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Spontaneous Packaging and Hypothermic Storage of Mammalian Cells with a Cell-Membrane-Mimetic Polymer Hydrogel in a Microchip

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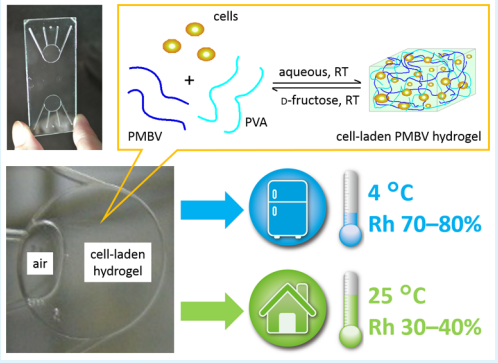
Supporting Information

ABSTRACT: Currently, continuous culture/passage and cryopreservation are two major, well-established methods to provide cultivated mammalian cells for experiments in laboratories. Due to the lack of flexibility, however, both laboratory-oriented methods are unable to meet the need for rapidly growing cell-based applications, which require cell supply in a variety of occasions outside of laboratories. Herein, we report spontaneous packaging and hypothermic storage of mammalian cells under refrigerated (4 °C) and ambient conditions (25 °C) using a cell-membrane-mimetic methacryloyloxyethyl phosphorylcholine (MPC) polymer hydrogel incorporated within a glass microchip. Its capability for hypothermic storage of cells was comparatively evaluated over 16 days. The results reveal that the cytocompatible MPC polymer hydrogel, in combination with the microchip structure, enabled hypothermic storage of cells with quite high viability, high intracellular esterase activity, maintained cell membrane integrity, and small morphological change for more than 1 week at 4 °C and at least 4 days at 25 °C. Furthermore, the stored cells could be released from the hydrogel and exhibited the ability to adhere to a surface and achieve confluence under standard cell culture conditions. Both hypothermic storage conditions are ordinary flexible conditions which can be easily established in places outside of laboratories. Therefore, cell packaging and storage using the hydrogel incorporated within the microchip would be a promising miniature and portable solution for flexible supply and delivery of small amounts of cells from bench to bedside.



KEYWORDS: *hypothermic storage, cell, 2-methacryloyloxyethyl phosphorylcholine (MPC), hydrogel, microchip*

INTRODUCTION

Currently, there are two major, well-established methods to supply cultivated mammalian cells for cell experiments. One is continuous culture and passage in culture media under a stringently controlled environment, typically, 37 °C, 5% CO_2, and 95% relative humidity. Cell culture incubators with highly sophisticated control technology are used to create such in vivo-like environment for maintaining cells. Another is freezing, namely, cryopreservation, which allows storage of cells for extended times in subzero temperatures using specialized equipment and cryoprotectants. While cryopreservation at −196 °C in liquid nitrogen is the most common method to store cells for research, cryopreservation at −80 °C or −20 °C is commonly used for short-term preservation or short-distance delivery of cells. However, these less flexible laboratory-oriented methods have been unable to meet the rapidly growing needs in cell-based assays,[1−3] cell therapies,[4−6] and regenerative medicine,[7−9] because the encouraging advances in these fields significantly expand the potential use of cells in a variety of occasions and places, where the sophisticated equipment and highly trained personnel for complicated cell culture and cryopreservation operations may not always be available. For example, these applications may be performed in hospitals, clinics, and even at home in the near future, rather than in laboratories, by using small amounts of cells delivered from certain manufacturing sites located in different cities or countries. Therefore, the establishment of simple, miniature, and portable methods for package and storage of mammalian cells under ordinary flexible conditions is urgently desired, but is greatly challenging.

Cell storage in the range of 1 to 35 °C, or so-called hypothermic storage, can provide significant flexibility for the supply of cells, but it is still a new field. Especially, hypothermic storage at ambient temperatures or refrigerated temperatures

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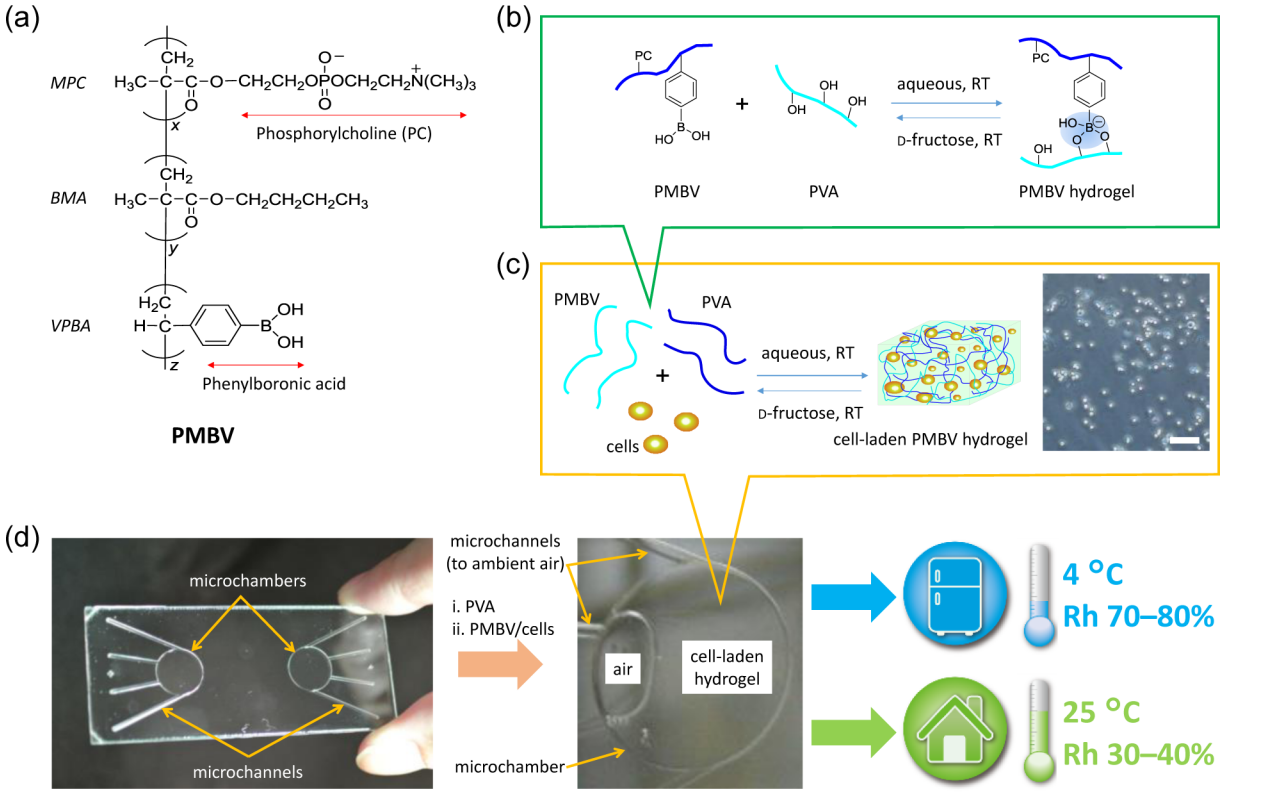

Figure 1. Spontaneous packaging and hypothermic storage (4 °C in a refrigerator and 25 °C in ambient air) of cells in the (a) PMBV hydrogel formed via (b, c) a cross-linking reaction between PMBV and PVA, within (d) a glass microchip. The inset in panel (c) shows a phase-contrast microscope image of L929 cells after packaging in the PMBV hydrogel within the microchip. Scale bar is 100 μm.

(2–8 °C) in widely available refrigerators holds great potential, but is poorly developed. A few groups have studied hypothermic storage of mammalian cells at 4 or 25 °C in standard cell culture media with addition of other ingredients.[10−14] Such liquid base hypothermic storage methods generally allow for the maintenance of cells for several hours to 1 or 2 days and hence are very useful in some emergency occasions, for example, during power failure when control of CO_2 and humidity of cultures is impossible. However, the short storage period and low cell viability are critical problems among various possible impediments to the widespread use of these methods.

In contrast to the liquid base hypothermic storage, in this study, a hydrogel was used to be a hypothermic storage material for the first time (Figure 1). The hydrogel is a 2-methacryloyloxyethyl phosphorylcholine (MPC) polymer-based hydrogel (Figure 1a,b). MPC polymers are kinds of phospholipid polymers and famous for their excellent biocompatibility and cytocompatibility owing to their capability to form cell-membrane-mimetic surfaces and structures from the bulk to the micro/nano scales.[15−21] The MPC polymer to form the hydrogel used in this study, referred to as PMBV (Figure 1a), is an MPC copolymer synthesized by a conventional radical polymerization of MPC, *n*-butyl methacrylate (BMA), and *p*-vinylphenylboronic acid (VPBA). Because PMBV is water-soluble, it dissolves in various aqueous cell culture media, such as the widely used Dulbecco's modified Eagle's medium (DMEM). PMBV spontaneously forms the hydrogel (referred to as PMBV hydrogel) with a small amount of high-molecular-weight poly(vinyl alcohol) (PVA) in aqueous solutions, via cross-linking between the boronic acid groups of PMBV and the hydroxyl groups of PVA (Figure 1b). When adding a low-molecular-weight sugar solution such as D-fructose and D-galactose, the formed hydrogel gradually dissociates to a solution state (i.e., dissolution), because the network of cross-linked polymer chains can be unlocked by the reaction between the boronic acid groups of PMBV and the hydroxyl groups of the sugar molecules (Figure 1b).[22] The gelation and dissolution can also take place in cell culture media. Accordingly, the PMBV hydrogel has shown capability to reversibly encapsulate/release a variety of mammalian cells (Figure 1c).[23] Other basic characterization of the PMBV hydrogel has been reported previously by us.[23] Recently, we have reported the capability of the PMBV hydrogel in bulk as well as in a microscale form to sustain cells under the standard cell culture conditions (i.e., 37 °C, 5% CO_2, and 95% relative humidity).[24,25]

In this study, the intriguing performance of the PMBV hydrogel in the hypothermic storage of cells in a microchip (Figure 1d) was reported. The PMBV hydrogel incorporated within the microchip enabled packaging and storage of mammalian cells at both refrigerated temperature (4 °C) and ambient temperature (25 °C) for several days to several weeks. The hypothermic storage method using the PMBV within the microchip offers a simple, flexible, miniature, and portable solution to cell packaging and storage for quite long extended times, and will be useful in the development of various cell-based applications on chip from bench to bedside.

MATERIALS AND METHODS

PMBV Synthesis. PMBV (Figure 1a) was synthesized by a conventional radical polymerization of MPC, BMA, and VPBA, according to a process previously reported.[23,25] First, MPC (Nippon Oil and Fats, Tokyo, Japan), BMA (Kanto Chemicals, Tokyo, Japan), VPBA (Tokyo Chemical Industry, Tokyo, Japan), and α,α'-azobis-

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(isobutyronitrile) (AIBN; as the initiator; Kanto Chemicals) with desired amounts were dissolved in ethanol in a glass ampule. After sealing the ampule, the copolymerization was performed at 60 °C for 6 h. A reprecipitation process in a solvent mixture of diethyl ether/chloroform (8/2, v/v) was applied to collect the product after polymerization. White powder of product was obtained by removing the remaining solvent via vacuum drying. The structure of the product was identified by ^{1}H NMR (a-300; JEOL, Tokyo, Japan) spectra as well as FT−IR (FT−IR 615; JASCO, Tokyo, Japan) spectra. The composition of MPC/BMA/VPBA in PMBV was 6/3/1. The molecular weight was determined by gel permeation chromatography (GPC; JASCO, Tokyo, Japan). The weight-average molecular weight (M_w) of PMBV was 5.4×10^4 (poly(ethylene oxide) standard). The polydispersity index, defined as the ratio between M_w and the number-average molecular weight (M_n), was 2.6.

Microchip Fabrication. The microchip (Figure 1d) was fabricated on Pyrex glass substrates (7.0×3.0 cm^2, 0.7-mm thick) by a process comprising standard photolithography, wet etching, and thermal bonding. First, holes for inlets and outlets were drilled on the upper plate by ultrasonic sandblasting. Afterward, the upper and lower plates were annealed at 570 °C for 5 h, and Cr/Au (20 nm/100 nm) thin films were sputtered on both plates for subsequent deep etching. Then, positive photoresist (2-μm thick) patterns of both microchannels (700-μm wide) and microchambers (5.0 mm in radius) were patterned on the upper plate, and photoresist patterns of only microchambers (5.0 mm in radius) were patterned on the lower plate, via photolithography. After Au and Cr etching processes with corresponding chemical etchants (I_2/NH_4I and $Ce(NH_4)_2(NO_3)_6$, respectively) to transfer the micropatterns to the glass substrates, 200 μm-deep micropatterns in both glass substrates were obtained by wet etching with 50% HF solution (etching rate = 13 μm/min). After removal of resist, Au, and Cr films, the perfectly cleaned upper and lower plates were finally bonded through a thermal bonding method.

Liquid Introduction. The microchip (Figure 1d) was sandwiched within a custom-made chip holder. Each microchamber with four microchannels worked independently. The introduction of liquid to each microchamber was described as follows. One Teflon capillary (inlet capillary) with one head immersed in a micro vial tube containing liquid to be introduced was connected to an inlet of the microchip. Another Teflon capillary (outlet capillary) with one head assembled to a syringe driven by a micro syringe pump (Model 210; KD Scientific, MA, U.S.A.) was connected to an outlet of the microchip. All connections were established by using Teflon screws, O-rings, and epoxy glue. The other two inlets/outlets were closed using Teflon blocking screws with O-rings. The microchip was positioned vertically to avoid liquid effluence from the outlet, when performing liquid introduction. The liquid was introduced from the inlet to the outlet by using a micro syringe pump under a withdraw mode.[26] After all liquid introduction, the microchip was positioned horizontally during hypothermic storage.

Cell Suspensions. L929 cells were cultured with DMEM (Invitrogen, NY, U.S.A.) supplemented with 10% fetal bovine serum (FBS; Sigma−Aldrich, MO, U.S.A.) in cell-culture dishes under a standard culture condition (37 °C, 5% CO_2, and 95% relative humidity). After L929 cell cultures were grown to 70%−80% confluence, the cells were trypsinized and adjusted to a density of 10^6 mL^{-1} with 5.0 wt % of PMBV which was dissolved in DMEM supplemented with 10% FBS, for subsequent cell packaging.

Cell Packaging. Before use, while microchips, capillaries, holders, and all other accessories were sterilized by autoclaving, all solutions were sterilized with sterilizing-grade filters (0.2-μm pore size). Cell packaging in the microchip (hydrogel/microchip format) was performed according to a process described as follows. To package the cells in the microchamber of the microchip, first, 5 μL of PVA (degree of polymerization, about 1500) solution (2.5 wt %) was introduced into the microchamber through a side microchannel. Then, 15 μL of PMBV solution (5.0 wt %) suspended with L929 murine fibroblast cell line cells was introduced through the same microchannel. While the PVA solution was prepared in 1× Dulbecco's phosphate buffer saline (D-PBS) and filtered with sterilizing-grade filters (0.2-μm pore size) before use, the PMBV/cell suspension was prepared in DMEM at a deliberately high cell density of 10^6 mL^{-1}. An optimum flow rate at approximately 5 μL s^{-1} was applied to introduce the PMBV/cell suspension to obtain quick and sufficient mixing between PMBV and PVA. The optimum flow rate was decided by observing morphology of the formed hydrogel and dispersion of the encapsulated cells. Under the optimum flow rate, a homogeneous hydrogel with cells uniformly dispersed could be formed. For cell packaging with the hydrogel in a 96-well microtiter plate (BD Bioscience, MA, U.S.A.), i.e,, the hydrogel/microplate format, first, 25 μL of 2.5 wt % PVA (Wako, Osaka, Japan) solution (Dulbecco's phosphate buffer saline, i.e., D-PBS, as solvent) was introduced to each well, and then 75 μL of the L929 cells/PMBV suspension was added. The cell-laden hydrogel formed in each well, by shaking the microtiter plate with a plate mixer (OPM-103; As One, Osaka, Japan) for 1 min. For cell storage in cell culture medium (culture medium/microplate format), after L929 cell cultures were grown to 70%−80% confluence, the cells were trypsinized and adjusted to a density of 7.5×10^5 mL^{-1} in DMEM supplemented with 10% FBS, and then 100 μL of the L929 cells/DMEM suspension was added to each well.

Viability Analysis. For the hydrogel/microchip format (total hydrogel volume 20 μL), after introducing 10 μL of calcein-AM/EthD-1 (2 μM/4 μM) mixture solution (Molecular Probes, Life Technologies, CA, U.S.A.) into the microchamber, the microchip was incubated in ambient air for 45 min, followed by fluorescence observation and analysis utilizing a fluorescence microscope (IX 71; Olympus, Tokyo, Japan) with a high-sensitivity digital CCD camera (Retiga EXi Fast1394; QImaging, BC, Canada). For the hydrogel/microplate format (total hydrogel volume 100 μL), the investigation was conducted using almost the same protocol as that in the hydrogel/microchip format, except that 50 μL of calcein-AM/EthD-1 (2 μM/4 μM) mixture solution was added to each well in the beginning. For the culture medium/microplate format, first, the cells in each well were washed with 100 μL of 1× PBS for five times; then, 100 μL of 1× PBS was added to each well, followed by adding 50 μL of calcein-AM/EthD-1 (2 μM/4 μM) mixture solution to each well; after that, the investigation was conducted using the same protocol as described above. For the hydrogel/chip format, three experimental repeats for every time point were performed simultaneously. For the other two formats, at least three experimental repeats for every time point were performed simultaneously. The number of live cells was counted from the green fluorescence (live; Ex/Em = 494/517 nm) image indicating intracellular esterase activity, by using an image processing and analysis software ImageJ (1.40g, NIH, U.S.A.). Intensity profiles of the green fluorescence of the cells on/near a randomly drawn horizontal plot line were analyzed with the same software. For easy comparison, fluorescence intensities were normalized with respect to the same maximal value, which represents the detectable maximal esterase activity (100%) with the method. The ratio between the detected intensity of green fluorescence of each live cell and the maximal fluorescence intensity is defined as relative esterase activity. The number of dead cells was counted from the red fluorescence (dead; Ex/Em = 528/617 nm) image indicating cell membrane integrity. For each format at every time point, viability, defined as the *number of live cells/number of cells in total (%)*, was evaluated from more than three merged live/dead images randomly taken in different areas of the samples of the corresponding experimental repeats. At least one image from every separate experiment was used to calculate the average and error bar of viability.

Morphology Analysis. Morphology of cells was analyzed from the phase-contrast images taken before viability analysis. For each image in each case, cells on/near five randomly drawn horizontal plot lines were evaluated in terms of percentage of cells with incomplete cell membranes and average cell diameter. The cells with obvious discontinuous boundary line are defined as cells with incomplete cell membranes. For each plot line, the percentage of cells with incomplete cell membranes is defined as the *number of cells with incomplete cell membranes/number of cells in total (%)*, on/near the plot line.

Cell Releasing and Recovery. A solution of D-fructose (0.2 M) dissolved in DMEM supplemented with 10% fetal bovine serum was

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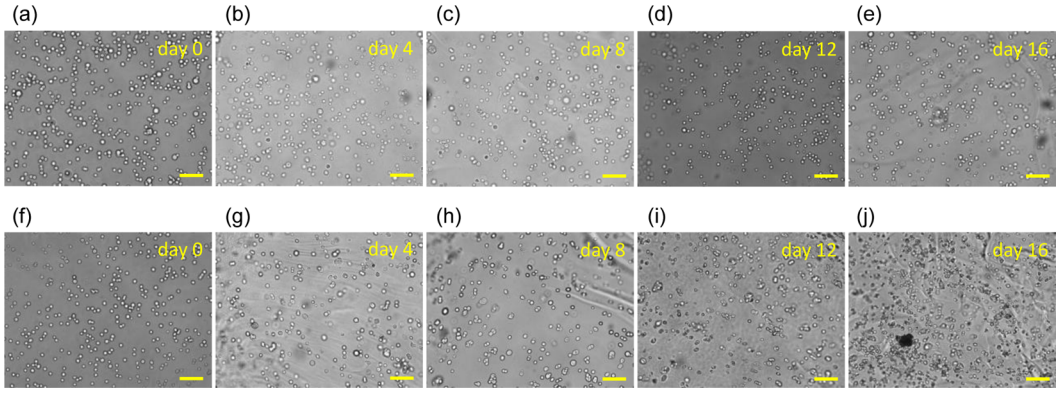

Figure 2. Phase-contrast images of L929 cells in the hydrogel/microchip format at (a–e) 4 °C and (f–j) 25 °C during the hypothermic storage. Scale bar is 100 μm.

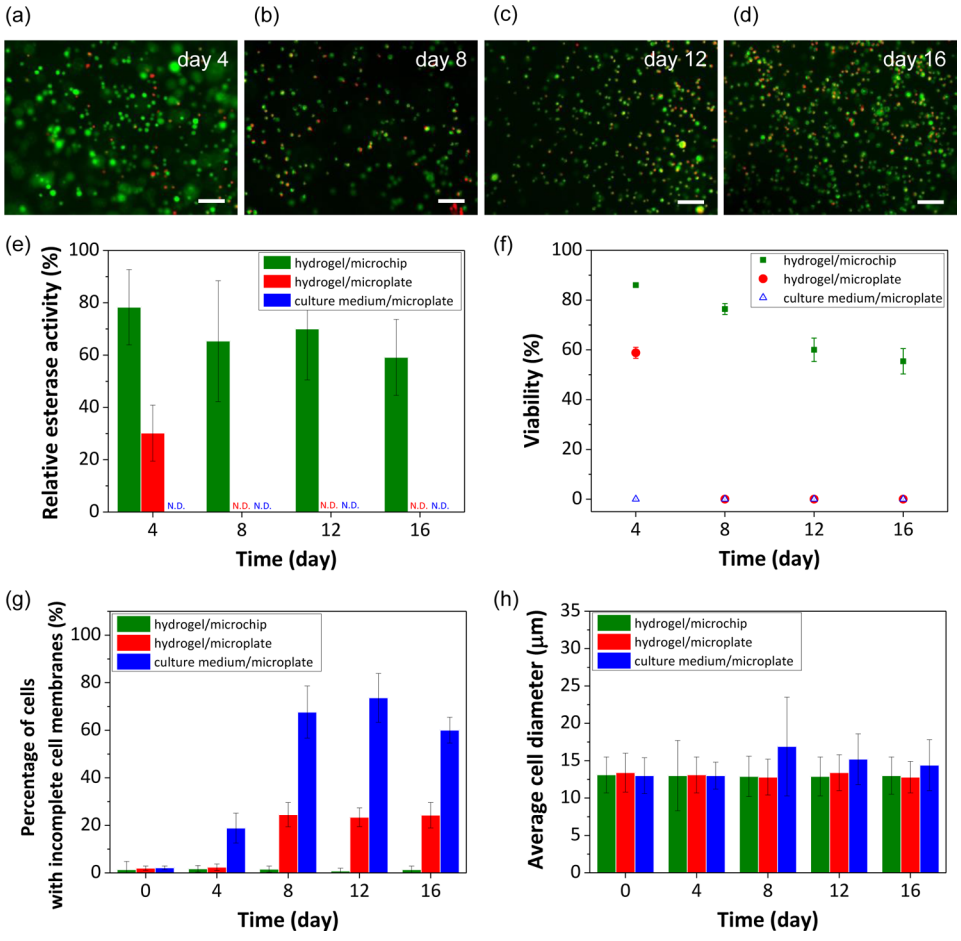

Figure 3. Viability and function analysis of L929 cells stored at 4 °C. (a–d) Merged live/dead (green/red) fluorescence images of the cells stored in the hydrogel/chip format for 4, 8, 12, and 16 days. (e) Relative intracellular esterase activity, (f) viability, (g) percentage of cells with incomplete membranes, and (h) average diameter of cells stored in three formats during hypothermic storage at 4 °C. "N.D." means no detectable signal. Data are mean ±SD from at least three separate experiments; $n \geq 6$ (i.e., cells on/near a randomly drawn horizontal plot line were analyzed) for (e), $n \geq 3$ (i.e., at least three merged live/dead images taken from experimental repeats were analyzed) for (f), $n = 5$ (i.e., percentage of cells with incomplete membranes on/near five randomly drawn horizontal plot lines were analyzed) for (g), and $n = 30$ (i.e, 30 randomly selected cells on/near five randomly drawn horizontal plot lines were analyzed) for (h), respectively. Scale bar is 100 μm.

prepared for cell releasing. First, 10 μL of the D-fructose solution was added to the cell storage microchamber. Then, the microchip was positioned vertically by the chip holder and fixed on a plate shaker (OPM-103, As One, Osaka, Japan). The microchip was shaken at approximate 100 rpm until the dissolution of the hydrogel was confirmed. After that, the chip was placed in a cell culture incubator (Model MCO-17A1C; Sanyo, Tokyo, Japan) to reculture the released cells.

RESULTS AND DISCUSSION

The microchip (Figure 1a) fabricated on Pyrex glass substrates comprises microchannels (700 μm wide and 200 μm deep) for

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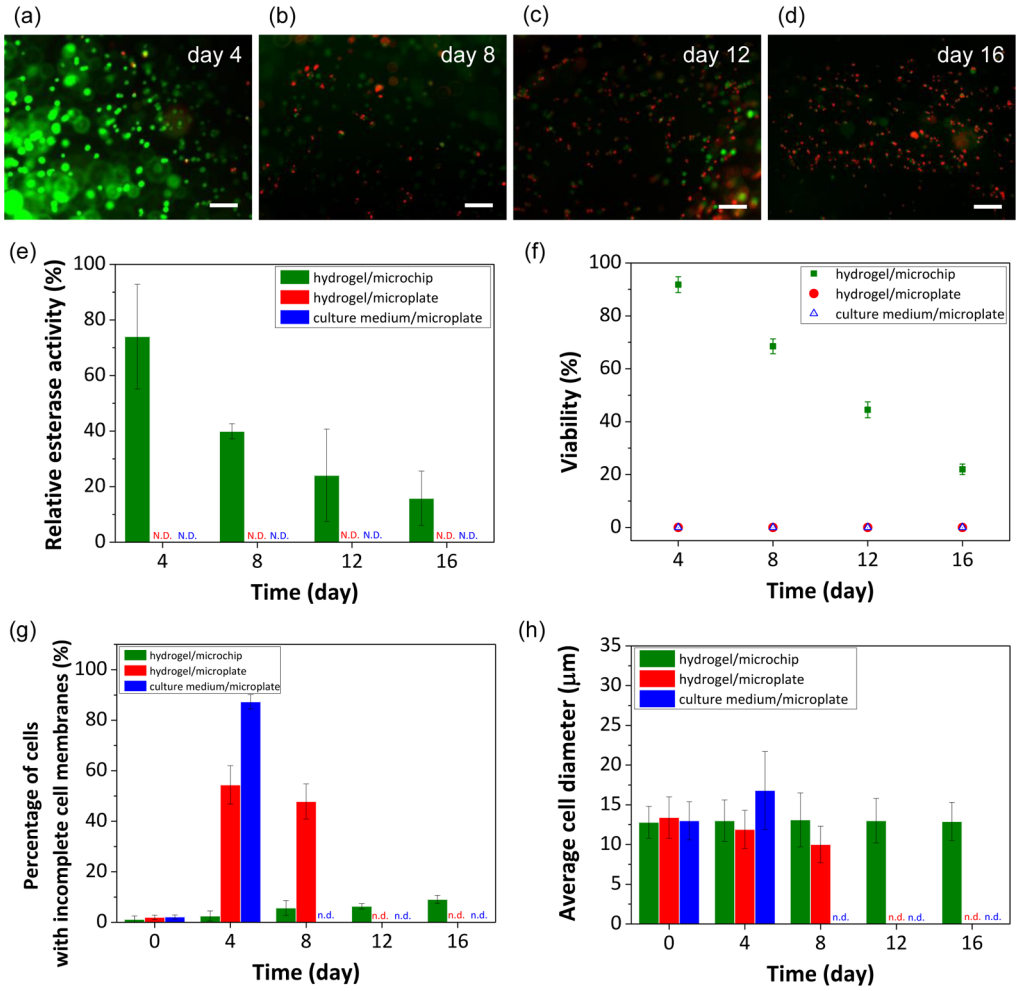

Figure 4. Viability and function analysis of L929 cells stored at 25 °C. (a–d) Merged live/dead (green/red) fluorescence images of the cells stored in the hydrogel/chip format for 4, 8, 12, and 16 days. (e) Relative intracellular esterase activity, (f) viability, (g) percentage of cells with incomplete membranes, and (h) average diameter of cells stored in three formats during hypothermic storage at 25 °C. "N.D." means no detectable signal and "n.d." means unable to determine. Data are mean ± SD from at least three separate experiments; $n \geq 6$ (i.e., cells on/near a randomly drawn horizontal plot line were analyzed) for (e), $n \geq 3$ (i.e., at least three merged live/dead images taken from experimental repeats were analyzed) for (f), $n = 5$ (i.e., percentage of cells with incomplete membranes on/near five randomly drawn horizontal plot lines were analyzed) for (g), and $n = 30$ (i.e, 30 randomly selected cells on/near five randomly drawn horizontal plot lines were analyzed) for (h), respectively. Scale bar is 100 μm.

liquid introduction and circular microchambers (5.0 mm in radius, 400 μm in depth, and approximately 30 μL in volume) for cell packaging and storage. L929 murine fibroblast cell line cells, which are model cells widely used in cell-based assays and tissue engineering, were packaged. The PMBV/cell suspension was prepared in DMEM at a deliberately high cell density of 10^6 mL^{-1} in order to investigate the capacity of cell packaging and storage. After introducing the PVA solution (5 μL), an optimum flow rate at approximately 5 μL s^{-1} was applied to introduce the PMBV/cell suspension (15 μL) allowed a quick and sufficient mixing between PMBV and PVA, and thereby the homogeneous hydrogel with uniformly dispersed cells could be formed in the microchip. All experimental steps for cell packaging were performed at ambient temperature (~25 °C) in a clean bench. A homogeneous cell-laden PMBV hydrogel spontaneously formed in the microchamber under the experimental condition (Figure 1d). As shown in Figure 1c and Figure 2a,f, during the gelation in the microchamber, the L929 cells at a density of 7.5×10^5 mL^{-1} were uniformly dispersed in the hydrogel with a volume of approximately 20 μL in total. In the microchamber, the remaining 10 μL empty space (Figure 1d) which was connected to ambient air through the central microchannels, provided a space for subsequent introduction of liquids for cell assays or cell release. After cell packaging, the inlets/outlets of all microchannels were kept open to ambient air during the whole period of the subsequent hypothermic storage. In addition, the microchip is reusable because the dissolved hydrogel with released cells can be easily discharged from the chamber after experiments.

Hypothermic storage of the packaged cells was performed under two conditions (Figure 1d). One was a refrigerated condition. In this case, the microchips were placed in a commonly used reagent storage refrigerator. The refrigerated temperature was set to 4 °C, and the average relative humidity in the refrigerator was 75% (with a range of 70–80%) at 4 °C. Another was an ambient condition. In this case, the microchips were placed in an ordinary room. The ambient temperature controlled by a room conditioner was about 25 °C and the relative humidity in the room was in a range of 30 to 40%. In addition, for the hypothermic storage under the refrigerated condition, sudden changes in both temperature and humidity might occur when opening and closing the door of the

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refrigerator in routine use. For the hypothermic storage under ambient conditions, the temperature might slightly fluctuate, and the humidity might change with the change of the surrounding environment. Furthermore, unlike conditions for standard cell cultures, there was no supply of CO_2 in both conditions. Additional complicated supports including capillaries and external pumps were not needed during the hypothermic storage in both conditions, because the continuous culture medium perfusion, which are generally required in most microchip-based cell applications,[27] was not necessary. Therefore, the hypothermic storage conditions in this study are not only quite flexible, but also easily reestablished because they are very close to actual life conditions.

Hypothermic storage of the packaged cells (referred to as "hydrogel/microchip") under both conditions was evaluated by measuring cell viability and function in terms of intracellular esterase activity, cell membrane integrity, and morphology, after 4, 8, 12, and 16 days (Figure 2−4). Meanwhile, the same evaluations were performed on hypothermic storage of cells in the PMBV hydrogel (100 μL) formed in a standard 96-well microtiter plate (referred to as "hydrogel/microplate") and hypothermic storage of cells in the standard liquid-base cell culture medium (DMEM; 100 μL) in a 96-well microtiter plate (referred to as "culture medium/microplate") under the same conditions, as controls (Figures S1 and S2 in the Supporting Information (SI), respectively). Hence, there were 8 experimental groups together with 16 control groups in this study, as summarized in Table S1. The hydrogel composition in the hydrogel/microplate format was the same as that in the hydrogel/microchip format. The cell densities (7.5×10^5 mL^{-1}) were identical for all three formats.

Figure 3a−d shows merged fluorescence images of viabilities of L929 cells in the hydrogel/microchip format at 4 °C for different storage periods. The viability was examined via two well-recognized parameters, namely, intracellular esterase activity and cell membrane integrity, by simultaneously using two well-established molecular probes, namely, calcein AM and ethidium homodimer-1 (EthD-1), respectively.[28] As a cell-permeant fluorogenic esterase substrate, the nonfluorescent calcein AM is enzymatically converted to green fluorescent calcein (excitation (Ex)/emission (Em) = 494/517 nm), upon intracellular hydrolysis of the ester moiety. The enzymatic conversion occurs in the presence of ubiquitous intracellular esterase activity, which is a distinguishing characteristic of live cells.[29] The intensity of the fluorescence has been used to roughly evaluate the level of intracellular esterase activity.[30] EthD-1, as a cell-impermeant fluorescent dye detecting cell membrane integrity, is only able to pass through the compromised membranes of dead cells, and consequently produces a 40-fold enhanced bright red fluorescence (Ex/Em = 528/617 nm) upon binding with high affinity to nucleic acids of dead cells.[29] EthD-1 stains all of the dead or dying cells that have undergone either necrosis or apoptosis.[31,32]

Green fluorescence was detected in most cells in the hydrogel/microchip format at 4 °C for 4 (Figure 3a), 8 (Figure 3b), 12 (Figure 3c), and even 16 days (Figure 3d), revealing that most cells possessed intracellular esterase activity (i.e., live cells) during more than two-weeks of extended storage. To facilitate comparison, green fluorescence intensities were normalized with respect to the same maximal value, which represents the detectable maximal esterase activity (100%) with the method. The ratio between the detected intensity of green fluorescence of each live cell and the maximal fluorescence intensity is defined as relative esterase activity. These live cells exhibited high levels of 80−60% relative esterase activity (Figure 3e). Furthermore, while some cells with compromised membranes were detected, as indicated by the red fluorescence (Figure 3a−d), the percentage of such cells (i.e., dead cells) gradually increased as the extension of the storage time (Table S2). Accordingly, the viability (Figure 3f), defined as the percentage of the number of cells with the green fluorescence (i.e., live cells) among all cells, was 86.0% at day 4, close to that of cells conventionally cultured in medium in a 96-well microtiter plate for 4 days (91.7%). The viability kept a high level of 76.4% at day 8, and gradually decreased to 60% at day 12 and further to 55.4% at day 16 (Figure 3f and Table S2). These results reveal that the PMBV hydrogel held the capability of hypothermic storage of cells with quite high viability for more than 1 week under the refrigerated condition (4 °C) in the microchip. In addition, the comparison of the merged live/dead images with their corresponding phase-contrast images revealed that all cells stored in the PMBV hydrogel were stained. Furthermore, the cell count revealed that the total number of living and dead cells obtained from every live/dead image was approximately equal to the number of cells in total obtained from every corresponding phase-contrast image (Tables S2 and S3). Because the conventional liquid base hypothermic storage commonly brings about a high level of cell lysis, resulting in dead cells being unaccounted for by membrane exclusion methods, the comparison and cell count suggest that the PMBV hydrogel within the microchip may protect cells from significant lysis during hypothermic storage.

In contrast, no viable L929 cells were detected in the culture medium/microplate format at 4 °C since day 4, indicating that the culture medium could not sustain cells in such a nonculture condition (Figure 3e,f and Figure S2a). Furthermore, L929 cells in the hydrogel/microplate format at 4 °C exhibited 58.8% viability with approximately 30% relative esterase activity at day 4, and no cells with esterase activity were detected since day 8 (Figure 3e,f and Figure S1a,b). The notable difference in viability between the hydrogel/microchip format and the hydrogel/microplate suggests that the structure of the microchip might assist the PMBV hydrogel in offering a sustainable environment for cell survival.

Cell death can occur through necrosis and apoptosis.[33] While both necrotic cells and apoptotic cells show compromised membranes, necrotic cells usually exhibit characteristic morphological features including incomplete membrane and increase of cell size.[34] Here, the cells with obvious discontinuous boundary line are defined as cells with incomplete cell membranes. In addition to analysis with the biochemical molecular probe EthD-1, cell membrane integrity was also analyzed by cell morphology from the phase-contrast images (Figure 2 and Figures S1 and S2). In contrast to the spread morphology of L929 cells in conventionally cultured medium (Figure S3), the cells in the hydrogel/microchip format showed round shapes after encapsulation, and kept almost uniformly round shapes during the whole period of storage at 4 °C (Figure 2a−e). While the percentage of cells with incomplete cell membranes was below 2% during the whole period of hypothermic storage at 4 °C for the hydrogel/microchip format, it was about 24% since day 8 for the hydrogel/microplate format, and about 19% on day 4, as well as above 70% since day 8 for the culture medium/microplate format (Figure 3g). Furthermore, the average cell diameter in both hydrogel formats at 4 °C did not change so much, in

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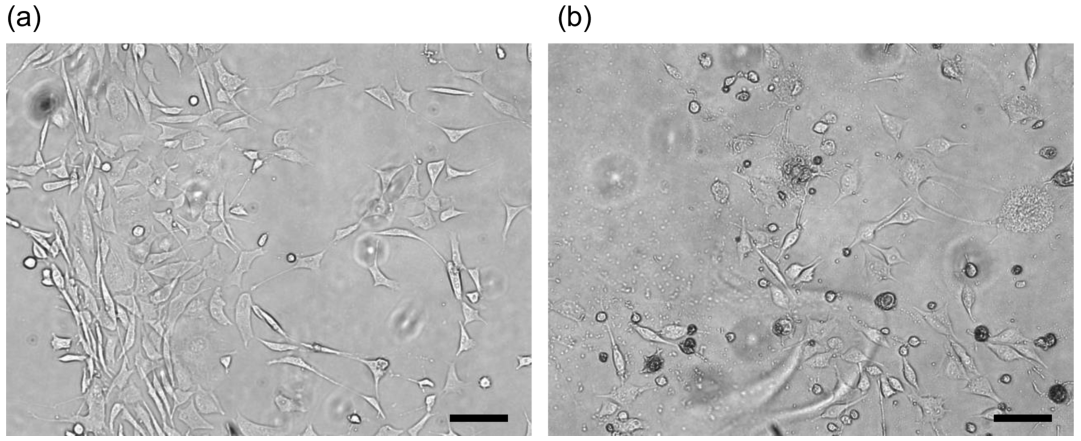

Figure 5. Phase-contrast images of L929 cells recultured for 24 h under standard cell culture conditions, after being released from the hydrogel in the microchip. Before release, the cells were stored in the hydrogel/chip format for (a) 8 days at 4 °C and (b) 4 days at 25 °C. Scale bar is 100 μm.

contrast to that in the culture medium/microplate format, which showed remarkable increases in the average cell diameter after day 8 (Figure 3h). The morphological information possibly suggests that the cell death during the period of storage at 4 °C might occur mainly through apoptosis for the hydrogel/microchip format, apoptosis/necrosis for the hydrogel/microplate format, and mainly necrosis for the medium/microplate format.

Figure 4a–d shows merged fluorescence images of viabilities of L929 cells in the hydrogel/microchip format at 25 °C for different storage periods. At day 4, most cells exhibited high relative intracellular esterase activity (74.0%; Figure 4e) and hence high viability (91.8%; Figure 4f and Table S3), with the same levels as those at 4 °C (Figure 3e,f and Table S2). At day 8, the cell viability was still high in the hydrogel/microchip format at 25 °C (Figure 4f and Table S3), but the relative intracellular esterase activity of the live cells significantly decreased to lower than 40% (Figure 4e). These results reveals that the PMBV hydrogel held the capability of hypothermic storage of cells with high viability for at least 4 days under the ambient condition (25 °C) in the microchip.

Overall, both the viability and the relative intracellular esterase activity of live cells in the hydrogel/microchip format decreased with larger slopes at 25 °C (Figure 4e,f) than 4 °C (Figure 3e,f). Such difference suggests that the PMBV hydrogel in the microchip had higher capability of hypothermic storage of cells in the refrigerated condition than the ambient condition. The PMBV hydrogel is considered to play a key role in cell maintenance owing to not only its well-known cytocompatibility but also its medium retention capacity. Actually, 95% (weight) content of the hydrogel is the cell culture medium that is composed of nutrients for cell maintenance. Considering the quite high average relative humidity (75%) in the refrigerator, one possible reason may be that the lower level of the PMBV hydrogel evaporation in the refrigerator than that in ambient air could provide a longer period of humidity and cytocompatible environment for cell maintenance. This hypothesis is supported by our observation that the hydrogel gradually shrank in the microchip at 25 °C but nearly did not at 4 °C during 16-days of storage (Figure 2). Furthermore, the elaborate semiclosed structure of the microchip is able to guarantee the exchange of gases between the miniature hydrogel and the outside environment through the microchannels, and meanwhile not only effectively prevent rapid evaporation of the hydrogel, but also reduce the contamination risk from the outside environment. No contamination of cells was observed during the hypothermic storage in both cases (Figure 2). Therefore, preventing evaporation of the hydrogel while maintaining gas exchange would be an effective strategy to improve the performance of the hypothermic storage using the PMBV hydrogel within the microchip at 25 °C in the future.

Serious loss of cell membrane integrity in both microplate formats at 25 °C (Figures S1h–j and S2e,f) was observed, making it difficult to measure the morphological features such as percentage of cells with incomplete cell membranes (Figure 4g) and average cell diameter (Figure 4h) in these cases. Accordingly, in contrast to the results in the hydrogel/microchip format, no viable cells were detected at day 4 in both the hydrogel/microplate format (Figure 4e,f and Figure S1g) and the culture medium/microplate format (Figure 4e,f), at 25 °C. The cell deaths in both microplate formats at 25 °C might occur mainly through necrosis, as supported by the aforementioned morphological information. Meanwhile, apoptosis might still be a dominant death type in the hydrogel/microchip format at 25 °C, but the number of necrotic cells gradually increased during the extended periods of storage, as implied from the cell morphological features (Figure 2f–j and Figure 4g,h). All these comparative results at both 4 °C and 25 °C suggest that both the cytocompatible PMBV hydrogel and the microchip were critical aspects to sustain cells for longer extended times.

After the hypothermic storage, the cells could be gradually released from the PMBV hydrogel by adding the D-fructose solution (0.2 M) to the microchamber of the microchip. The released cells in the microchamber were further recultured in a cell culture incubator under the standard cell culture condition (37 °C, 5% CO_2, and 95% relative humidity). As shown in Figure 5a, the viable cells (8 day hypothermic storage in the hydrogel/microchip format at 4 °C) adhered to the glass surface of the microchamber and became confluent after being reculturing for 24 h. A similar result was also obtained for the case of cells stored for 4 days in the hydrogel/microchip format at 25 °C (Figure 5b). These preliminary results suggested that the released cells might be functionally recovered under standard cell culture conditions. To gain a much clearer insight into the cell function of cells after release, further investigation from the perspective of cell biology including cell cycles, gene

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expression, and metabolic activity is very necessary in the future.

CONCLUSIONS

We presented spontaneous packaging and hypothermic storage of mammalian cells under refrigerated (4 °C) and ambient conditions (25 °C) using a cell-membrane-mimetic polymer (PMBV) hydrogel incorporated within a microchip. The capability of hypothermic storage of cells in the hydrogel/microchip format during 4, 8, 12, and 16 days was comparatively evaluated with two control groups (hydrogel/microplate format and culture medium/microplate), by measuring cell viability and function in terms of intracellular esterase activity, cell membrane integrity, and morphology. The results reveal that, in contrast to the other two control groups, the cytocompatible PMBV hydrogel, in combination with the microchip structure, allowed hypothermic storage of cells with quite high viability, high intracellular esterase activity, maintained cell membrane integrity, and small morphological change for more than 1 week at 4 °C and at least 4 days at 25 °C. The stored cells could be released from the hydrogel and exhibited the ability to adhere to a surface and achieve confluence under standard cell culture conditions. The storage conditions are flexible and easily established in places outside of laboratories, because they are ordinary conditions and do not need continuous complicated professional support. The extended storage time frames allow time for using current worldwide express delivery services. Therefore, the cell packaging and storage using the PMBV hydrogel incorporated within the microchip may offer a potentially alternative method allowing flexible supply and delivery of small amounts of cells locally and globally, and would be very useful for the development of various cell-based applications from bench to bedside, after a large number of elaborated case studies using a variety of types of cells.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.5b06796.

Experimental section, summary of experimental groups and control groups (Table S1), experimental data sheets for evaluation of viability of L929 cells stored in the hydrogel/chip format at 4 °C (Table S2) and at 25 °C (Table S3), merged live/dead fluorescence and phase-contrast images of L929 cells in the hydrogel/microplate format at 4 and 25 °C (Figure S1), merged live/dead fluorescence and phase-contrast images of L929 cells in the culture medium/microplate format at 4 and 25 °C (Figure S2), and a phase-contrast image of L929 cells conventionally cultured in DMEM in a cell culture incubator (Figure S3) (PDF)

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Notes

The authors declare no competing financial interest.

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