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Contracting cardiomyocytes in hydrophobic room-temperature ionic liquid

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ABSTRACT

Room-temperature ionic liquids (RTILs) are drawing attention as a new class of nonaqueous solvents to replace organic and aqueous solvents for chemical processes in the liquid phase at room temperature. The RTILs are notable for their characteristics of nonvolatility, extremely low vapor pressure, electric conductivity, and incombustibility. These distinguished properties of RTILs have brought attention to them in applications with biological cells and tissue in vacuum environment for scanning electron microscopy, and in microfluidic devices for micro-total analysis system (micro-TAS). Habitable RTILs could increase capability of nonaqueous micro-TAS for living cells. Some RTILs seemed to have the capability to replace water in biological applications. However, these RTILs had been applied to just supplemental additives for biocompatible test, to fixed cells as a substitute for an aqueous solution, and to simple molecules. None of RTILs in which directly soaks a living cell culture. Therefore, we demonstrated the design of RTILs for a living cell culture and a liquid electrolyte to stimulate contracting cardiomyocytes using the RTILs. We assessed the effect of RTILs on the cardiomyocytes using the beating lifetime to compare the applicability of RTILs for biological applications. Frequent spontaneous contractions of cardiomyocytes were confirmed in amino acid anion RTILs $[P_{8,8,8,8}][Leu]$ and $[P_{8,8,8,8}][Ala]$, phosphoric acid derivatives $[P_{8,8,8,8}][MeO(H)PO_2]$, and $[P_{8,8,8,8}][C_7CO_2]$. The anion type of RTILs had influence on applicable characteristics for the contracting cardiomyocyte. This result suggested the possibility for biocompatible design of hydrophobic group RTILs to achieve biological applications with living cells.

1. Introduction

Room-temperature ionic liquids (RTILs) are drawing attention as a new class of non aqueous solvents to replace organic and aqueous solvents for chemical processes in the liquid phase at room temperature [1–3]. The RTILs are notable for their characteristics of nonvolatility, extremely low vapor pressure, electric conductivity, and incombustibility. They are synthesized from the combination of an organic cation and various anions and form a third class of solvents with interesting characteristics of their component parts. Stability of biomolecules in RTILs and some biological applications have gained increasing attention after some recent reports presented some noteworthy results [4–7]. Some RTILs seemed to have the capability to replace water in biological applications [8]. Hydrated IL which is a hydrophilic RTIL with a small amount of water solvated cytochrome c protein and retained the protein activity [4]. The nonvolatility and extremely low vapor pressure of RTILs has brought attention to them in applications with biological cells and tissue in vacuum environment for scanning electron microscopy [7,8]. Microfluidic devices for micro-total analysis system (micro-TAS) have employed the RTILs as an electric conductive hydraulic fluid which did not evaporate to get an electric circuit in micro fluidic channels [9,10] because of difficulty to protect micro liquid in conventional PDMS micro channels from evaporation [11,12]. These individual biological and micro-TAS challenges implied us RTIL could have a directly connecting interface to truly living cell. However, these RTILs were applied to just supplemental additives for biocompatible test, to fixed cells as a substitute for an aqueous solution, and to simple molecules. And no application has been reported which directly soaks a living cell culture in the RTILs.

Habitable RTILs could increase capability of nonaqueous micro-TAS for living cells. Therefore, we demonstrated the design of RTILs for a living cell culture and a liquid electrolyte to stimulate cardiomyocytes using the RTILs on the beating cardiomyocytes. Beating behavior of the cardiomyocytes was compared to evaluate the applicability of the RTILs for biological applications, since cardiomyocyte is the micro living kinetic system containing receptors and motility. The beating of the cardiomyocytes could respond rapidly to changes of the cultivation environment [13]. Thus, we as-

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sessed the effect of RTILs on the cardiomyocytes using the beating lifetime to compare the applicability of RTILs for biological applications. To determine the design of habitable RTIL, ion species dependent lifetime was investigated in this study. The applications to the living system utilized the electric conductivity and nonvolatilization of the RTILs as an evaporation-resistant liquid electrolyte interface which was layered on the cardiomyocytes.

2. Materials and methods

2.1. Ionic liquid

Functional groups are generally affected to cellular behavior on functionalized materials [14–18]. We, therefore, evaluated the applicability of hydrophilic and hydrophobic RTILs with various types of anions containing amino acids, phosphoric acid derivatives, and fluorinated compounds, as shown in Table 1. Hydrophilic fluorinated IL, [C4mim][BF_4] was used as commercially received from Kanto Chemical Co. Another hydrophilic RTIL, [ch][dhp] [4] and phosphoric acid derivative RTIL, [$P_{8,8,8,8}$][$MeO(H)PO_2$] [19] were synthesized according to the literature. Hydrophobic amino acid RTILs, [$P_{8,8,8,8}$][Leu], [$P_{8,8,8,8}$][Ala] and [$P_{8,8,8,8}$][Lys] were synthesized by a modified method [20]. Other hydrophobic RTILs, [$P_{8,8,8,8}$][Tf_2N], [$P_{8,8,8,8}$][TFA] and [$P_{8,8,8,8}$][C_7CO_2] were donated from Hokko Chemical Industry Co., Ltd.

To evaluate changes of osmotic pressure and pH of exposed culture medium, a 200 μL volume of hydrophobic RTILs was spread onto 200 μL of the conventional culture medium (CM1) in a 1-mL micro tube, and left to stand for 30 min. The exposed culture media samples were taken using a needle syringe from the lower layer of the two-phase separation.

2.2. Preparation of cardiomyocyte

Rat primary cardiomyocytes were isolated from neonatal rat (Wister, 1-day old; Saitama Experimental Animals Supply Co., Ltd.) heart at 37 °C in Dulbecco's phosphate buffered saline (D-PBS (−), Gibco) containing 25 μg/mL collagenase (301617; Gibco) during magnetic stirring. Isolated cells were suspended in culture medium (CM1) for cardiomyocyte culture, which contained Dulbecco' modified eagle medium (DMEM, 043-30085, Wako), 10% fetal bovine serum (SV30014.03, Hyclone), and 1% penicillin–streptomycin (168-23191, Wako). Then the cell suspension was seeded in wells. The CM1 was replaced every 2 days during culturing.

P19.CL6 (RCB2318, Riken cell bank) cells were differentiated to autonomous beating cardiomyocytes [21,22]. P19.CL6 cells were grown less than 70% confluent in culture medium (CM2) which was α-minimal essential medium (Gibco) supplemented with 10% fetal bovine serum (Gibco), penicillin (100 U/mL), and streptomycin (100 μg/mL), and were maintained in a 5% CO_2 atmosphere at 37 °C. To induce differentiation, 5 mL cell suspension of P19.CL6 cells was fed at a density of 2×10^5 cells/mL into a 60-mm low cell binding dish (Nunc) with differentiation medium (DM), which was the CM2 containing 1% DMSO. The cells formed a spheroid aggregation on the low cell binding dish during 4 days culturing. At day 4, the spheroid P19.CL6 cells were centrifuged and replaced in a 60-mm tissue culture grade dish with fresh DM. The DM was changed every two days. The differentiated P19.CL6 cells contracted autonomously at 11 days after differentiation.

2.3. Evaluation of lifetime of the cardiomyocytes exposed to RTILs

All RTILs listed in Table 1 were exposed to the cardiomyocyte culture to evaluate the affect of the change of the culture medium and applied to the surface coverage and the interface material to stimulate cardiomyocytes using a small well culture assay. The lifetimes of the cardiomyocytes which frequently contracted during RTIL exposure were compared among the listed RTILs in Table 1. Primary rat cardiomyocytes were cultured into 24-well plate for more than 3 days to evaluate the lifetime of the cardiomyocytes exposed to the RTILs. 100 μL of fresh CM1 was fed into each well after all of the original medium was removed. A 100 μL amount of RTILs was spread into the wells and the temperature in the wells was kept at 37 °C using a heated stage (Tokai-hit) on a phase contrast inverted microscope (Ti-S, Nikon). The frequent contractions of the cardiomyocytes were tracked using motion tracking software (Ditect, Dipp-motion) on a captured video by a 60-fps CMOS camera mounted on the microscope.

2.4. Evaporation resistance of RTILs

Evaporation resistance of [$P_{8,8,8,8}$][C_7CO_2] for the culture medium was compared to resistance of non-covered culture medium and paraffin. The most applicable RTIL, [$P_{8,8,8,8}$][C_7CO_2] was applied for the cardiomyocyte culture. Cardiomyocytes from P19.CL6 were seeded to 5-mm PDMS wells on a tissue culture dish. 40 μL of [$P_{8,8,8,8}$][C_7CO_2] was fed into each well after all of the original medium was removed, and then the temperature in the wells was kept at 37 °C using the heated stage on the microscope. Sustention of frequent contractions of the cardiomyocytes were compared among [$P_{8,8,8,8}$][C_7CO_2] covered, non-covered, and liquid paraffin covered cultures.

2.5. Stimulation of cardiomyocytes via RTILs

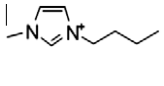
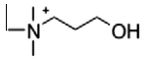
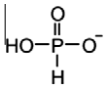
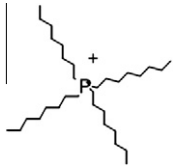
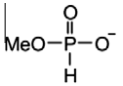
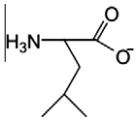
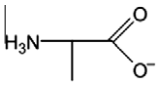
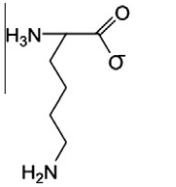
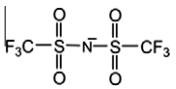
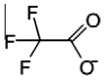
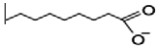
An electrochemical cell was prepared to demonstrate the liquid electrolyte using [$P_{8,8,8,8}$][C_7CO_2]. Fig. 1 shows the 5-mm PDMS well with an ITO electrode on the bottom of the well. The well had a half glass cover over it. The cardiomyocytes from P19.CL6 were cultured in the well. 20 μl of CM2 was filled in the well, then [$P_{8,8,8,8}$][C_7CO_2] was supplied into the well inlet. An electrical stimulator (Nihonkhoden) was connected to the Pt electrode and ITO layer of the well. The Pt electrode was inserted into the [$P_{8,8,8,8}$][C_7CO_2] droplet. Five electrical pulses of 30–50 V in amplitude, 50 ms in duration, and separated by 500 ms intervals were applied to the electrochemical cell. This pulse train was repeated with a 5-s interval. The responses of the cardiomyocyte contraction to the electric stimulation via [$P_{8,8,8,8}$][C_7CO_2] and liquid paraffin were recorded using a high-speed camera (SA3, Photoron) with 125 fps on a phase contrast inverted microscope (Ti-U, Nikon). The contraction behaviors were detected using motion tracking software.

3. Results

3.1. Lifetime of the cardiomyocytes exposed to RTILs

Spontaneous contractions of cardiomyocytes were counted to compere the lifetime of the cardiomyocytes exposed to RTILs. The hydrophobic RTILs and the culture medium were clearly separated to cover the culture. The hydrophobic RTILs formed an upper layer on the culture medium, since all the studied hydrophobic RTILs were lighter than the culture medium. Hydrophilic RTILs had a critical affect to cardiomyocyte contraction. Hydrophobic RTILs with fluorine compound anions also had a short lifetime. These contractions of cardiomyocytes were rapidly stopped as soon as adding RTILs with fluorine compound anions. In contrast, spontaneously contracting cardiomyocytes were confirmed in hydrophobic RTILs containing amino acid anions [Leu] and [Ala], and the phosphoric acid derivative anion [$MeO(H)PO_2$], and [C_7CO_2]. Fig. 2(A) shows the lifetime of the cardiomyocytes during

Table 1
List of hydrophilic and hydrophobic RTILs for the applicability evaluation testThe osmotic pressure and pH were measured in the culture medium (CM1), which was exposed to the respective IL for 30 min.

Cation	Anion	Name	Abbreviation	Hydrophilicity	Categorized property	Osmoticpressure at water layer[osmol/kg]	pH
–	–	Culture medium (CM1) (DMEM, 10% HS, 1%penicillin-streptobacillosis)	–	–	–	322–331	8.2
		1-butyl-3-methylimidazolium tetrafluoroborate	[C4mim][BF_4]	Hydrophilic	Fluorine compound	–[a]	–[a]
		Cholinedihydrogenphosphate	[ch][dhp]	Hydrophilic	Phosphoric acid derivative	–[a]	–[a]
		Tetraoctylphosphoniummethylphosphonate	[$P_{8,8,8,8}$][$MeO(H)PO_2$]	Hydrophobic	Phosphoric acid derivative	332	8
		Tetraoctylphosphoniumleucine	[$P_{8,8,8,8}$][Leu]	Hydrophobic	Amino acid	334	9.2
		Tetraoctylphosphoniumalanine	[$P_{8,8,8,8}$][Ala]	Hydrophobic	Amino acid	332	8.8
		Tetraoctylphosphoniumlysine	[$P_{8,8,8,8}$][Lys]	Hydrophobic	Amino acid	290	9.2
		Tetraoctylphosphonium bis(trifluoromethansulfonyl) imide	[$P_{8,8,8,8}$][Tf_2N]	Hydrophobic	Fluorine compound	337	8.8
		Tetraoctylphosphonium trifluoroacetate	[$P_{8,8,8,8}$][TFA]	Hydrophobic	Fluorine compound	346	8.6
		Tetraoctylphosphonium *n*-octanoate	[$P_{8,8,8,8}$][C_7CO_2]	Hydrophobic	Fatty acid	288–297	8.6

[a] Not measured.

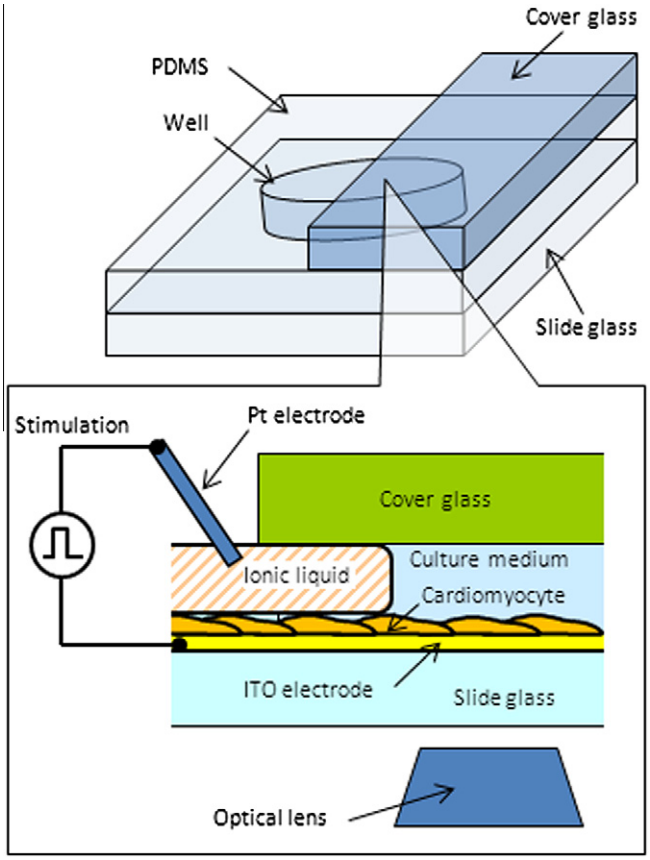


Fig. 1. Liquid electrolyte interface for contracting cardiomyocyte via RTIL. RTIL was dropped in a PDMS micro well to make interface to contracting cardiomyocytes which were cultured in the micro well on ITO-coated glass. The electrochemical well was used to demonstrate the liquid electrolyte based on the RTIL electric conductivity. Cardiomyocyte cells were cultured on the ITO electrode. The micro well was half-covered by a cover-glass to be a micro channel. The RTIL was supplied into the well from an inlet. Electrical stimulus via RTIL evoked cardiac contraction in the narrow fluid channel. RTIL $[P_{8,8,8,8}][C_7CO_2]$ and liquid paraffin was compered the electrical conductivity whether these liquids conducted the electrical stimulus.

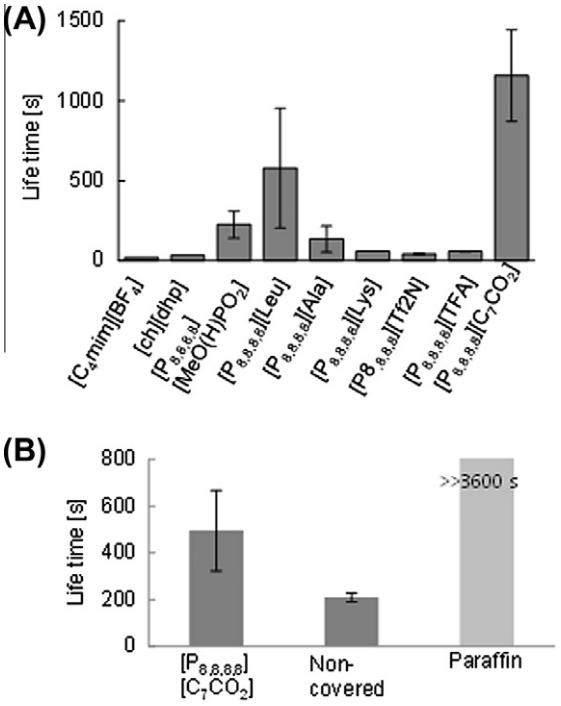


Fig. 2. Lifetime evaluation of RTILs for exposing contracting cardiomyocytes. (A) Comparison of beating lifetime of the cardiomyocyte during the culture exposed to the respective IL. Since the lifetime was longer for $[P_{8,8,8,8}][Leu]$ and $[P_{8,8,8,8}][C_7CO_2]$ than other ILs, they had a low effect on the cultured cardiomyocytes. ILs of the amino acid anion group except for $[P_{8,8,8,8}][Lys]$ showed the second longest lifetime. (B) Resistivity for evaporation of the cardiomyocyte culture. $[P_{8,8,8,8}][C_7CO_2]$ covered cells had a longer lifetime than non-covered cells.

RTIL exposure. The hydrophobic RTILs containing amino acid anions except lysine, and the phosphoric acid derivative anion $[MeO(H)PO_2]$, and $[C_7CO_2]$ had a longer lifetime than the hydrophilic RTILs and the hydrophobic RTILs with fluorine compound anions. Non-amino acid anion RTIL, $[P_{8,8,8,8}][C_7CO_2]$, had the longest lifetime of the cardiomyocytes among the RTILs, which was about more than 1000 s.

Especially, RTILs with anions of $[C_7CO_2]$ and leucine had beating lifetimes longer than about 10 min during the RTIL exposure, although $[C_7CO_2]$ induced a high pH and a significant drop in osmotic pressure in the culture medium and leucine also induced a high pH. The anion type was not correlated to the hydrophobicity of the RTIL, the pH, or the osmotic pressure.

3.2. Evaporation resistance of RTILs

The cardiomyocytes were frequently contracted till evaporation of the culturing medium in all materials for coverage. $[P_{8,8,8,8}][C_7CO_2]$ had an advantage in better resistance to evaporation of the culture medium (CM2) than the non-covered culture since the lifetime using $[P_{8,8,8,8}][C_7CO_2]$ was statistically significant longer (Fig. 2(B)). $[P_{8,8,8,8}][C_7CO_2]$ successfully protected the cultivation condition from water evaporation. In contrast, non-covered cardiomyocytes were stopped the contraction after <200 s left because of the dehydration of the cells from the surface.

3.3. Liquid electrolyte interface for contracting cardiomyocytes via RTIL

Fig. 3(A, B) are the responses of the cardiomyocyte to the electrical stimulation via $[P_{8,8,8,8}][C_7CO_2]$ and liquid paraffin, respectively. Both cardiomyocytes in wells remained frequent and spontaneous contractions during the evaluation. Electrical stimulation evoked contractions were synchronized to the stimulation via $[P_{8,8,8,8}][C_7CO_2]$. In contrast, the electrical stimulation via liquid paraffin could not contribute to the evoked contraction of the cardiomyocytes, since liquid paraffin does not have an electrical conductivity. Therefore, the applicable RTILs had a capability to be a liquid electrolyte interface to stimulate living biological cells and tissue.

4. Discussion

The low-density hydrophobic RTILs [23] used in this study had lower density than the culture medium. Because a preliminary result showed that the RTILs formed a liquid phase at the bottom of the water phase, when high-density hydrophobic RTILs were spread on the water phase, and then beating behavior of cardiomyocytes grew adhesively on the bottom of the dish immediately disappeared and flaked cell cultures were observed.

The RTILs in contact with the culture media affected to the conditions of the culture medium. Osmotic pressure and pH of the culture medium were measured to investigate the changes of the culture conditions when the RTILs would be applied as a liquid electrolyte on the culture cells. Table 1 shows the differences of osmotic pressure and pH between the exposed culture medium and the original culture medium. Osmotic pressure, the change of which indicated the migration of ionic components between the

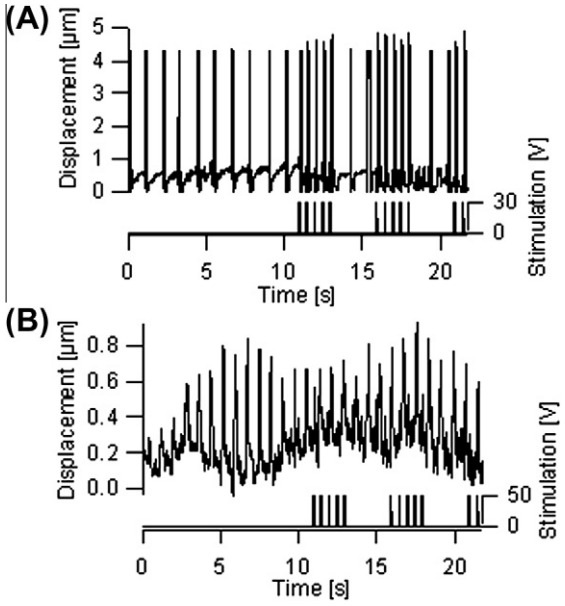


Fig. 3. Comparison of capability of liquid electrolyte interface for contracting cardiomyocyte via RTIL $[P_{8,8,8,8}][C_7CO_2]$ and liquid paraffin. Contraction response to electric stimulations for cardiomyocytes via (A) $[P_{8,8,8,8}][C_7CO_2]$ and (B) paraffin. $[P_{8,8,8,8}][C_7CO_2]$ transmitted well the stimulation pulses to the cardiomyocytes. The bottom timelines show the stimulation pattern used. The electrochemical well in Fig. 1 was used to demonstrate the liquid electrolyte based on the RTIL electric conductivity.

RTILs and the culture medium, increased slightly except for $[P_{8,8,8,8}][Lys]$ and $[P_{8,8,8,8}][C_7CO_2]$. All pH values increased after exposure to the hydrophobic RTILs except for $[P_{8,8,8,8}][MeO(H)PO_2]$.

The cardiomyocytes were spontaneously contracting in RTILs with anions of $[C_7CO_2]$ and leucine. RTILs with anions of $[C_7CO_2]$ and leucine had beating lifetimes longer than about 10 min during the RTIL exposure, although $[C_7CO_2]$ induced a high pH and a significant drop in osmotic pressure in the culture medium and leucine also induced a high pH. The anion type was not correlated to the hydrophobicity of the RTIL, the pH, or the osmotic pressure. These results indicated that the lifetime of the cardiomyocytes depended on hydrophobicity of RTIL and also anion toxicity. The hydrophobic RTILs inhibited toxic ion migration toward the living cardiomyocytes due to a thin interlayer of the culture medium, and also less toxic anion types could increase the beating lifetime of the cardiomyocytes.

A longer lifetime of the cardiomyocytes was recorded for the RTILs with no functional group on alkyl chain on their anion forms ($[C_7CO_2]$), while the RTILs $[P_{8,8,8,8}][Lys]$ with an amino group on long alkyl chain in the anion immediately induced fatal condition changes for cardiomyocytes. Amino groups are generally utilized to functionalize the culture dish surface for cell adhesion culturing [14–16], and long alkyl chain for amino group induced cell adhesion on its functionalized surface [16]. Thus an amino group on long alkyl chain of the anion of the RTILs would interfere with the function of the cell membrane. This indicated that the applicability of the RTILs for applications to living system could be designed taking into consideration the hydrophobic property and the functional group on the anion of the RTILs.

We could demonstrate that the $[P_{8,8,8,8}][C_7CO_2]$ was suitable as an evaporation-resistant liquid electrolyte for stimulation bioapplications. The evaporation-resistant liquid electrolyte would be useful for small scale cell analysis with less evaporation in long term procedures. Liquid paraffin only provides protection from evaporation, with an easy accessibility to the cells through the liquid paraffin cover in a small culture environment [24]. Liquid paraffin cannot be utilized as a sensing interface materials for electrochemical activity and stimulation due to its nonconductive characteristics, although the liquid paraffin covered culture had an extremely long lifetime of more than 3600 s.

Applicable hydrophobic RTIL covers can additionally provide an electric interface for such tiny biological devices as a cardiomyocyte driven microfluidic pump [25] and bioactuators [26–29]. The electrical conductivity of the RTILs could also contribute capability to be applied a nano processing using charged beam microscope for the living cells. The nonvolatility and extremely low vapor pressure of RTILs has brought attention to them in applications with biological cells and tissue in vacuum environment for scanning electron microscopy [7,8]. The vacuum technology using beam techniques is able to fabricate a nano scale structure in the RTIL, [30] which would assist in the nanotechnology biological analysis using RTIL. Low energy electron beam had less damaged for a living cell [31].

In summary, the applicable design of habitable RTIL was related to anion species. The anion type had significant influence on applicable characteristics for the contracting cardiomyocytes. Spontaneous contractions of cardiomyocytes were confirmed in RTILs with no functional group on alkyl chain on their anion forms. Applicable RTIL $[P_{8,8,8,8}][C_7CO_2]$ could be a good electrolyte for electrical stimulation to contracting cardiomyocytes. The applicability of the RTILs should be widespread owing to their nonvolatility and electric conductivity; we expect them to be used for bioactuators and in living cell analysis in a small droplet in microfluidic devices.

Acknowledgments

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