The gelatin-based liquid marbles for cell cryopreservation

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A B S T R A C T

As an alternative and a straightforward cryopreservation biotechnological tool, liquid marble provides a promising cryopreservation approach. Currently, effective cell preservation mainly based on the addition of dimethyl sulfoxide (DMSO) and fetal bovine serum (FBS). As state-of-the-art cryoprotectant (CPA), DMSO, has intrinsic toxicity, which is the bottleneck of its widespread application. The complex compositions of FBS have the potential risks of pathogenic microorganism contamination. However, efficient cell cryopreservation using liquid marbles, a platform independent of DMSO and FBS, has not been well investigated yet. Herein, we explore the cryopreservation role of liquid marbles based on gelatin solution. Gelatin has a superior biocompatibility, which is incomparable. During a freeze-thaw cycle, gelatin produces negligible osmotic pressure, and has high ice recrystallization inhibition (IRI) activity to induce the formation of smaller and smooth ice crystals. Moreover, the specific structure of liquid marble also provides favorable supports for cell survival. The cryopreservation efficiency of mouse fibroblasts cells 1929 via the gelatin-based liquid marble was as high as 90%, and the recovered cells could maintain their normal functionalities. This work opens a new window of opportunity for non-toxic and efficient cryopreservation of liquid marbles without the need of DMSO and FBS addition.

1. Introduction

Cryopreservation of living cells is indispensable in the field of biomedicine, including assisted reproduction, cell therapy, tissue engineering, drug screening, etc [1–5]. Trillions of cells are preserved worldwide for daily clinical use [6–8]. Although ultra-low temperature enables long-term preservation of biological samples, the cryogenic temperatures to which cells are exposed inevitably lead to ice formation and growth, causing irreversible mechanical damage and osmotic shock on a cellular level [9]. The solution to this is using significant volumes of cryoprotectants (CPAs) to protect them from damages during freezing and thawing [10]. It is a common sense that glycerol and dimethyl sulfoxide (DMSO) have been incorporated with fetal bovine serum (FBS)/proteins (like albumin) for cell cryopreservation [1,11]. However, these CPAs come with some problems. Such as, glycerol, as the ‘gold standard’ CPA for red blood cells, can easily leads to hemolysis [12]. DMSO is intrinsically toxic, affects the epigenetic profile of cells and induces uncontrolled differentiation of stem cells, which results in phenotypic changes [13–15]. In addition, FBS from different donor species in the preservation solution would be an undesirable component for cell cryopreservation, which may introduce xenogeneic factors and cause spontaneous differentiation [15–17].

Recently, researchers have designed and synthesized CPAs for DMSO-free/FBS-free cryopreservation of cells [16,18–20]. Meanwhile, various innovative cryopreservation techniques, such as, cell printing, micro-fluidic platforms, and liquid marbles (LMs), have also been used to improve the viability and efficiency of cell cryopreservation [21–24]. Among these, LM, first proposed by Aussillous and Quéré in 2001 [25], could provide a promising cryopreservation approach. LMs are nonsticky droplets wrapped by micro/nano-scale particles and are highly sought after in the field of biomedicine. As microbiological reactors, Shen and colleagues first employed LMs for the preparation of embryonic bodies, and they also achieved rapid identification of blood types using LMs [26, 27]. Li et al. advanced the functionality of LM microreactors by subjecting LMs to vertical oscillations to achieve sufficient mixing with variable dynamic modes [28]. This strategy sets a solid foundation for the development of advancing the functional LM microreactors, and may also provide insights for the research study about mixing in soft-elastic

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The use of LMs as a three-dimensional niche environment for cell culture deserves to be emphasized. For example, Wang et al. used LMs as the reactor for 3D culture of stem cell spheroids. In this LM system, not only the cell microenvironment was well maintained, but also the medium/drugs and spent culture medium could be mixed/removed in a precise manner [30]. In addition, LMs have been used for high-throughput drug screening and tumor growth as well [31,32]. However, the reported work on cryopreservation using LM is currently only conducted by Serrano et al. and Vadivelu et al. [23,24]. Using LMs, Serrano et al. successfully achieved the preservation of murine L929 fibroblasts without adding CPA for the first time. On this basis, Nguyen et al. used agarose hydrogel-containing FBS embedded inside the LM to weaken the complete dependence on FBS and achieved cell cryopreservation with well-controlled serum concentrations. However, the efficient cell cryopreservation of LMs independent of FBS has not been well investigated yet. While LM-based cell cryopreservation is still in its early age, it promises to be a scalable platform for traditional cryopreservation. Thus, there is an urgent need to improve the application of LMs with high-efficency and low-toxicity CPAs.

Here, we selected biocompatible gelatin as CPA, the building block of collagen, which is often chosen as matrix material for cellular scaffolds [33–36]. Meanwhile, gelatin has growing applications in the food and beverage, nutraceuticals industries, and pharmaceuticals [37]. Moreover, it plays an important role in maintaining the water balance inside and outside the cell membrane [38]. Therefore, the gelatin was used as the CPA to develop LM for the cell cryopreservation without DMSO and FBS in this study. The formation and properties of gelatin-based LMs were investigated in detail. During a freeze-thaw cycle, gelatin as CPA produces negligible osmotic pressure. In addition, the gelatin could induce the formation of smaller and smoother-shaped ice crystals but without detrimental dynamic ice shaping (DIS), which prevents the formation of needle-like ice crystals that would pierce cell membranes and damage structural integrity. This is extremely beneficial for the survival of cryopreserved cells over the whole cryopreservation process. Moreover, the use of gelatin-based LMs could offer additional benefits for outstanding efficiency of cell cryoprotection via a barrier provided by hydrophobic PTFE shell structure and a small volume cryopreservation. When thawing, the LMs could burst instantly, making cells achieve super-fast recovering in a few seconds, which is beneficial for reducing cell damage induced by ice recrystallization. Therefore, the gelatin-based LMs cryopreservation system is much interesting and meaningful compared to the recovery of traditional cryopreservation.

2. Materials and methods

2.1. Materials

Poly (tetrafluoroethylene) (PTFE) and gelatin were obtained from Sigma-Aldrich. Roswell Park Memorial Institute-1640 (RPMI-1640) was purchased from Gibco. Fetal bovine serum (FBS) was purchased from Shanghai Yeasen. DMSO, phosphate-buffered saline (PBS), penicillin-streptomycin (P/S), and trypsin-EDTA (0.025–0.01 wt%) were all purchased from Shanghai Solarbio Science and Technology Co., Ltd. Live/dead kits were obtained from Invitrogen. The culture medium for L929 cells contained RPMI-1640 with 10% FBS and 1% PS. Milli-Q water (18.2 MΩ cm) was used in all experiments.

2.2. Characterization of PTFE

The PTFE sample was sprayed with gold for 60 s using an ion sputter, and the microstructure of the PTFE powder was observed using a scanning electron microscope (S-4800). The contact angle of the PTFE powder was measured by depositing water droplets on its surface using a JC2000D1 system (Shanghai Zhongchen Digital Technology Apparatus).

### Table 1

| Cell Density and Volume of the Different cell-containing LMs. |
|-----------------------------|-----------------------------|-----------------------------|
| volume (µL)                  | 5                          | 10                         | 15                          | 20                          | 30                          |
| Cell density (cell µL−1)     | 25,000                     | 25,000                     | 25,000                     | 25,000                     | 25,000                     |
| Number of cells per LM       | 125,000                    | 250,000                    | 375,000                    | 500,000                    | 750,000                    |
| LM                          | 250,000                    | 500,000                    | 750,000                    | 1,000,000                  | 1,500,000                  |

Three different positions of the sample were measured to obtain the average contact angle value. The optical images were obtained using a microscope.

2.3. Cell culture

L929 cells were incubated at 37 °C in a sterile incubator (Heracell VIOS 160i) with an environment of CO2 atmosphere (5%), Trypsin-EDTA was used to separate confluent cultures of L929 cells from the flask substrates. Then, L929 cells were collected by centrifugation for 4 min using a low-speed centrifuge (TD4) at 1000 rpm. Finally, the supernatant was completely sucked out to obtain cell pellet, the cells were counted using a hemocytometer (Qiujing XB. K. 25) and re-suspended for the preparation of LMs with a certain cell density and volume in cryopreservation solution.

2.4. Preparation and cryopreservation of cell-containing LMs

Gelatin was dissolved in PBS-free RPMI-1640 medium to prepare different concentrations of cryopreservation solutions (0, 2.5, 5, or 7.5 wt%). Then, the collected cell pellets were re-suspended in certain cell density using the prepared gelatin cryopreservation solution. To prepare the gelatin-based LMs, the PTFE powder was placed on Parafilm. Afterward, the re-suspended cell stock suspension with different volumes (5–30 µL) and cell concentrations was pipetted, and the cell suspension was placed on top of the PTFE powder bed. Using a small spoon to draw the powder carefully approach to the droplet until the surface of the droplet was completely covered with PTFE powder to obtain cell-containing LMs. Subsequently, the obtained gelatin-based LMs were introduced into the cryovials (Corning, 1.8 mL) using a small spoon. Finally, these cryovials containing LMs were transferred to freezing container (Nalgene 5100-0001C) for cell stepwise freezing. To simplify, gelatin-based LMs were abbreviated with three terms referring to the (1) LM or CS (cell suspensions), (2) concentrations of gelatin (0, 2.5, 5, or 7.5 wt%), and (3) drop volume (15 µL). For example, sample LM-5-15, LM represented the LM structure, 5 represented 5% gelatin, and 15 represented the liquid volume inside the marble, which was 15 µL. Furthermore, LMs with different drop volume cells and cell seeding densities were prepared. Table 1 gives diverse range of both parameters.

2.5. Distribution of cells in the LM

The cells were stained with calcein-AM before preparation of LMs. After cryovials containing LMs were removed from liquid nitrogen, the LMs were embedded in optimal cutting temperature (OCT) compound at −20 °C. The samples were cut into 6 µm sections. Subsequently, images were observed by an inverted optical microscope.

2.6. Investigation on the powder dosage of LM shell

The prepared LMs were placed separately in EP tubes (Axygen 1.5 mL) of known weight. Then the EP tubes were left at room temperature for 24 h until the liquid inside the LMs completely evaporated. At this time the weight of the EP tubes was obtained separately. The dosage of
PTFE powder in the LM shell layer can be obtained by counting the difference between the corresponding weights before and after of the EP tubes.

2.7. Ice formation inhibition assay

Ice formation inhibition assay was performed using a cold stage system (MK2000, INSTEC). A 10 μL of gelatin solution was dropped onto a coverslip, resting on a stainless sheet placed over liquid nitrogen vapor. When the droplets hit the stainless sheet, the wafers will form instantaneously. The coverslip was quickly transferred to the pre-chilled sample chamber, and cryomicrograph of ice crystals were obtained using a polarizing microscope (Nikon, C-SP) after quenching at −6 °C for 30 min. The ice crystal cryomicrographs of gelatin solutions were compared to that of a pure water negative control.

2.8. Differential scanning calorimetry (DSC) tests

The heat flow (mW/mg) of gelatin solutions were was measured and recorded using a DSC system (DSC204F1). During the heating process, the endothermic peak of the gelatin aqueous solution system will appear along with the melting of ice, and the freezing point of water also was determined as a negative control.

2.9. Cytotoxicity of cell CPAs tests

Suspended L929 cells were exposed in medium containing 2% gelatin, 3% gelatin, 4% gelatin, 2% DMSO, 3% DMSO, 4% DMSO and fresh medium, respectively. After 1, 2, or 3 days, the L929 cell attachment and morphology were observed using an inverted microscope (Nikon Eclipse Ti–S).

2.10. Rapid thawing of cell-containing LMs

Take the cryovials containing the LMs out of the liquid nitrogen, and then pour the LMs into the centrifuge tube containing the culture medium preheated at 37 °C. Rapid cell thawing was achieved by gently shaking the centrifuge tube to make LMs burst in 3–5 s. Finally, cells were collected by centrifugation as previously described. The cell numbers of samples after cryopreservation were counted using a hemocytometer.

2.11. Cell survival efficiency assays

The cell survival efficiency of the thawed L929 cells was detected by using Live/dead staining. First, the mixed solution of calcein-AM/ethidium homodimer-1 reagent was added to a 96-well TCPS plate as a dye solution, and then the thawed L929 cell suspension was added to the
well plate. The samples were incubated for 30 min in the dark at room temperature, photographs of live-dead staining of cells were taken using an inverted fluorescence microscope. The number of live cells (green) and dead cells (red) were counted to evaluate the post-thaw survival efficiency of L929 cells.

2.12. Cell attachment and proliferation tests

The recovered L929 cell was resuspended in fresh medium, and then the cell suspension was added into 12-well plate. After 24 h (37 °C, 5% CO₂ culture), the cell morphology was observed under a microscope and photographed for recording. Thawed L929 cells and fresh cells were cultured separately in 12-well TCPS plates for 6 days. The number of cells was counted daily for 1–6 days using microscopy.

3. Results and discussion

3.1. Formation and properties of gelatin-based LMs

The recent advancement in cell-based applications in biomedicine leads to an increasing demand for novel biotechnological tools to achieve effective cryopreservation of living cells. LMs as innovative and alternative biotechnological tools for cell cryopreservation is a promising cryopreservation approach, which could be further improved and broadened. Poly (tetrafluoroethylene) (PTFE), a hydrophobic polymer without cytotoxic, has been widely used in cardiovascular surgery. Our results suggested that the PTFE shows regular morphology and uniform size (Fig. 1A), and it has high hydrophobic properties with a water contact angle of 131° (Fig. 1B). Thus, PTFE was selected in this study for the preparation of the cell-containing LMs.

Scheme 1 shows the procedure for the preparation of gelatin-based LM for cryopreservation of cells. Briefly, when a gelatin droplet containing cells was dropped onto the bed of the PTFE powder, the droplet would be wrapped with the PTFE particles during rolling around owing to the tendency to minimize the surface free energy, forming the cell-containing LM. Namely, the reduction of surface free energy of the system leads to the formation of LMs [39,40]. A series of LMs with different volume were prepared via adjusting the volume of gelatin solution (Fig. 1C). The PTFE layer blocked the microscopic contact between the internal liquid and the substrate, and the non-wetting and stable presence of trace liquids on the surface of the substrate was achieved, which was similar to leidenfrost droplets, but different from the preparation of superhydrophobic surfaces by chemical modification or construction special surface microstructure [41–43]. When the volume of internal liquid increased, the LMs will gradually change from a spherical to an ellipsoidal shape. Meanwhile, the diameter and the height of LMs increase as the increase of the inner liquid volume (Fig. 1D). The liquid volume of LMs increased from 5 to 100 μL, the diameter of LM increased from 2.4 to 6.5 mm, and the height of LM increased from 2.3 to 4.62 mm. When the volume of the marble was small (<20 μL), the height of LMs tended to the diameter. With the increase of the gelatin solution, the disparity between diameter and height of LMs increased, which was due to the prominent gravity effect, so that the LMs became oval. The statics of these LMs stem from the balance between gravity and capillary action [25]. The resultant LMs shown high stable. The stability of the prepared LMs was preserved not only while moved around on a hydrophobic plastic substrate (Fig. 1C) but also when transferred to a cryoscope for cell cryopreservation (Fig. 1E) and even after cryopreservation removed from liquid nitrogen environment (Fig. 1E). This is due to the barrier effect of an air layer formed between the coated particles and the supporting liquid surface [44]. Moreover, we investigated the dosage of PTFE powder in the shell layer of LMs, as shown in Fig. S1. The prepared LMs were placed separately in the EP tubes (Fig. S1A). After the internal liquid of LMs completely evaporated, the shell powder remained at the bottom of the EP tube (Fig. S1B). We found that for LMs formed by a certain volume of liquid, after sufficient rolling, the PTFE progressively distributed on the surface of any droplet until the entire liquid-air interface was coated, and there was no significant difference in the dosage of powder coated on the surface of the LMs.

3.2. The capacity of gelatin to prevent ice injuries

The ice formation and growth are highly detrimental to most biological systems [45,46]. Ice formation and growth not only lead to solute injury on a cellular level as the concentration of extracellular solutes rises forming an increasingly hypertonic environment, but also mechanical damage when intracellular ice would quickly form at a higher cooling rate [9]. Therefore, the effects of gelatin medium in LMs on the ice formation and growth take very important roles for cell cryopreservation. These two types of cryo-injuries cause a problem in cell cryopreservation.

Scheme 1. Schematic of using gelatin-based LM for cryopreservation of cells.
Gelatin is a macromolecular hydrocolloid, which is the building block of collagen. In addition to α, β and γ subunits, gelatins also have some molecular chain fragments [47]. In an ice cream mix matrix, gelatin can act as a stabilizer to prevent the formation of larger ice crystals [48,49]. During a freeze-thaw cycle, gelatin also produces negligible osmotic pressure because most of the osmotic pressure is attributed to colloidal. Therefore, cells would not be damaged by osmotic shock during a slow cooling process, even as extracellular ice precipitation gradually increases. Meanwhile, slow-freezing method not only allows sufficient time for intracellular water to gradually diffuse out of cells to reduce the formation of intracellular ice crystals, but also minimize the osmotic pressure damage [16]. In this work, the ice inhibition capacity of gelatin was investigated using a standard splat assay and DSC tests. During cooling, residual solution containing the cells becomes viscous as the concentration of solute increases [50]. In general case, the extracellular ice is unstable and the small ice crystals begin to spontaneously merge and form larger crystals to minimize surface energy, which is the phenomenon of ice recrystallization (IR) that can cause mechanical damage to cell membranes [51]. Fig. 2A showed that the gelatin has high ice recrystallization inhibition (IRI) activity. Meanwhile, ice crystals formed in the gelatin solution are relatively round and smooth, rather than sharp needle-like crystals, which prevented deadly mechanical damage to the cells. Fig. 2B showed that the mean largest grain sizes (MLGS) of the gelatin solutions were decreased compared with pure water (148 nm) (p < 0.01). This behavior also dependent on the gelatin concentration. The gelatin solution (5 wt%) showed the smallest MLGS (23 μm), while the MLGS in 7.5 wt% gelatin solution slightly improved to 43 μm, which may be mainly due to the reduction of interactions between gelatin chain with ice crystal surface [52,53]. Previous study suggested that the IRI activity of hydrocolloid is based on an interaction of hydrocolloid molecules with the ice crystal surface [52,54]. As a common hydrocolloid, the formation capacity of hydrocolloid gel would increase with the increases of gelatin concentration. The three-dimensional gel structure can effectively prevent the formation of large ice crystals [54,55], therefore, the micro-viscosity or steric barrier will retard the diffusion of water to the detriment of bigger crystal regeneration with the increase of gelatin concentration [54,56,57]. Meanwhile, hydrodynamic interactions could affect the diffusion of CPAs (e.g., antifreeze proteins) to the ice surfaces before effective binding [58]. Therefore, the 5 wt% gelatin showed higher IRI activity compared water and 2.5 wt% gelatin (Fig. 2A and B). However, a more rigid gel would lead to the reduction of IRI activity [52,54] owing to the water holding capacity of the hydrocolloid was reduced, it caused some syneresis and increased the water mobility of the system. Therefore, there was a reduced interaction in 7.5 wt% gelatin than that in 5 wt% gelatin and further showed a related low IRI activity. These results were consistent with previous reports, Gaukel et al. found that the formation of hydrocolloid gel led to the reduction of IRI activity [52]. Goff et al. thought that a firm system was not always been effective at retarding ice crystal growth [54]. The thermodynamic behavior of gelatin solutions during the thawing process was also investigated, and the endothermic peaks of pure water and gelatin solutions were intrinsically related to the results of corresponding IRI activity (Fig. 2C). During the thawing process, owing to Ostwald ripening, IR causes the ice crystals to grow larger, being one of the major causes of cell death [50, 59]. However, IRI inhibitors such as CPAs can weaken the dependence of permeating CPAs and provide a new promising candidate for cell cryopreservation. Several studies also have reported that short collagen/gelatin polypeptides would have the ability to prevent ice crystal growth in a manner comparable to antifreeze proteins under super-cooled systems [60–62]. Since gelatin has the same chemical composition as short

![Fig. 2.](Image)

**Fig. 2.** The effects of gelatin on the formation of ice. (A) The optical microscopic images of recrystallized ice crystals grown in pure water and gelatin solutions with different concentration (after annealing at −6 °C for 30 min). Scale bar = 100 μm. (B) Mean largest grain size (MLGS) of pure water and gelatin solutions. (C) Differential scanning calorimetry (DSC) melting thermograms of pure water and gelatin solutions. G: gelatin. Value = mean ± standard deviation, n ≥ 3. ***P < 0.001.
collagen/gelatin peptide, the superior IRI activity of gelatin during cell cryopreservation could be similar to the binding of gelatin peptide to ice surface. Overall, gelatin could as the CPA to prepare the LM and inhibit ice formation/growth and thus protects cells from ice damage.

3.3. Cytotoxicity of CPA

The biocompatibility of CPA is essential and crucial during the cell cryopreservation. It has been reported that CPA’s toxicity would cause cell damages and induce cellular dysfunctions [63]. The cytotoxicity of current CPA is the bottleneck in clinical cell-based applications. Researchers have been working on the development of new low-toxic/non-toxic CPAs to reduce/get rid of the damage [18,20]. To verify the nontoxicity of gelatin as the CPA, L929 cells were exposed in medium containing 2–4% of gelatin or 2–4% of DMSO. As shown in Fig. 3, after 1–3 days of exposure, cell viability in 2–4% of gelatin solution was the same as that in the control (Fig. 3B), and the cells could attach to substrates with normal morphology (Fig. 3C). In contrast, some

![Figure 3](image-url)
dead cells (red) can be observed in DMSO groups, and cell viability decreased with increasing exposure time and DMSO concentrations (Fig. 3A and B). When exposed to 2–4% of DMSO for 3 days, the cell viability of L929 cells decreased from 85.6% (2%) to 68.6% (4%). After 3 days of exposure to high concentration of 4% DMSO, the cell viability of L929 cells had begun to decrease significantly, from 91.4% to 68.6%. However, when exposed to the same concentration of gelatin for the same time, the number of dead cells did not increase, and the cell viability of L929 cells was basically unchanged. Meanwhile, the DMSO would affect the cell morphology and make the cell morphology abnormal with shrinkage appearance. As shown in Fig. 3C, the morphology of L929 cells in the gelatin samples were similar to that in the control, while L929 cells in 2–4% of DMSO were shrunken and abnormal in morphology, changing from a spindle to a spherical shape. As the concentration of DMSO increased from 2% to 4%, the number of cells with abnormal morphology increased, and when the cells were exposed to DMSO from 1–3 days, the morphological shrinkage of the cells also became severe. After 3 days of 4% DMSO exposure, a large number of shrunken cells appeared, and some cells with normal morphology were smaller in area compared with the control, presenting a fine spindle shape. The results of cell-attachment efficiency were consistent with those when cells were directly exposed in medium containing 2–4% of gelatin, and 2–4% of DMSO for cell attachment culture at 37 ºC (Fig. S2). These results demonstrated gelatin has a superior biocompatibility compared with DMSO.

3.4. Cell cryopreservation of gelatin-based LMs

Despite massive studies in other areas, the use of LMs in cell cryopreservation has been very limited to date. Based on previous studies of LMs for cell cryopreservation [23,24], we aimed to further improve this platform as novel and alternative biotechnological tools for traditional cell cryopreservation, independent of DMSO and FBS. In this study, we used biocompatible gelatin as the CPA to construct the LM system for cell cryopreservation. Fig. 4 presented the post-thaw survival efficiency of L929 cells at different concentrations of gelatin using two freezing procedures. Results suggested that L929 cells were dead when using conventional cryopreservation (1.5 mL of solutions in cryovials) without any
As expected, the proportion of viable cells increased in LM system. After the introduction of gelatin as CPA, the number of viable cells in both CS and LM increased significantly, and the proportion of viable cells was highest when the gelatin concentration was 5 wt% (Fig. 4A). The LM-5-15 provided almost the same proportion of viable cells as DMSO (10 wt%) using traditional stepwise protocol (Fig. 4A, C). Meanwhile, the cell number in LM-5-15 after cryopreservation did not decrease significantly compared with that before cryopreservation (Fig. S3). Since LMs could burst instantly, cells could achieve super-fast recovering and release in 3–5 s when thawed (Movie S1). However, the post-thaw survival efficiency of L929 cells was negligible in all groups when using ultrarapid freezing protocol (Fig. 4B), which was similar to

Fig. 5. The adhesion behaviors of recovered cells. (A) The microscope photos of recovered L929 cells and (B) the adhesion ratio of L929 cells after recovery for 24 h. Scale bar = 100 μm. Value = mean ± standard deviation, n ≥ 3. ***P < 0.001.

Fig. 6. Cryopreservation efficiency of LMs at different cell densities and LM volumes. (A) Live/dead fluorescence images of L929 cells after recovered. (B) Post-thaw survival efficiency of L929 cells evaluated at different cell densities and liquid volume. Scale bar = 100 μm. Value = mean ± standard deviation, n ≥ 3.
that of the blank control group. In comparison, a bell-shaped relationship could be observed between gelatin concentration and L929 cell survival efficiency in LMs and CS using stepwise freezing protocol (Fig. 4C). Thus, the stepwise freezing process was appropriate for the cell cryopreservation based on gelatin-based LM because the lower cooling rate not only could maintain the LM structure, but also allow water to sufficiently flow out of the cell to reduce the formation of cellular ice crystals [16, 64]. Notably, the cell cryoprotection efficiency of in gelatin-based LMs was significantly higher than CS (p < 0.001). And even cells in LM-0-15 still resulted in a ~30% survival efficiency, as shown in Fig. 4C, because the encapsulation of cells by the LM could provide a physical barrier to protect them from adverse environmental conditions [65, 66]. This LM encapsulation exhibited such good results for cell cryopreservation. Compared with the widely used hydrogel encapsulation cell cryopreservation, this technique does not require the construction of a complex three-dimensional spatial network and the addition of DMSO and other CPAs [67, 68]. Moreover, the efficiency of CS was higher than that 1.5 mL of gelatin solutions in cryovials, and the blank samples of CS have ~15% survival efficiency. It could be deduced that preserving cells with a small volume would be beneficial. Overall, the gelatin-based LMs could provide non-toxic and efficient preservation of mammalian cells without DMSO and FBS.

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We also evaluated the cell attachment of post-thaw cells to further confirm the difference between LM and CS cryopreservation. As shown in Fig. 5, cells from LMs and CS were then cultured in regular polystyrene plates for 24 h, and the recovered cells from gelatin-based LM manner could attach to the substrates and have similar cell morphology to fresh cells. In the CS sample after recovery, only part of the cells adhered to the bottom of the plate, and most of the cells existed in the form of cell debris. Even the normal adherent cells just had a fine spindle shape, which was different from the spindle shape of fresh cells. For example, in the CS-7.5-15 sample, the cells were mostly spherical with severe shrinkage, floating in the medium, and the adherent cells with abnormal cell morphology. A bell-shaped relationship could be observed between gelatin concentration and the adhesion ratio of L929 cells after recovery in LMs and CS. With increasing gelatin concentrations (0–5 wt%), the adhesion ratio of L929 cells increased, and then decreased (7.5 wt%). Meanwhile, the normal L929 cells optimum adherence rate of LM was increased to ~98.0% (LM-5-15). However, the adhesion rate of CS was only 42.6% under the same conditions (Fig. 5B). These results also suggested that cell cryopreservation using LMs, independent of DMSO and FBS, was feasible.

To further validate the cell cryopreservation efficiency of gelatin-based LM, we study the LM performance at different droplet volumes and cell seeding densities. The diverse range of both parameters used to prepare LMs were given in Table 1. As presented in Fig. 6, we found that LM volumes from 5 to 30 μL and cell densities of (2.5–5) × 10^4 cells μL are in a safe range, within which cell cryopreservation efficiency without observable difference (the survival efficiency of all the samples keep around 85%) could match the performance of traditional stepwise protocol.

Cryoinjuries will not only affect cell viability, but also further affect intracellular enzyme and mitochondrial functions, which will further affect cell behavior including attachment and proliferation [63]. Therefore, after cryopreservation using LMs containing gelatin, we cultured the post-thaw survival cells to assess cell attachment or proliferation behavior, as shown in Fig. 7. It can be observed that the recovered L929 cells could maintain membrane integrity and attach to the substrates with normal cellular morphology (Fig. 7A), which is similar to the behavior of the fresh cells. Meanwhile, Fig. 7B presented that the proliferation curves of the recovered L929 cells (24–144 h) almost overlap.

![Fig. 7. Functionalities of recovered L929 cells. (A) Cell morphology after cryopreservation using LMs containing gelatin compared with fresh cells. Scale bar = 100 μm. (B) Proliferation curves of recovered L929 cells after cryopreservation using LMs containing gelatin compared with that of fresh cells. Value = mean ± standard deviation, n ≥ 3.](image-url)
that of fresh sample, indicating that these L929 cells could maintain the ability of proliferation. Therefore, after cryopreservation using LMs containing gelatin, the normal functionalities of post-thaw L929 cells were not affected.

3.5. Cryopreservation mechanisms of gelatin-based LMs

Based on the above results, a potential mechanism is presented in Fig. 8. Firstly, as an alternative biotechnology to traditional cell cryopreservation, the structure of LMs is obviously different and could provide additional benefits to cell cryopreservation. This was because encapsulation of cells could provide a better barrier to protect internal cells against adverse environmental conditions [65,66]. And hydrophobic coatings also inhibit ice during freezing [41,69–71]. Due to the presence of the PTFE shell, the cells in the LMs tend to distribute away from the shell, which may further reduce the damage caused by ice formation/growth (Fig. S4) [24]. Meanwhile, when used for cell cryopreservation, the droplet volumes inside LMs usually could be appropriate from 5 to 30 μL. A small volume is advantageous, not only increasing the degree of under cooling, but also facilitating the heat transfer in the cryopreservation system. In this case, it is assumed that heat transfer benefits from the surface-to-volume ratio. Secondly, during freeze-thaw process, cells are protected from damage owing to the above-mentioned gelatin CPA to prevent ice injuries by inhibiting ice formation/growth. Gelatin is a macromolecular hydrocolloid with strong hydrophilicity, which could form a gel structure at low temperature and further prevent the formation of large ice crystals [54,55]. Many studies showed that short collagen/gelatin peptides can prevent ice crystal growth in a manner comparable to antifreeze proteins under super-cooled systems [60–62]. These behaviors were because the microviscosity or steric barrier of the gel structure will hinder the diffusion of water to the detriment of bigger crystal regeneration [54,56, 57]. Meanwhile, the interaction between hydrocolloid molecules and ice crystal surface can also inhibit recrystallization [52,54]. As expected, we found that gelatin has a high IRI activity, the ice crystals formed in the gelatin solution are relatively round and smooth, rather than sharp needle-like crystals, which prevented deadly mechanical damage to cells. At a lower cooling rate, the intracellular water gradually diffused out of cells to reduce the formation of intracellular ice crystals and minimize osmotic pressure damage [16]. In addition, the gradual ice precipitation in the process is beneficial to the maintenance of the marble structure, but not causes cell solute damage. Finally, the LMs could burst instantly, making cells achieve super-fast recovering in 3–5 s when thawed, which could minimize IRI in narrow working window in the thawing process (Movie S1). Taken together, this technique is straightforward, and timesaving compared to the recovery method in traditional cryopreservation.

4. Conclusions

By investigating the cryoinjuries during the whole cryopreservation process, we demonstrated the excellent regulation of ice crystals by LMs containing gelatin in DMSO-free and FBS-free cell cryopreservation. It was found that gelatin has a high IRI activity to induce the formation of smaller size and smooth ice crystals. The superior cell survival efficiency of L929 cells was the same as that of DMSO using traditional freezing protocol. More interestingly, LMs could burst instantly, which is straightforward, and timesaving. The major cellular parameters of the post-thaw cells such as viability, adhesion, morphology, and proliferation are not changed. This work extended the platform of LM-based
biotechnological tools for cell cryopreservation, which will revolutionize the traditional cryopreservation methods.

Credit author statement

Min Liu: Conceptualization, Methodology, Investigation, Validation, Data curation, Formal analysis, Roles/Writing original draft. Changhong Chen: Methodology, Validation, Jiajun Yu: Methodology, Validation. Haitao Zhang: Methodology, Validation. Lei Liang: Methodology, Validation. Bingyan Guo: Methodology, Validation. Yuwei Qiu: Methodology, Validation. Fanglian Yao: Methodology, Conceptualization, Supervision, Funding acquisition. Hong Zhang: Methodology, Writing review & editing. Junjie Li: Conceptualization, Methodology, Writing review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmbio.2022.100477.

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