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Mammalian Cell Cryopreservation by Using Liquid Marbles

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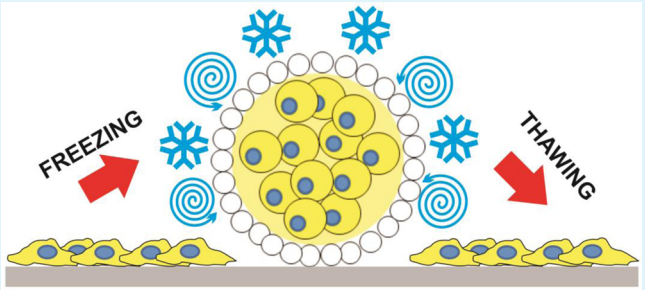
ABSTRACT: Liquid marbles (LMs) are nonsticky droplets covered by micro- or nanometrically scaled particles and obtained by simply rolling small amounts of a liquid in a very hydrophobic powder. Since pioneer work by Aussillous and Quéré, a wide palette of hydrophobic materials for the preparation of LMs, as well as potential applications, have been reported. Because of the bioinspired origin of this concept, the applicability of LMs in biomedicine is gaining increasing attention, with remarkable advances in their use as microbioreactors for blood typing, drug screening, and tumor growth, among others. Herein, we explore the novel use of LMs as a biotechnological tool for the cryopreservation of mammalian cells as an alternative to conventional methods, which typically require the use of cryopreservant agents that commonly associate with some degree of cell toxicity. Murine L929 fibroblasts, a reference cell line for cytotoxicity studies, and poly(tetrafluoroethylene), a hydrophobic polymer widely used in cardiovascular surgery, were selected for the preparation of the cell-containing LMs. Our results reveal that there is a safe range of droplet volumes and cell densities that can be successfully used to cryopreserve mammalian cell lines and recover them after thawing without significantly affecting major cellular parameters such as adhesion, morphology, viability, proliferation, and cell cycle. We envision that progress in the exploration of cell-containing LMs could also open their impact as microreactors for the miniaturization of cytotoxicity procedures of drugs and materials in which powerful tools for cell evaluation such as flow cytometry could be used because of the elevated amount of cells handled.



KEYWORDS: *cryopreservation, L929 fibroblast, liquid marble, mammalian cell, PTFE*

1. INTRODUCTION

The concept of liquid marbles (LMs), also named as "nonstick droplets" encapsulated by micro- or nanometrically scaled particles,[1] was first described by Aussillous and Quéré in 2001.[2] By rolling small amounts of a liquid (typically 1−10 mm^3) in a very hydrophobic powder (e.g., lycopodium grains of 20 μm diameter covered with fluorinated silanes), these authors pioneered the encapsulation of an aqueous liquid droplet so it behaved like a soft solid with dramatically reduced adhesion to a solid surface. Since then, a wide palette of more or less hydrophobic materials have been reported for the preparation of LMs, including poly(tetrafluoroethylene) (PTFE),[3] calcium carbonate,[4] polystyrene latexes,[5] poly(lactic acid),[6] TiO_2,[7] silica nanoparticles,[8,9] carbon black,[10] graphite powder,[11] octadecyltrimethoxysilane-modified halloysite nanotubes,[12] or graphene,[13] among many others. As a new technological system, LMs have attracted remarkable attention for application in areas as diverse as gas sensing,[14] water pollution,[15] heavy-metal ion sensing,[16] and microfluidics,[17,18] to cite a few. Additionally, interesting properties such as rapid self-recovery[19] and responsiveness to pH,[5,20] light,[7] or magnetic fields[21] have recently been described for nonsticky droplets.

As a bioinspired concept (e.g., aphids produce marbles with a secreted powdery wax), the use of LMs in biological applications is gaining more and more attention these days. Efforts in this field include work by Shen and colleagues, who first described LMs as microbiological reactors for the formation of embryonic bodies (EBs).[22] Interestingly, these authors found that the use of PTFE-based LMs allowed the preparation of more homogeneous EBs in terms of size and shape in comparison to those formed by the liquid suspension method. More recently, these authors demonstrated the feasibility of using these LMs to support spontaneous cardiac mesoderm differentiation of EBs.[23] Other advances in the area comprise their use as microbioreactors for rapid blood typing,[4] drug screening of anchorage-dependent cells,[24] tumor growth,[25] and culturing of microorganisms.[26] Readers are referred to recent reviews on the properties and applications of LMs for further details on the topic.[1,27,28]

Searching for more and more efficient methodologies for the cryopreservation of cells and tissues has been a major need for the maintenance of biological entities alive,[29] with dramatic relevance in the case of assisted reproduction.[30] Since the ground-breaking discovery of glycerol preserving cells from the injury caused by freezing,[31] an extensive collection of procedures have been introduced in the regular cell culture

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practice in an attempt to maintain healthy and growing cell cultures and to prevent contamination-, environmental-, and, more importantly, age-related risks responsible for cell loss in culture.[32,33] Interestingly, these protocols have been optimized for the cryopreservation of specially sensitive cells such as pluripotent stem cells.[34] Optimized cell freezing protocols currently applied are the result of a careful examination of the intracellular and extracellular events occurring during cell freezing (e.g., progressive slowdown of cellular metabolism, extracellular ice crystal formation linked to solute concentration, cell dehydration, and shrinkage).[35] In this scenario, survival after a freezing/thawing cycle is almost negligible in most eukaryotic cells unless cryoprotective agents are used, which typically aid dehydration and shrinkage processes in slow freezing conditions, but are unable to prevent cell damage in those based on fast freezing. Although still under debate, the mechanisms underlying the impact in cell survival of these cryopreservant agents seem to be related to alterations in the physical conditions of both the ice and extracellular solution. Among the wide palette of compounds that have proven cryoprotective effects (e.g., methylacetamide, methyl alcohol, and ethylene glycol), dimethyl sulfoxide (DMSO) and glycerol are those more conventionally used.[36] Nonetheless, most of these molecules cause cytotoxic effects above a certain dose, so their successful performance requires their use at low concentrations.

Herein, we explore the novel use of LMs as biotechnological tools for the cryopreservation of mammalian cells in the absence of any cryopreservant agents. These studies have been performed with murine L929 fibroblasts, a reference cell line for cytotoxicity studies of materials in vitro.[37] As a hydrophobic polymer for the preparation of the droplets, we selected PTFE, a material previously reported for LM formation[3,38] and widely used in the clinic (e.g., cardiovascular grafts). The effects of volume and cell density on the efficacy of the cryopreservation process were investigated in the cell-containing LMs (cc-LMs) prepared. Specifically, two commonly used freezing protocols were compared: rapid immersion in liquid nitrogen (LN) and slow cooling up to −80 °C with a standard freezing container and posterior maintenance at such a temperature in an ultracold freezer (F). The following cellular parameters were monitored over time to identify significant alterations induced by the use of a LM-based cryopreservation procedure: viability, morphology, proliferation, size, complexity, and cell cycle.

2. EXPERIMENTAL SECTION

2.1. Materials. All reagents were purchased from Sigma-Aldrich and used as received unless otherwise indicated. Murine L929 fibroblasts were gifted by Dr. M. Teresa Portolés. Cell culture media and supplements were purchased from Lonza and used following manufacturer's indications.

2.2. Cell Culture. Murine L929 fibroblasts were cultured in regular polystyrene culture flasks (75 cm^2) with Dulbecco's Modified Eagle Medium (DMEM) supplemented with fetal bovine serum (FBS; 10%), streptomycin (100 UI mL^{-1}), penicillin (100 UI mL^{-1}), and L-glutamine (1 mM) (culture media). Cultures were maintained at 37 °C in a sterile incubator under a CO_2 atmosphere (5%) until confluence was reached prior to cryopreservation studies. The morphology of the cultures was regularly monitored by using an inverted Axiovert CFL-40 optical microscope coupled with an Axiocam ICC-1 digital camera (Zeiss).

2.3. Preparation and Cryopreservation of cc-LMs. Confluent cultures of murine L929 fibroblasts were incubated with a trypsin/ethylenediaminetetraacetic acid (EDTA) solution for 5 min at 37 °C. Once cells detached from the flasks, as evidenced by observation under the optical microscope, an equal volume of FBS was added to neutralize trypsin and the suspension immediately centrifuged at 300*g*. The obtained pellet was then resuspended in a small volume of FBS (typically 100 μL for the pellet of two confluent 75 cm^2 flasks). At this point, the cell density was measured by using a Neubauer chamber and the viability tested with trypan blue (typically, 98 ± 2%). Then the volume of this highly concentrated cell suspension (stock) was adjusted with more FBS to permit the preparation of the desired cc-LMs in terms of both cell density and volume. Briefly, PTFE powder (from Sigma-Aldrich, product number 430935, particle size <1 μm) was first placed on top of a piece of Parafilm "M" properly fixed to the working area inside the hood. Afterward, drops of different volumes (5, 10, and 30 μL) and cell concentrations (0.5×10^5, 1×10^5, and 2×10^5 cells μL^{-1}) were pipetted from the cell stock suspension and placed on top of the PTFE powder bed. By using a spoon-shaped spatula, PTFE particles were carefully approached to the cell drop, which was forced to roll until its surface was completely covered by the polymer powder. These cc-LMs were then immediately introduced into regular cryotubes and frozen under two different conditions: (1) fast freezing by immersion in LN or (2) slow freezing in a ultracold freezer (−80 °C) by using a standard freezing container (Nalgene Mr Frosty, Sigma-Aldrich). After 15 days, the cryotubes were thawed and the cells contained in the LMs immediately released by rapid contact of the cc-LMs with warm culture media in the polystyrene plate. After the cell viability in these suspensions was tested, the thawed cells were allowed to grow in regular polystyrene six-well culture plates for different times. To simplify, the cc-LMs were abbreviated with three terms referring to the (1) drop volume (5, 10, or 30), (2) cell density in thousands (50, 100, or 200), and (3) freezing procedure used (LN or F). Control samples included cell suspensions (10^6 cells in 1 mL) with or without DMSO in either LN (DMSO-LN and LN, respectively) or F (DMSO-F and F, respectively) and concentrated cell suspensions (0.5×10^6 cells in 10 μL) without LM structure or DMSO in both freezing conditions (CS-10−50-LN and CS-10−50-F). A set of cc-LMs of 10 and 20 μL in volume were additionally prepared to investigate the lifetime of this particular type of LM. Briefly, cc-LMs were placed on top of a piece of Parafilm "M" and photographed every 5 min for a total period of 1 h by using an Olympus MVX10 magnifying glass coupled with a digital camera (Olympus DP71). The images acquired were then calibrated and processed with *ImageJ* software to estimate the reduction in the cc-LM size (diameter) over time (expressed as μm min^{-1}).

2.4. Cell Viability. The viability in cell suspensions was evaluated by using labeling with trypan blue. Because viable cells are able to exclude this vital colorant from the cytoplasm, only cells either dead or damaged at their membranes appear stained in blue. The viability in cell cultures was analyzed with a Live/Dead Viability kit (Invitrogen) following the manufacturer's instructions. After staining, samples were visualized by using a Leica SP5 confocal laser scanning microscope. The fluorescence of both probes (i.e., calcein and ethidium homodimer-1, EthD-1) was excited by an argon laser tuning to 488 nm. After excitation, emitted fluorescence was separated by using a triple dichroic filter 488/561/633 and measured at 505−570 nm for green fluorescence (calcein) and 630−750 nm for red fluorescence (EthD-1).

2.5. Flow Cytometry Studies. Cell suspensions were obtained by incubation with trypsin/EDTA and posterior fixation with ethanol (70% in distilled water) at −20 °C for 5 min. After centrifugation, cell pellets were washed in phosphate buffer saline three times and stained with propidium iodide (1 mg mL^{-1} in distilled water containing RNase) for 30 min at 37 °C in darkness. After staining, cell cycles were analyzed by using a Cytomic FC500 flow cytometer (Beckman Coulter). Additionally, the light-scattering properties of these cells were examined by measuring the forward angle (FSC) and the 90° side angle (SSC) light scatters as indicators of the cell size and complexity, respectively.[39] In each case, at least 10000 events were analyzed for statistical significance.

2.6. Cell Proliferation Studies. Cell cultures at different time points (1, 2, 4, and 7 days) were incubated with a trypsin/EDTA solution for 5 min to allow cell detachment. The total number of cells

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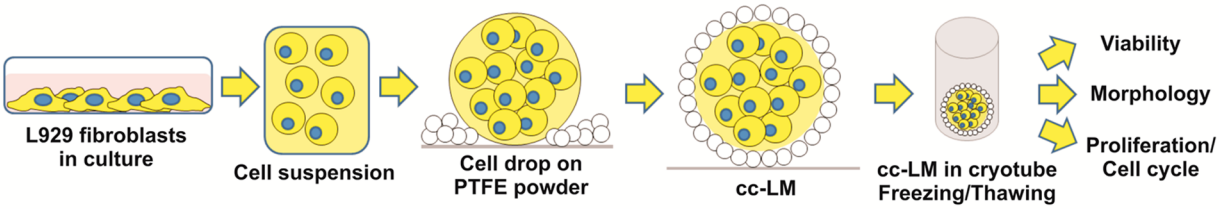
Scheme 1. Schematic of the Procedure Followed for the Preparation and Testing of cc-LMs



in each case was then obtained by counting the suspensions in a Neubauer chamber under an inverted Axiovert CFL-40 optical microscope.

2.7. Statistics. Statistical analysis was performed using the Statistical Package for the *Social Sciences* software, version 17.0. Comparisons among groups were done by analysis of variance (ANOVA), followed by either Scheffé or Games-Howell post hoc tests depending on the variance homogeneity (previously evaluated by the Levene test). In all comparisons, $p < 0.05$ was considered statistically significant.

3. RESULTS AND DISCUSSION

Despite extensive studies in other areas, the use of LMs in biological applications has been very limited to date. On the basis of previous findings demonstrating LMs as compatible systems for mammalian cells,[23] we herein present the use of PTFE-based LMs as novel and alternative biotechnological tools for mammalian cell cryopreservation. Importantly, this approach lacks the use of any cryoprotective agent, thus preventing the above-mentioned cytotoxicity problems typically associated with the use of high concentrations of these agents (see the Introduction).[36] We selected PTFE as the polymer of choice for the fabrication of cc-LMs because of both its hydrophobic and noncytotoxic properties, which enabled the formation of stable liquid droplets containing viable cells, as previously reported by others.[22] Scheme 1 shows the general procedure followed for the preparation of cc-LMs and the different stages of their posterior evaluation. Briefly, each stable cc-LM was prepared by simply rolling a drop of a highly concentrated cell suspension on a PTFE powder bed (Figure 1A). By careful rolling of the droplet, the polymer particles were progressively distributed on the surface of any drop until the complete liquid−air interface was coated. The selection of FBS as the medium contained in a cc-LM was based on the well-known fact that the serum helps cell survival during freezing.[40] Because previous work by Sarvi et al. revealed the droplet volume and cell seeding density as critical factors for the use of LMs as biological microreactors,[22] we herein tested a diverse range of both parameters for the preparation of cc-LMs (Table 1). Other aspects such as the surface free energy and hydrophilicity of the encapsulating microparticles have also been identified decisive in determining the effective surface tension and lifetime of a LM floating on water.[11] In this sense, work by Arbatan et al. reported the effective surface tension of PTFE-based LMs being lower than that of water, within the range of 57.6−70.2 mN m^{-1}.[3] The stability of the so-prepared cc-LMs was preserved not only while moved around on a hydrophobic substrate but also when placed inside a cryotube for preservation (Figure 1B) and even after inoculation of biological colorants such a trypan blue or alizarin red (Figure 1C). Specific lifetime studies of cc-LMs demonstrated a significant reduction in size (diameter) over time at standard laboratory conditions (i.e., 21 ± 2 °C, 40% of relative humidity). Particularly, cc-LMs of 10 μL in volume diminished their diameter at an average rate of 20 ± 7 μm min^{-1} for the first hour (Figure 1D), while those of 20 μL did at 15 ± 5 μm min^{-1}. Although key for other applications of LMs, we do not anticipate these findings regarding the cc-LM lifetime to represent a limitation for their practical use in cell freezing procedures because handling live cells usually necessitates working efficiently but rapidly to minimize cell damage. In this sense, a typical sequence of cc-LM preparation and handling for freezing procedures requires less than 5 min. Cryotubes containing the so-prepared cc-LMs were then frozen through two conventional freezing procedures: (1) direct immersion in LN or (2) slow freezing up to −80 °C by using a cell freezing container filled with isopropyl alcohol and maintenance in an ultracold freezer, both standard protocols in the common cell culture practice.

The resulting cc-LMs were maintained in frozen conditions for 15 days. Subsequently, samples were thawed, and the cell

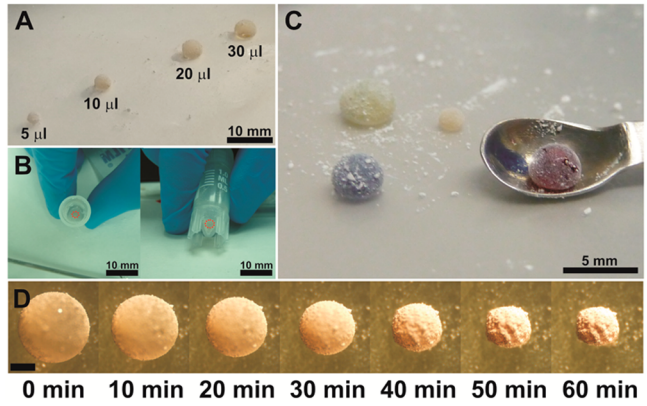


Figure 1. (A) Macroscopic aspect of cc-LMs with different volumes. (B) Stability of a representative cc-LM inside a cryotube from the top (left) or side (right). The dashed red line indicates the contour of the cc-LM. (C) Preservation of the cc-LM integrity after inoculation of the cell colorants (e.g., trypan blue and alizarin red). (D) Representative series of images illustrating the progressive shrinking of a cc-LM (10 μL in volume) over 1 h at room temperature. The scale bar in this sequence represents 1 mm.

Table 1. Cell Density and Volume of the Different cc-LMs Tested

	volume (μL)		
	5	10	30
cell density (cells μL^{-1})	50000	50000	50000
	100000	100000	
	200000	200000	
total number of cells per drop	250000	500000	1500000
	500000	1000000	
	1000000	2000000	

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viability was tested in suspension by trypan blue staining. In all cases, the integrity of the cc-LMs was confirmed to be preserved after both freezing and thawing. As observed in Figure 2, only cc-LMs with the smallest volumes and cell

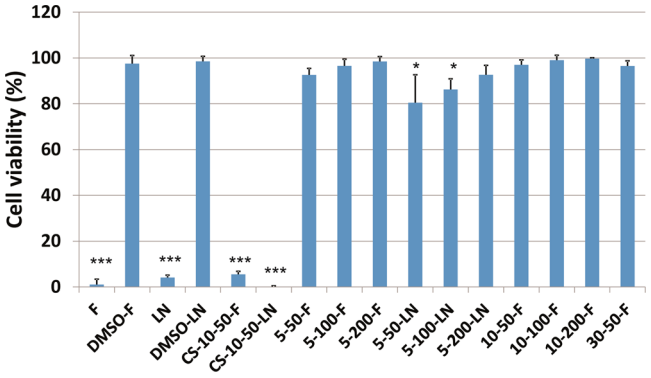

Figure 2. Cell viability after a freezing/thawing cycle in cc-LMs. Control samples are plotted for comparison (F, freezing at −80 °C; DMSO-F, freezing at −80 °C with 10% DMSO; LN, freezing in LN; DMSO-LN, freezing in LN with 10% DMSO; CS-10−50-F, concentrated cell suspension containing 0.5 × 10^6 cells in 10 μL at −80 °C; CS-10−50-LN, concentrated cell suspension of 0.5 × 10^6 cells in 10 μL frozen in LN). Statistics: (***) $p < 0.005$ and (*) $p < 0.05$.

densities and frozen in LN (i.e., 5−50-LN and 5−100-LN) showed a slight but significant reduction in the cell viability. In the rest of cases, cell suspensions displayed high percentages of viable cells, similar to those found in control samples frozen under standard conditions (DMSO-F and DMSO-LN), thus encouraging the use as cryopreservation microdevices of those LMs containing any cell density from 0.5 × 10^5 to 2 × 10^5 cells μL^{-1} and 10−30 μL in volume. Cells from all of the conditions were then cultured in regular polystyrene plates, and their viability was tested in culture for up to 96 h (Figure 3). No significant differences were observed in any conditions with respect to control cells, thus indicating that the alterations detected right after thawing were only transient and reversed by subsequent culture. The morphology of these cultures was also

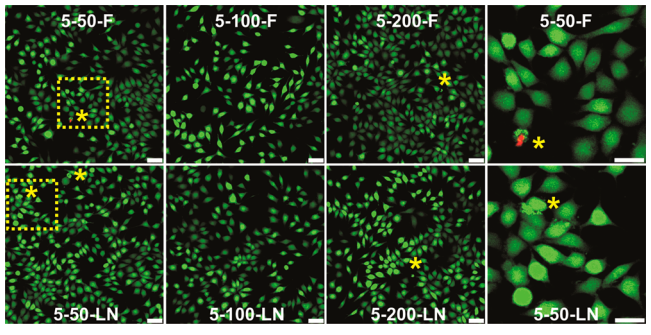

Figure 3. Cell viability of L929 fibroblasts in culture after thawing from different cc-LMs. Representative images of cultures at 24 h are shown, except for cc-LMs 5−50-F and 5−50-LN in which 96 h of culture was selected to reach similar levels of confluence. Zoomed images on the fourth column correspond to regions delimited by yellow dashed lines in 5−50-F and 5−50-LN cc-LMs to illustrate details of both healthy and apoptotic cells in these cultures. Yellow asterisks indicate cells undergoing apoptosis. Scale bars represent 50 μm in all images, except for zoom-in ones (20 μm).

monitored over time both after thawing (subculture 1) and after the first subsequent passage (subculture 2). As illustrated in Figure 4 and according to findings in viability studies, only

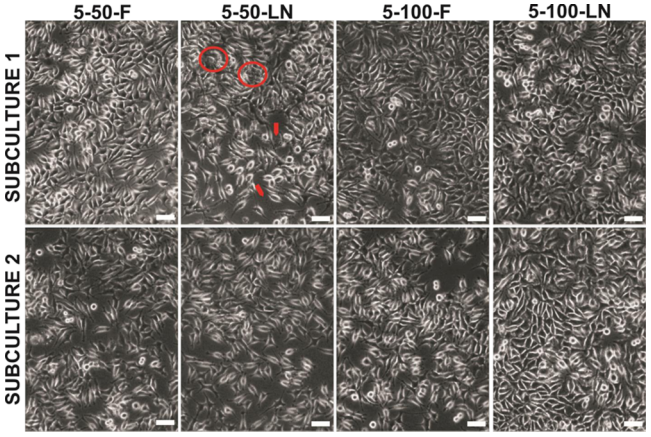

Figure 4. Morphological studies of L929 fibroblasts in culture after thawing from cc-LMs (subculture 1) and after an additional passage (subculture 2). Representative images of cultures at 48 h are shown. Red circles and arrows indicate cellular debris indicative of cell damage. Scale bars represent 10 μm in all images.

cultures from cc-LMs containing the lowest density and volume and frozen in LN (i.e., 5−50-LN) showed some cell debris in subculture 1, indicative of a certain degree of cell damage at the early time points after thawing. We additionally evaluated whether these slight morphological alterations observed in 5−50-LN cc-LMs correlated with changes in the cell size or complexity by using flow cytometry.[39] As observed in Figure 5,

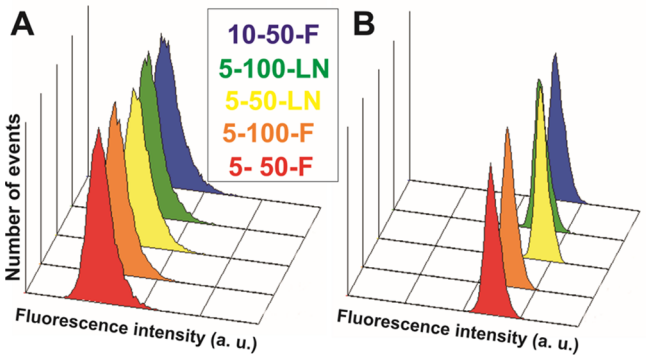

Figure 5. Flow cytometry studies with L929 fibroblasts cultured from cc-LMs submitted to a freezing/thawing cycle. Histograms show FSC (A) and SSC (B) properties, indicative of the cell size and internal complexity, respectively.

the cell population obtained from thawing 5−50-LN cc-LMs experienced a light displacement toward higher values of SSC, i.e., higher intracellular complexity, while preserving their size (i.e., similar FSC values). Because the cell size was preserved, the increase in the cell complexity found could not be ascribed to a reduction in the cell volume caused by dehydration and shrinkage during freezing. Moreover, these changes might reflect some minor alterations in the intracellular machinery or the accumulation of some cytoplasmic components that only further molecular studies would help to identify.

We next explored the effect of the freezing/thawing process on the ability of L929 fibroblasts to properly proliferate in

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culture by evaluating the growth kinetics up to 7 days (Figure 6). As can be observed, fibroblasts in all cultures significantly

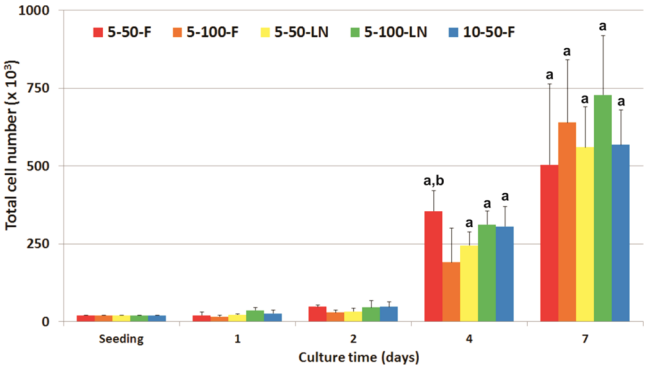

Figure 6. Cell proliferation studies of L929 fibroblasts cultured after a freezing/thawing cycle from cc-LMs. Statistically significant differences among time points for a particular type of cc-LM are indicated as "a" and those among treatments for a particular time point as "b". In all comparisons, the significance level was $p < 0.05$.

proliferated over time, as expected from healthy cells in subconfluence culture conditions. Equal to the rest of cellular parameters previously tested, similar trends were found in all cc-LMs with respect to control cells except for 5−50-LN, again confirming the applicability of cc-LMs as efficient cryopreservation vehicles. In the particular case of 5−50-LN, the slight cell damage identified at the early time points after thawing likely promoted stimulation of cell proliferation at later time points in order to compensate for cell loss. Finally, in an attempt to corroborate that the use of cc-LMs as novel freezing platforms did not cause any further deleterious cell effects, we studied cell cycle profiles in the different cell populations derived from thawed cc-LMs (Figure 7). As expected for cell cultures at a subconfluence stage, those cell cycle fractions attributed to the G_0 (i.e., resting phase assigned to cells that are not dividing and are therefore temporarily out of the cell cycle) and G_1 (i.e., interphase, for cells that are preparing cell division and start synthesizing proteins) phases were in the range of 80−85% in all conditions tested. The remaining cells (15−20%) corresponded to those in the S (i.e., synthesis) and G_2/M (i.e., premitotic and mitosis, respectively) phases to guarantee culture turnover and colonization of the entire culture substrate until contact inhibition is reached. Importantly, negligible sub-G_0/G_1 fractions (e.g., entities with a smaller fluorescence intensity than that assigned to cells in the G_0/G_1 phases) were detected in any cases, thus evidencing no significant activation of apoptotic mechanisms.

The proof-of-concept herein presented focused on the exploration of cc-LMs as novel and alternative biotechnological platforms for mammalian cell cryopreservation. According to previous work by others reporting the relevance of the droplet volume and cell density in the behavior of LMs,[22] we found that droplet volumes from 5 to 30 μL and cell densities of (0.5−2) $\times$ 10^5 cells μL^{-1} are in a safe range within which cell cryopreservation matches the performance of standard freezing procedures (i.e., DMSO-F and DMSO-LN) and significantly exceeds direct freezing of a cell suspension without cryopreservant agents (viability values of 1.05 $\pm$ 2.35 and 4.13 $\pm$ 1.03% in F and LN samples, respectively; Figure 2). Moreover, the conformation of the cell-containing sphere in the shape of a LM is revealed to be critical as small droplets containing high cell densities, but in the absence of either cryopreservants or a LM structure, were unable to preserve the cell viability after cryopreservation (i.e., CS-10−50-F and CS-10−50-LN). Nonetheless, on the basis of the results obtained, the combination of smaller LM sizes with lower cell densities was more likely to cause cell damage under more harsh freezing conditions such as rapid cooling by direct immersion in LN (i.e., 5−50-LN). Despite the fact that one could consider volumes from 10 to 30 μL as ideal to work with, the constraint on the use of cc-LMs with larger volumes comes from the difficulties derived from the mechanical stability of the drops (e.g., a larger size typically correlates with faster mobility). On the basis of our findings, we suggest sizes of around 10 μL ideal in terms of both droplet handling/stability and cellular performance after a freezing/thawing cycle. Besides the size, the preparation of more concentrated cc-LMs will mainly be limited by the availability of cells and the volume restrictions derived from achieving the initial stock cell suspension. It is worth noting that the undesired liquid evaporation that typically occurs in LM and may limit their practical use in certain applications[41,42] is not a critical issue in our case because evaporation under frozen conditions is negligible.

4. CONCLUSIONS

We herein described the pioneering use of PTFE-based LMs as suitable and alternative biotechnological tools for mammalian cell cryopreservation without the use of any cryoprotective

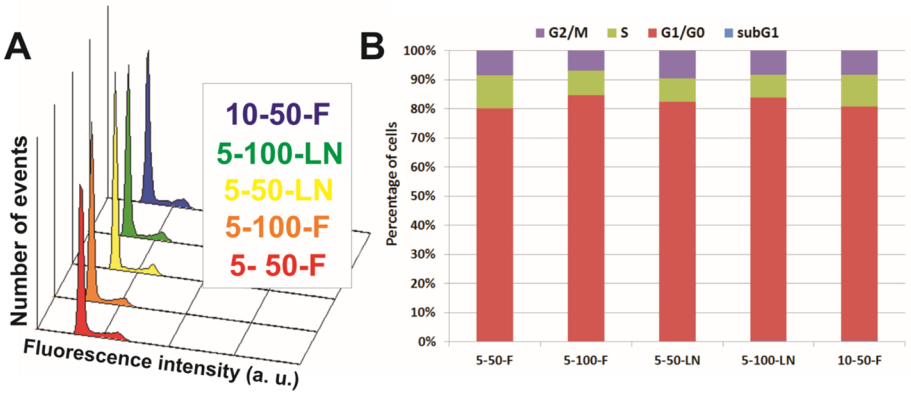

Figure 7. Cell cycle studies of L929 fibroblasts cultured after a freezing/thawing cycle from cc-LMs: (A) cell cycle profiles at 96 h by flow cytometry; (B) percentage of cells on each phase of the cycle.

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agents. Our results revealed that there is a safe range of droplet volumes and cell densities that can be successfully used to cryopreserve mammalian cell lines and recover them after thawing without significantly affecting the major cellular parameters such as adhesion, morphology, viability, proliferation, and cell cycle. Progress in the exploration of cc-LMs might open their impact as biotechnological tools not only for cryopreservation but also for miniaturization of the cytotoxicity procedures of drugs and materials. In this sense, cc-LMs behave as versatile bioreactors of a few millimeters in dimensions that would permit screening of the behavior of a wide range of cell types in contact with almost any compound able to be injected and dispersed in an aqueous droplet. Interestingly, the high number of cells typically handled in cc-LMs would easily allow the use of powerful tools for cell evaluation such as flow cytometry.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

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REFERENCES

(1) Bormashenko, E. New Insights into Liquid Marbles. *Soft Matter* **2012**, *8*, 11018−11021.
(2) Aussillous, P.; Quéré, D. Liquid Marbles. *Nature* **2001**, *411*, 924−927.
(3) Arbatan, T.; Shen, W. Measurement of the Surface Tension of Liquid Marbles. *Langmuir* **2011**, *27*, 12923−12929.
(4) Arbatan, T.; Li, L.; Tian, J.; Shen, W. Liquid Marbles as Micro-Bioreactors for Rapid Blood Typing. *Adv. Healthcare Mater.* **2012**, *1*, 80−83.
(5) Dupin, D.; Thompson, K. L.; Armes, S. P. Preparation of Stimulus-Responsive Liquid Marbles Using a Polyacid-Stabilised Polystyrene Latex. *Soft Matter* **2011**, *7*, 6797−6800.
(6) Matsukuma, D.; Watanabe, H.; Minn, M.; Fujimoto, A.; Shinohara, T.; Jinnai, H.; Takahara, A. Preparation of Poly(lactic-acid)−Particle Stabilized Liquid Marble and the Improvement of its Stability by Uniform Shell Formation Through Solvent Vapor Exposure. *RSC Adv.* **2013**, *3*, 7862−7866.
(7) Tan, T. T. Y.; Ahsan, A.; Reithofer, M. R.; Tay, S. W.; Tan, S. Y.; Hor, T. S. A.; Chin, J. M.; Chew, B. K. J.; Wang, X. Photoresponsive Liquid Marbles and Dry Water. *Langmuir* **2014**, *30*, 3448−3454.
(8) Bhosale, P. S.; Panchagnula, M. V. Sweating Liquid Micro-Marbles: Dropwise Condensation on Hydrophobic Nanoparticulate Materials. *Langmuir* **2012**, *28*, 14860−14866.
(9) Zang, D.; Chen, Z.; Zhang, Y.; Lin, K.; Geng, X.; Binks, B. P. Effect of Particle Hydrophobicity on the Properties of Liquid Water Marbles. *Soft Matter* **2013**, *9*, 5067−5073.
(10) Dandan, M.; Yildirim Erbil, H. Evaporation Rate of Graphite Liquid Marbles: Comparison with Water Droplets. *Langmuir* **2009**, *25*, 8362−8367.
(11) Cengiz, U.; Yildirim Erbil, H. The Lifetime of Floating Liquid Marbles: The Influence of Particle Size and Effective Surface Tension. *Soft Matter* **2013**, *9*, 8980−8991.
(12) Wu, H.; Watanabe, H.; Ma, W.; Fujimoto, A.; Higuchi, T.; Uesugi, K.; Takeuchi, A.; Suzuki, Y.; Jinnai, H.; Takahara, A. Robust Liquid Marbles Stabilized with Surface-Modified Halloysite Nanotubes. *Langmuir* **2013**, *29*, 14971−14975.
(13) Hu, H.; Allan, C. C. K.; Li, J.; Kong, Y.; Wang, X.; Xin, J. H.; Hu, H. Multifunctional Organically Modified Graphene with Super-Hydrophobicity. *Nano Res.* **2014**, *7*, 418−433.
(14) Tian, J.; Arbatan, T.; Li, X.; Shen, W. Liquid Marbles for Gas Sensing. *Chem. Commun.* **2010**, *46*, 4734−4736.
(15) Bormashenko, E.; Musin, A. Revealing of Water Surface Pollution with Liquid Marbles. *Appl. Surf. Sci.* **2009**, *255*, 6429−6431.
(16) Sivan, V.; Tang, S.-Y.; O'Mullane, A. P.; Petersen, P.; Eshtiaghi, N.; Kalantar-Zadeh, K.; Mitchell, A. Liquid Metal Marbles. *Adv. Funct. Mater.* **2013**, *23*, 144−152.
(17) Zhao, Y.; Fang, J.; Wang, H.; Wang, X.; Lin, T. Magnetic Liquid Marbles: Manipulation of Liquid Droplets Using Highly Hydrophobic Fe_3O_4 Nanoparticles. *Adv. Mater.* **2010**, *22*, 707−710.
(18) Bormashenko, E.; Pogreb, R.; Bormashenko, Y.; Musin, A.; Stein, T. New Investigations on Ferrofluidics: Ferrofluidic Marbles and Magnetic-Field-Driven Drops on Super-Hydrophobic Surfaces. *Langmuir* **2008**, *24*, 12119−12122.
(19) Hu, Y.; Jiang, H.; Liu, J.; Li, Y.; Hou, X.; Li, C. Highly Compressible Magnetic Liquid Marbles Assembled from Hydrophobic Magnetic Chain-Like Nanoparticles. *RSC Adv.* **2014**, *4*, 3162−3164.
(20) Dupin, D.; Armes, S. P.; Fujii, S. Stimulus-Responsive Liquid Marbles. *J. Am. Chem. Soc.* **2009**, *131*, 5386−5387.
(21) Zhang, L.; Cha, D.; Wang, P. Remotely Controllable Liquid Marbles. *Adv. Mater.* **2012**, *24*, 4756−4760.
(22) Sarvi, F.; Arbatan, T.; Chan, P. P. Y.; Shen, W. A Novel Technique for the Formation of Embryonic Bodies Inside Liquid Marbles. *RSC Adv.* **2013**, *3*, 14501−14508.
(23) Sarvi, F.; Jain, K.; Arbatan, T.; Verma, P. J.; Hourigan, K.; Thompson, M. C.; Shen, W.; Chan, P. P. Y. Cardiogenesis of Embryonic Stem Cells with Liquid Marble Micro-Bioreactor. *Adv. Healthcare Mater.* **2014**, DOI: 10.1002/adhm.201400138.
(24) Oliveira, N. M.; Correia, C. R.; Reis, R. L.; Mano, J. F. Liquid Marbles for High-Throughput Biological Screening of Anchorage-Dependent Cells. *Adv. Healthcare Mater.* **2015**, *4*, 264−270.
(25) Arbatan, T.; Al-Abboodi, A.; Sarvi, F.; Chan, P. P. Y.; Shen, W. Tumor Inside a Pearl Drop. *Adv. Healthcare Mater.* **2012**, *1*, 467−469.
(26) Tian, J. F.; Fu, N.; Chen, X. D.; Shen, W. Respirable Liquid Marble for the Cultivation of Microorganisms. *Colloids Surf., B* **2013**, *106*, 187−190.

DOI: 10.1021/acsami.5b00072
ACS Appl. Mater. Interfaces 2015, 7, 3854−3860

(27) Bormashenko, E. Liquid Marbles: Properties and Applications. *Curr. Opin. Colloid Interface Sci.* **2011**, *16*, 266−271.
(28) McHale, G.; Newton, M. I. Liquid Marbles: Principles and Applications. *Soft Matter* **2011**, *7*, 5473−5481.
(29) Karlsson, J. O. M.; Toner, M. Long-Term Storage of Tissues by Cryopreservation: Critical Issues. *Biomaterials* **1996**, *17*, 243−256.
(30) Rall, W. F.; Fahy, G. M. Ice-Free Cryopreservation of Mouse Embryos at −196 °C by Vitrification. *Nature* **1985**, *313*, 573−575.
(31) Polge, C.; Smith, A. U.; Parkes, A. S. Revival of Spermatozoa after Vitrification and Dehydration at Low Temperatures. *Nature* **1949**, *164*, 666−666.
(32) Hay, R. J. Preservation of Cell Culture Stocks in Liquid Nitrogen. *Tissue Cult. Assoc. Man.* **1978**, *4*, 787−790.
(33) Schroy, C. B.; Todd, P. A Simple Method for Freezing and Thawing Cultured Cells. *Tissue Cult. Assoc. Man.* **1976**, *2*, 309−310.
(34) Cohen, R. I.; Thompson, M. L.; Schryver, B.; Ehrhardt, R. O. Standarized Cryopreservation of Pluripotent Stem Cells. *Curr. Protoc. Stem Cell Biol.* **2014**, *28*, 1C.14.
(35) Mazur, P. Freezing of Living cells: Mechanisms and Implications. *Am. J. Physiol.* **1984**, *247* (3 Pt 1), C125−142.
(36) Klebe, R. J.; Mancuso, M. G. Identification of New Cryoprotective Agents for Cultured Mammalian Cells. *In Vitro* **1983**, *19*, 167−170.
(37) Serrano, M. C.; Pagani, R.; Vallet-Regí, M.; Peña, J.; Rámila, A.; Izquierdo, I.; Portolés, M. T. In Vitro Biocompatibility Assessment of Poly(ε-caprolactone) Films Using L929 Mouse Fibroblasts. *Biomaterials* **2004**, *25*, 5603−5611.
(38) Gao, L.; McCarthy, T. J. Ionic Liquid Marbles. *Langmuir* **2007**, *23*, 10445−10447.
(39) Serrano, M. C.; Pagani, R.; Peña, J.; Portolés, M. T. Transitory Oxidative Stress in L929 Fibroblasts Cultured on Poly(ε-caprolactone). *Biomaterials* **2005**, *26*, 5827−5834.
(40) Ryan, J. Cryopreservation of Animal Cells. In *Cell Culture Manual*; Sigma-Aldrich: St. Louis, MO, 2011−2014; pp 314−319. Online link: http://www.sigmaaldrich.com/life-science/cell-culture/learning-center/cell-culture-manual.html. Last retrieved: 01/19/2015.
(41) McHale, G.; Herbertson, D. L.; Elliot, S. J.; Shirtcliffe, N. J.; Newton, M. I. Electrowetting of Non-Wetting Liquids and Liquid Marbles. *Langmuir* **2007**, *23*, 918−924.
(42) Laborie, B.; Lachausée, F.; Lorenceau, E.; Rouyer, F. How Coatings with Hydrophobic Particles May Change the Drying of Water Droplets: Incompressible Surface Versus Porous Media Effects. *Soft Matter* **2013**, *9*, 4822−4830.

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