluidic printhead of the RX1 bioprinter was used to bioprint oxygen-releasing scaffolds. This was achieved by simultaneously extruding a bioink containing liposomal catalase along with cells side by side with a bioink containing liposomal H_2O_2 .

The bioprinted oxygen-releasing scaffolds effectively preserved the viability of ovarian stromal cells in hypoxia, resulting in a viability similar to that observed in bioprinted scaffolds without liposomes under normoxic conditions (control). Notably, the oxygen-releasing liposomes improved cell viability in normoxia samples compared with the control (Fig. 3a–e). Moreover, the results of the mitochondrial activity in different samples indicated higher activity in scaffolds containing oxygen-releasing liposomes compared with samples without liposomal catalase and H_2O_2 (Fig. 3f). These results demonstrate the suitability of using oxygen-releasing systems not only in hypoxic conditions but also for culturing tissue-engineered scaffolds under normoxia. This is especially relevant owing to the limitations in oxygen diffusion imposed by medium height and scaffold thickness, where cells placed at the bottom of scaffolds experience the lowest oxygen pressure.¹⁶ Indeed, oxygen diffusion in the tissue exhibits a direct correlation with surface area and an inverse relationship with thickness.⁶⁶

Hypoxia is known to induce apoptosis.^{67–69} Once apoptosis is initiated, PS translocates, becoming exposed on the outer surface of cells.⁷⁰ Annexin V has a high affinity to interact with PS,⁷¹ and annexin V labeling of PS on the cell surface is indicative of cells in the early apoptosis stage.⁷⁰ Annexin V immunostaining analyses demonstrated that the bioprinted oxygen-releasing scaffold provides a suitable system for mitigating hypoxic stress on ovarian stromal cells, with the number of annexin V-positive cells being comparable with those cultured under normoxic conditions (control).

Conversely, bioprinted cells without a liposomal oxygen-releasing system experienced more apoptosis due to the hypoxic stress on the ovarian stromal cells. Kim et al.¹⁸ reported that oxygen delivery under hypoxic conditions moderated cell apoptosis compared with samples exposed to hypoxia without oxygen delivery, which had a higher rate of apoptotic activity. Moreover, Suvarnapathaki et al.⁷² provided oxygen-releasing scaffolds by synthesizing microparticles containing different concentrations of calcium peroxide (CaO_2) in polycaprolactone and encapsulated them in gelatin methacrylate. Their results showed that low levels of oxygen provided by low concentrations of CaO_2 led to an increase in apoptosis levels. Therefore, incorporating an appropriate oxygen-releasing system into tissue-engineered scaffolds holds the potential to safeguard cell survival.

Conclusion

In summary, our study effectively used bioprinting technology to replicate the intricate architecture of natural ovarian tissue, specifically its two-layered structure

comprising the cortex and the medulla. This was achieved by simultaneously extruding two different bioinks using a microfluidic RX1 bioprinter, resulting in the creation of parallel filaments with different mechanical properties, achieved through the usage of high and low concentrations of alginate. Moreover, the persistent challenge of hypoxia in tissue-engineered scaffolds was addressed through the incorporation of an oxygen-releasing system. Liposomal catalase and liposomal H_2O_2 were synthesized and incorporated into the two bioinks. Our observations underscored the positive impact of the bioprinted oxygen-releasing scaffold on the viability of ovarian stromal cells, both in hypoxic and in normoxic environments. Notably, the liposomal oxygen-releasing system demonstrated the ability to protect cells from apoptosis induced by low oxygen levels and hypoxic stress as well as support cellular metabolism according to the mitochondrial activity.

In conclusion, our study illustrates the potential for bioprinting technology to replicate the complex architecture of natural tissues and offers a promising solution to mitigate hypoxia-related challenges in tissue engineering. The liposomal oxygen-releasing system shows promise as a valuable tool for enhancing cell viability under adverse conditions, providing new avenues for the development of functional tissue-engineered constructs.

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Authors' Contributions

A.D. and S.M.: Conceptualization, methodology, software, validation, formal analysis, investigation, writing original draft, and visualization; C.A.A.: Resources, review and editing, supervision, project administration, and funding acquisition.

Disclosure Statement

The authors declare no competing interests.

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References

1. Donnez J, Dolmans M-M. Fertility preservation in women. *Nat Rev Endocrinol* 2013;9(12):735–749.
2. Dolmans M-M, Donnez J, Cacciottola L. Fertility preservation: The challenge of freezing and transplanting ovarian tissue. *Trends Mol Med* 2020;27(8):777–791.

3. Amorim CA, Shikanov A. The artificial ovary: Current status and future perspectives. *Future Oncol* 2016;12(20):2323–2332.
4. Dolmans M-M, Amorim CA. Fertility preservation: Construction and use of artificial ovaries. *Reproduction* 2019;158(5):F15–F25.
5. Sonmezer M, Oktay K. Fertility preservation in female patients. *Hum Reprod Update* 2004;10(3):251–266.
6. Du Y, Carranza Z, Luan Y, et al. Evidence of cancer therapy-induced chronic inflammation in the ovary across multiple species: A potential cause of persistent tissue damage and follicle depletion. *J Reprod Immunol* 2022;150:103491.
7. Wu T, Gao Y, Su J, et al. Three-dimensional bioprinting of artificial ovaries by an extrusion-based method using gelatin-methacryloyl bioink. *Climacteric* 2022;25(2):170–178.
8. Jafari H, Dadashzadeh A, Moghassemi S, et al. Ovarian cell encapsulation in an enzymatically crosslinked silk-based hydrogel with tunable mechanical properties. *Gels* 2021;7(3):138.
9. Laronda MM, Rutz AL, Xiao S, et al. A bioprosthetic ovary created using 3D printed microporous scaffolds restores ovarian function in sterilized mice. *Nat Commun* 2017;8(1):15261.
10. Luyckx V, Dolmans M-M, Vanacker J, et al. A new step toward the artificial ovary: Survival and proliferation of isolated murine follicles after autologous transplantation in a fibrin scaffold. *Fertil Steril* 2014;101(4):1149–1156.
11. Vanacker J, Luyckx V, Dolmans M-M, et al. Transplantation of an alginate–matrigel matrix containing isolated ovarian cells: First step in developing a biodegradable scaffold to transplant isolated preantral follicles and ovarian cells. *Biomaterials* 2012;33(26):6079–6085.
12. Nason-Tomaszewski CE, Thomas EE, Matera DL, et al. Extracellular matrix-templating fibrous hydrogels promote ovarian tissue remodeling and oocyte growth. *Bioact Mater* 2024;32:292–303.
13. Ouni E, Vertommen D, Chiti MC, et al. A draft map of the human ovarian proteome for tissue engineering and clinical applications. *Mol Cell Proteomics* 2019;18(Suppl 1):S159–S173.
14. Lee H-Y, Kim H-W, Lee JH, et al. Controlling oxygen release from hollow microparticles for prolonged cell survival under hypoxic environment. *Biomaterials* 2015;53:583–591.
15. Dadashzadeh A, Moghassemi S, Shavandi A, et al. A review on biomaterials for ovarian tissue engineering. *Acta Biomater* 2021;135:48–63.
16. Place TL, Domann FE, Case AJ. Limitations of oxygen delivery to cells in culture: An underappreciated problem in basic and translational research. *Free Radic Biol Med* 2017;113:311–322.
17. Khorshidi S, Karkhaneh A, Bonakdar S. Fabrication of amine-decorated nonspherical microparticles with calcium peroxide cargo for controlled release of oxygen. *J Biomed Mater Res A* 2020;108(1):136–147.
18. Kim HY, Kim SY, Lee H-Y, et al. Oxygen-releasing microparticles for cell survival and differentiation ability under hypoxia for effective bone regeneration. *Biomacromolecules* 2019;20(2):1087–1097.
19. Oh SH, Ward CL, Atala A, et al. Oxygen generating scaffolds for enhancing engineered tissue survival. *Biomaterials* 2009;30(5):757–762.
20. Yeong W-Y, Chua C-K, Leong K-F, et al. Rapid prototyping in tissue engineering: Challenges and potential. *Trends Biotechnol* 2004;22(12):643–652.
21. Nillesen ST, Geutjes PJ, Wismans R, et al. Increased angiogenesis and blood vessel maturation in acellular collagen–heparin scaffolds containing both FGF2 and VEGF. *Biomaterials* 2007;28(6):1123–1131.
22. Zhang W, Wray LS, Rnjak-Kovacina J, et al. Vascularization of hollow channel-modified porous silk scaffolds with endothelial cells for tissue regeneration. *Biomaterials* 2015;56:68–77.
23. Li Z, Guo X, Guan J. An oxygen release system to augment cardiac progenitor cell survival and differentiation under hypoxic condition. *Biomaterials* 2012;33(25):5914–5923.
24. Abdi SIH, Choi JY, Lau HC, et al. Controlled release of oxygen from PLGA-alginate layered matrix and its *in vitro* characterization on the viability of muscle cells under hypoxic environment. *Tissue Eng Regen Med* 2013;10(3):131–138.
25. Choi J, Hong G, Kwon T, et al. Fabrication of oxygen releasing scaffold by embedding H₂O₂-PLGA microspheres into alginate-based hydrogel sponge and its application for wound healing. *Applied Sciences* 2018;8(9):1492.
26. Abdi SIH, Ng SM, Lim JO. An enzyme-modulated oxygen-producing micro-system for regenerative therapeutics. *Int J Pharm* 2011;409(1-2):203–205.
27. Pedde RD, Mirani B, Navaei A, et al. Emerging biofabrication strategies for engineering complex tissue constructs. *Advanced Materials* 2017;29(19):1606061.
28. Choi JK, Agarwal P, Huang H, et al. The crucial role of mechanical heterogeneity in regulating follicle development and ovulation with engineered ovarian microtissue. *Biomaterials* 2014;35(19):5122–5128.
29. Markstedt K, Mantas A, Tournier I, et al. 3D bioprinting human chondrocytes with nanocellulose–alginate bioink for cartilage tissue engineering applications. *Biomacromolecules* 2015;16(5):1489–1496.
30. Arslan-Yildiz A, El Assal R, Chen P, et al. Towards artificial tissue models: Past, present, and future of 3D bioprinting. *Biofabrication* 2016;8(1):014103.
31. Skardal A, Atala A. Biomaterials for integration with 3-D bioprinting. *Ann Biomed Eng* 2015;43(3):730–746.
32. Dhawan A, Kennedy PM, Rizk EB, et al. Three-dimensional bioprinting for bone and cartilage restoration in orthopaedic surgery. *J Am Acad Orthop Surg* 2019;27(5):e215–e226.
33. Ramesh S, Harrysson OL, Rao PK, et al. Extrusion bioprinting: Recent progress, challenges, and future opportunities. *Bioprinting* 2021;21:e00116.
34. Ng WL, Huang X, Shkolnikov V, et al. Polyvinylpyrrolidone-based bioink: Influence of bioink properties on printing performance and cell proliferation during inkjet-based bioprinting. *Bio-Des Manuf* 2023;6(6):676–690.
35. Li Y, Zhang X, Zhang X, et al. Recent progress of the vat photopolymerization technique in tissue engineering: A brief review of mechanisms, methods, materials, and applications. *Polymers (Basel)* 2023;15(19):3940.
36. Jiang T, Munguia-Lopez JG, Flores-Torres S, et al. Extrusion bioprinting of soft materials: An emerging technique for biological model fabrication. *Applied Physics Reviews* 2019;6(1):11310.
37. Vijayavenkataraman S, Fuh JY, Lu WF. 3D printing and 3D bioprinting in pediatrics. *Bioengineering* 2017;4(3):63.
38. Ashammakhi N, Hasan A, Kaarela O, et al. Advancing frontiers in bone bioprinting. *Adv Healthc Mater* 2019;8(7):e1801048.

39. Levato R, Dudaryeva O, Garciamendez-Mijares CE, et al. Light-based vat-polymerization bioprinting. *Nat Rev Methods Primers* 2023;3(1):47.
40. Abelseh E, Abelseh L, De la Vega L, et al. 3D printing of neural tissues derived from human induced pluripotent stem cells using a fibrin-based bioink. *ACS Biomater Sci Eng* 2018;5(1):234–243.
41. Beyer ST, Walus K, Mohamed T, et al. System for additive manufacturing of three-dimensional structures and method for same. Google Patents: 2021.
42. Beyer S, Bsoul A, Ahmadi A, et al. 3D alginate constructs for tissue engineering printed using a coaxial flow focusing microfluidic device. *IEEE*: 2013.
43. Gallardo M, Paulini F, Corral A, et al. Evaluation of a new freezing protocol containing 20% dimethyl sulphoxide concentration to cryopreserve human ovarian tissue. *Reprod Biomed Online* 2018;37(6):653–665.
44. Dadashzadeh A, Moghassemi S, Peaucelle A, et al. Mind the mechanical strength: Tailoring a 3D matrix to encapsulate isolated human preantral follicles. *Hum Reprod Open* 2023;2023(2):hoad004.
45. Moghassemi S, Dadashzadeh A, Jafari H, et al. Liposomal oxygen-generating hydrogel for enhancing cell survival under hypoxia condition. *Colloids Surf B Biointerfaces* 2023;231:113562.
46. Fisher-Wellman KH, Gilliam LA, Lin C-T, et al. Mitochondrial glutathione depletion reveals a novel role for the pyruvate dehydrogenase complex as a key H₂O₂-emitting source under conditions of nutrient overload. *Free Radic Biol Med* 2013;65:1201–1208.
47. Komlódi T, Sobotka O, Gnaiger E. Facts and artefacts on the oxygen dependence of hydrogen peroxide flux using Amplex UltraRed. *Bioenergetics Communications* 2021; 2021:4–4.
48. Moghassemi S, Dadashzadeh A, Camboni A, et al. Photodynamic therapy using OR141-loaded nanovesicles for eradication of leukemic cells from ovarian tissue. *Photodiagnosis Photodyn Ther* 2022;40:103139.
49. Hakamivala A, Moghassemi S, Omidfar K. Modeling and optimization of the niosome nanovesicles using response surface methodology for delivery of insulin. *Biomed Phys Eng Express* 2019;5(4):045041.
50. Dadashzadeh A, Moghassemi S, Amorim C. Evaluation of PEGylated fibrin as a three-dimensional biodegradable scaffold for ovarian tissue engineering. *Materials Today Chemistry* 2021;22:100626.
51. Singh P, Bodycomb J, Travers B, et al. Particle size analyses of polydisperse liposome formulations with a novel multispectral advanced nanoparticle tracking technology. *Int J Pharm* 2019;566:680–686.
52. Vilela J, Moghassemi S, Dadashzadeh A, et al. Safety of lavender oil-loaded niosomes for *in vitro* culture and biomedical applications. *Nanomaterials* 2022;12(12):1999.
53. Marín-García J. Chapter 14—Oxidative Stress and Cell Death in Cardiovascular Disease: A Post-Genomic Appraisal. In: *Post-Genomic Cardiology (Second Edition)*. (Marín-García J. ed.) Academic Press: Boston; 2014; pp. 471–498.
54. Catalán V, Frühbeck G, Gómez-Ambrosi J. Chapter 8 - Inflammatory and oxidative stress markers in skeletal muscle of obese subjects. In: *Obesity*. (del Moral AM, Aguilera García CM. eds.) Academic Press: Boston; 2018; pp. 163–189.
55. Frimer AA, Forman A, Borg DC. H₂O₂-diffusion through liposomes. *Israel Journal of Chemistry* 1983;23(4):442–445.
56. Song X, Xu J, Liang C, et al. Self-supplied tumor oxygenation through separated liposomal delivery of H₂O₂ and catalase for enhanced radio-immunotherapy of cancer. *Nano Lett* 2018;18(10):6360–6368.
57. Yoshimoto M, Sakamoto H, Yoshimoto N, et al. Stabilization of quaternary structure and activity of bovine liver catalase through encapsulation in liposomes. *Enzyme and Microbial Technology* 2007;41(6–7):849–858.
58. Muzykantov VR. 14 Immunotargeting of Catalase to the Pulmonary Vascular Endothelium. *Drug Targeting: Strategies, Principles, and Applications* 2000:241–254.
59. Howard MD, Greineder CF, Hood ED, et al. Endothelial targeting of liposomes encapsulating SOD/catalase mimetic EUK-134 alleviates acute pulmonary inflammation. *J Control Release* 2014;177:34–41.
60. Moghassemi S, Dadashzadeh A, Azevedo RB, et al. Photodynamic cancer therapy using liposomes as an advanced vesicular photosensitizer delivery system. *J Control Release* 2021;339:75–90.
61. Mancini M, Moresi M, Rancini R. Mechanical properties of alginate gels: Empirical characterisation. *Journal of Food Engineering* 1999;39(4):369–378.
62. Gladukh I, Podorozhna M. Study of structural and mechanical properties of sodium alginate gels. *Eureka: HS* 2021(2): 82–89.
63. Girón-Hernández J, Gentile P, Benlloch-Tinoco M. Impact of heterogeneously crosslinked calcium alginate networks on the encapsulation of β -carotene-loaded beads. *Carbohydr Polym* 2021;271:118429.
64. Liling G, Di Z, Jiachao X, et al. Effects of ionic crosslinking on physical and mechanical properties of alginate mulching films. *Carbohydr Polym* 2016;136:259–265.
65. Naghie S, Karamooz-Ravari MR, Sarker M, et al. Influence of crosslinking on the mechanical behavior of 3D printed alginate scaffolds: Experimental and numerical approaches. *J Mech Behav Biomed Mater* 2018;80: 111–118.
66. Maina JN, West JB. Thin and strong! The bioengineering dilemma in the structural and functional design of the blood-gas barrier. *Physiol Rev* 2005;85(3):811–844.
67. Greijer A, Van der Wall E. The role of hypoxia inducible factor 1 (HIF-1) in hypoxia induced apoptosis. *J Clin Pathol* 2004;57(10):1009–1014.
68. Touri M, Moztaazadeh F, Abu Osman NA, et al. Oxygen-releasing scaffolds for accelerated bone regeneration. *ACS Biomater Sci Eng* 2020;6(5):2985–2994.
69. Shimizu S, Eguchi Y, Kamiike W, et al. Induction of apoptosis as well as necrosis by hypoxia and predominant prevention of apoptosis by Bcl-2 and Bcl-XL. *Cancer Res* 1996;56(9):2161–2166.
70. Pietkiewicz S, Schmidt JH, Lavrik IN. Quantification of apoptosis and necroptosis at the single cell level by a combination of Imaging Flow Cytometry with classical Annexin V/propidium iodide staining. *J Immunol Methods* 2015; 423:99–103.
71. Van Engeland M, Nieland LJ, Ramaekers FC, et al. Annexin V-affinity assay: A review on an apoptosis detection system based on phosphatidylserine exposure. *Cytometry: The Journal of the International Society for Analytical Cytology* 1998;31(1):1–9.

72. Suvarnapathaki S, Nguyen A, Gouloupoulos A, et al. Oxygen-generating scaffolds for cardiac tissue engineering applications. *ACS Biomater Sci Eng* 2022;9(1):409–426.

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