# Cryoprotective Properties of Completely Synthetic Polyampholytes via Reversible Addition-Fragmentation Chain Transfer (RAFT) Polymerization and the Effects of Hydrophobicity

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***Abstract***

A completely synthetic polyampholyte cryoprotectant was developed with cationic and anionic monomers by reversible addition-fragmentation chain transfer polymerization. The neutralized random polyampholyte, which had an equal composition ratio of monomers, showed high cryoprotective properties in mammalian cells. Introduction of a small amount of hydrophobic monomer enhanced cell viability after cryopreservation, indicating the importance of hydrophobicity. Leakage experiments confirmed that these polyampholytes protected the cell membrane during cryopreservation. Due to low cytotoxicity, this polyampholyte has the potential to replace the convention cryoprotective agent dimethyl sulfoxide. The present study is the first to show that we can design a polymeric cryoprotectant that will protect the cell membrane during freezing using appropriate polymerization techniques.

**Keywords: biocompatibility; cryopreservation; polyampholyte; reversible addition-fragmentation chain transfer (RAFT)**

## 1. Introduction

Cryopreservation is a process through which different types of cells, tissues, or organs are preserved at very low temperatures in such a way as to allow them to be restored with all their original functions whenever required. Cryopreservation is of paramount importance in various medicinal and biological contexts. Polge et al. were the first to report the preservation of living cells at very low temperatures after the accidental discovery of the cryoprotective properties of glycerol on fowl sperm [1]. Some years later, the cryopreservation of red blood cells was achieved using dimethyl sulfoxide (DMSO) as a cryoprotectant [2]. These common cell membrane-penetrating cryoprotectants (i.e., glycerol and DMSO) protect cells from lethal damage caused by the formation of intracellular ice during freezing and thawing. However, the cryoprotective properties of glycerol are relatively weak, and DMSO shows high toxicity [3] and affects the differentiation of various types of cells [4-6]. Thus, there is a great need to develop newer cryoprotective agents, especially in applications of regenerative medicine.

The polyampholyte carboxylated poly-L-lysine (COOH-PLL) shows excellent post-thaw survival efficiency [7-9] and cryoprotective properties against human mesenchymal stem cells, while retaining the cells' full differentiation capacity without

the addition of any other low-molecular-weight cryoprotectants or proteins [10]. However, the mechanisms through which a non–membrane-penetrating polymer, such as COOH-PLL, could exhibit substantial cryoprotective properties are still not clear. From our previous studies demonstrating that polyampholytes are absorbed on the cell membrane during freezing [10], we hypothesized that the mechanisms of such polyampholytes are likely related to the induction of membrane protection against mechanical damage from ice formation.

Controlled radical polymerization (CRP) techniques, such as atom transfer radical polymerization (ATRP) [11,12], nitroxide-mediated polymerization (NMP) [13,14], and reversible addition-fragmentation chain transfer (RAFT) [15-17] polymerization, have been studied extensively in recent years. Among them, RAFT polymerization has the advantage that it can be applied to a wide range of functional and nonfunctional monomers under a variety of conditions and solvents to yield polymers with predetermined molecular weights, narrow molecular weight distributions, and complex architecture [18,19]. Moreover, it does not require the use of any toxic organometallic catalysts. Recently, many researchers have attempted to achieve RAFT polymerization in aqueous media [20-23].

In the present study, we sought to examine whether a completely synthetic polyampholyte (different from COOH-PLLs), synthesized using RAFT polymerization, could exhibit cryoprotective properties in murine L929 cells, which would allow us to elucidate the molecular mechanisms of these effects by synthesizing various polymers. We also investigated the membrane-protective properties of the newly synthesized polyampholyte and evaluated the effects of hydrophobicity on enhanced membrane protection and cryopreservation. This report is the first to reveal that a completely synthetic polymer possesses cryoprotective properties and to demonstrate the relationship between the cryoprotective properties of a polymer and the cell membrane protection of these polyampholytes.

## 2. Materials and Methods

### 2.1 Materials

2-(Dimethylamino)ethyl methacrylate (DMAEMA), methacrylic acid (MAA), and n-butyl methacrylate (Bu-MA) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). N-Octyl methacrylate (Oc-MA) was purchased from NOF Corporation (Tokyo, Japan). All of these monomers were distilled under reduced pressure prior to use to remove inhibitors. 2-(Dodecylthiocarbonothioylthio)-2-methylpropionic acid (RAFT agent) and

carboxyfluorescein (CF), obtained from Sigma Aldrich (St. Louis, MO, USA), were used as provided without further purification. 4-4'-Azobis-(4-cyanovaleric acid) (V-501, initiator) was purchased from TCI (Tokyo, Japan). All other reagents were reagent grade and were used without further purification.

### 2.2 Synthesis of Polyampholytes

We synthesized various amphoteric copolymers and terpolymers by changing the monomer ratios of the components added or by changing the molar ratio of total monomer/RAFT agent/initiator to obtain polymers with various molecular weights. To synthesize a 1:1 copolymer, DMAEMA (4 mmol), MAA (4 mmol), 2-(dodecylthiocarbonothioylthio)-2-methylpropionic acid (0.2 mmol), and V-501 (0.04 mmol) were added to a reaction vial, and 20 mL of water-methanol mixture (1:1 [v/v]) was then added. To introduce hydrophobic moieties to the polyampholytes, 1%–10% of the total monomer amount of Bu-MA or Oc-MA was added in the reaction mixture. Alternatively, 5% HE-MA was added to the reaction mixture to introduce hydrophilicity. The solution was then purged with nitrogen gas for 1 h and stirred at 70°C. Samples were removed periodically (25 μL), and the conversion at each reaction time was obtained by $^{1}$H-NMR (400 MHz, Bruker). After 24 h, the reaction mixture was precipitated using 2-propanol, the precipitates were collected by centrifugation, and the

compound was dried over vacuum. The molecular weight and distribution (polydispersity index, PDI) of the polymers was determined by gel permeation chromatography (GPC, column, BioSep-s2000, Phenomenex, Inc., CA, USA) and was measured on a Shimadzu high-performance liquid chromatography data system using a refractive index detector. Phosphate buffer solution (pH 7.4, 0.1 M) was used as the mobile phase (flow rate, 1 mL/min) and pullulan was used as the standard. The chemical structures of the compounds were confirmed by $^{1}H$ NMR using $D_2O$ as solvent.

**2.3 Cell Culture**

L929 cells (American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma Aldrich) supplemented with 10% fetal bovine serum (FBS). Cells were cultured at 37°C in a $CO_2$ incubator in a humidified atmosphere. When the cells were confluent, they were washed with phosphate buffered saline (PBS) and then treated with trypsin solution (0.25% [w/v] trypsin containing 0.02% [w/v] ethylenediaminetetraacetic acid [EDTA] in PBS) to detach the cells. The cell pellet was then collected by centrifugation, mixed with fresh DMEM, and subsequently transferred to a new culture plate for subculture.

**2.4 Cryopreservation of Cells**

Polyampholyte solutions were prepared in DMEM without FBS at 5%–15% concentrations. The pH of the solution was adjusted to 7.4, and the osmotic pressure was adjusted to 500 mmol/kg by the addition of sodium chloride using a vapor pressure Osmometer (VAPRO Model 5660, WESCOR Biomedical Systems, UT, USA). These solutions were filter sterilized using a MILLEX GP Filter Unit 0.22 μm (Millipore Corp., Billerica, MA, USA). One million L929 cells were suspended in 1 mL of this cryopreservation solution and stored at -80°C without controlling the cooling rate [7,8].

### 2.5 Cell Viability and Proliferation Assay

After 24 h, the cells were thawed by immersing the vial into a water bath at 37°C. The cell suspension was then diluted 10-fold with DMEM followed by centrifugation at 1000 rpm for 5 min. The supernatant was discarded, and fresh DMEM was added. The cells were then centrifuged again, and the cell pellet was suspended in a small amount of fresh DMEM. A portion of the suspension was then removed to determine cell viability, which was determined by staining with trypan blue. The remaining cells were plated in 6-well culture plates at a cell density of $1 \times 10^4/cm^2$ (n = 5). To determine cell survival, the medium, including dead floating cells, was collected, and the attached cells were trypsinized. All cells were stained with trypan blue and counted using a hemocytometer immediately post-thawing (0 h) and at 6 h after thawing. The reported values are the ratios of living cells to the total number of cells. To study cell

proliferation capacity after thawing, the cells were seeded in 24-well culture plates at a cell density of $5.0 \times 10^4/cm^2$ with 2 mL of DMEM (n = 3). Cell numbers were counted at 2, 4, 5, and 7 days after seeding [7].

### 2.6 Cytotoxicity Assay

L929 cells suspended in 0.1 mL medium at a concentration of $1.0 \times 10^4$/mL were placed in 96-well culture plates. After 72 h of incubation at 37°C, 0.1 mL medium containing different concentrations of polyampholyte solution was added to the cells, followed by incubation for 48 h. To evaluate cell viability, the supernatant was discarded from each of the wells, and 10 μL of Cell Counting Kit-8 reagent (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) and 90 μL DMEM were added to the cultured cells. Cells were then further incubated for 3 h at 37°C. The resulting color intensity, which was proportional to the number of viable cells, was recorded at 450 nm using a microplate reader (MTP-300 Corona Electric). The cytotoxicity was represented as the concentration of copolymers that caused a 50% decrease in the reduction of WST-8 (in CCK-8) by dehydrogenases present in the cell and was compared with the untreated control culture ($IC_{50}$).

### 2.7 Liposome Preparation

Lecithin (12 mg) was isolated from egg (Wako), dissolved in diethyl ether, and poured into a glass test tube. Organic solvent was then evaporated under a gentle stream of $N_2$, and the precipitate was dried under a vacuum overnight. The resulting lipid film was hydrated in 300 μL of 0.1 M CF/PBS solution, and liposomes were formed using a mini-extruder set (Avanti Polar Lipids) and membranes with a 0.1-μm pore size [24].

### 2.8 Leakage Experiment

The external CF from the liposomes was removed by passing the solution obtained after extrusion through a Sephadex G-25 column (NAP-5, GE Healthcare). Liposomes were eluted from the column using PBS and collected in a small vial. The liposomes were then suspended in polyampholyte solutions of various concentrations and stored at -80°C. After 24 h, the solution was thawed by immersing the vial in a 37°C water bath. The solution was then diluted 1000-fold. CF fluorescence was measured using a Fluololog-3 instrument (Horiba Jobin-Yvon, Japan) with an excitation wavelength of 450 nm and a detection wavelength of 520 nm at 25°C. When liposomes remain intact, the fluorescence is strongly quenched, but when liposomes are damaged, the fluorescence increases as CF is released into the surrounding buffer. The maximum CF fluorescence (around 100% leakage) was determined after lysis of the liposomes with Triton X-100, and the percent leakage was calculated relative to these values [25].

### 2.9 Statistical Analysis

All data are expressed as the mean ± standard deviation (SD). All experiments were conducted in triplicate. To compare data among more than 3 groups, the Tukey-Kramer test was used. To compare data between 2 groups, Student's t-test was used.

## 3. Results and Discussion

### 3.1 Characterization

MAA, DMAEMA, and other monomers were polymerized in water-methanol (1:1 [v/v]) solvent. The results of the polymerization are summarized in Table 1. The compositions of each monomer in the polymers were well controlled and similar to the feed ratio. Figure S1 (Supplementary material) shows representative $^{1}$H-NMR charts of poly (DMAEMA-r-MAA) and 5% Bu-MA and Oc-MA incorporated into poly (DMAEMA-r-MAA). The kinetic plots of conversion to polymerization time of poly (DMAEMA-r-MAA) indicated that 80% monomer conversion had been reached after 6 h (Figure 1a). We selected the RAFT agent for this polymerization according to a previous report [26] and succeeded in obtaining not only amphoteric copolymers, but also amphoteric terpolymers harboring hydrophobic moieties in the same solvent and with the same initiator. Although we also tested another RAFT agent (4-cyano-4-(phenylcarbonothioylthio)pentanoic acid) for the same polymerization, the

reaction did not proceed, even after 24 h. These results suggested that the combination of monomers, RAFT agents, and solvents is important to achieve successful living polymerization. The molecular weight and polydispersity (Mw/Mn) are shown in Figure 1b. The Mn values of the 1:1 DMAEMA-MAA copolymer increased linearly with time, while the Mw/Mn ratio remained below 1.4. Furthermore, GPC curves showed that all copolymers had a unimodal distribution with Mn in the range of 2–15 kDa and low polydispersities of 1.2–1.3 (Table 1). The PDIs were relatively high, but comparable with those in another study [26] for polymers synthesized using the same RAFT agent. After 24 h, the NMR charts of all the copolymers and terpolymers showed no peaks ascribed to vinyl protons, indicating that the reaction was completed in 24 h. Figure 1c depicts the relationships among the RAFT agent to monomer ratio, molecular weight, and degree of polymerization of the 1:1 DMAEMA-MAA copolymer. The degree of polymerization was proportionally decreased with the increase in RAFT agent/monomer ratio. These results suggested that these amphoteric polymers were successfully synthesized with controlled radical polymerization.–

### 3.2 Cryoprotective Properties of These Polyampholytes

#### 3.2.1 Effects of Monomer Ratios

The ratio of monomers (MAA and DMAEMA) in the copolymer was varied in order to optimize conditions for cryoprotection. For this, 3 different types of copolymers with different ratios of monomers were prepared, and cell viability after cryopreservation with 15% polymer/DMEM solutions without FBS was calculated. The copolymer synthesized with a 1:1 ratio of MAA to DMAEMA showed the highest cell viability (Figure 2), as compared to copolymers synthesized with 2:3 and 3:2 ratios. This result was similar to the results presented in our previous report [7,8], which showed that appropriate amounts of positively and negatively charged groups were needed to confer high cryoprotective properties. A cell viability of over 90%, as achieved in the current study, was similar to that achieved with the commonly used cryoprotectant DMSO. After 6 h of culture, cell viability did not decrease, indicating that live cells were well protected immediately after thawing and that the evaluation by trypan blue staining was appropriate for viability measurement. Thus, for subsequent experiments, we prepared and modified the polyampholyte based on that synthesized with positive and negative monomers in a 1:1 ratio.

### 3.2.2 Effects of Hydrophobicity

In our previous report, when cell were frozen in a polyampholyte solution, the polyampholyte molecules were attached onto the cell membrane, as determined by

fluorescent-conjugated polyampholyte detection with confocal laser microscopy [10]. This indicates that the mechanism through which the polyampholyte acts as a cryoprotectant probably differs from that of DMSO. Other nonpenetrating polymeric cryoprotectants, such as hydroxymethyl starch (HES), have also been reported [27]. Previous studies have shown that HES attracts and absorbs water. Its viscosity is then reduced, and the rate of dehydration increases, which allows cells to be cooled rapidly, thereby avoiding intracellular ice crystal formation as well as chilling injury. However, HES showed various cryopreservation properties that depended on the type of cell line used, and therefore, its use as a cryoprotectant is limited by its weak cryoprotective properties [28]. Thus, the cryoprotective mechanism of HES may be similar to that of polyampholytes by providing extracellular protection of the cell membrane. To enhance the cryoprotective properties of these membrane-protective cryoprotectants, we sought to increase the cell membrane interaction of the polyampholytes by introduction of hydrophobic group(s) in the polymer backbone. The alkyl chain is highly hydrophobic, and amphiphilic polymers, such as polyethylene glycol and polyvinyl alcohol, in which the alkyl chain was introduced, show cell membrane attachment via hydrophobic interactions between membrane lipids and the alkyl chain [29]. To identify the effects of hydrophobicity, a small amount of a hydrophobic alkyl chain monomer, i.e., n-butyl methacrylate (Bu-MA) or n-octyl methacrylate (Oc-MA), was introduced into the 1:1

copolymer. Different amounts of Bu-MA were introduced, and cell viability after cryopreservation was measured. As shown in Figure 3a, the viability of L929 cells cryopreserved with 15% poly (DMAEMA-co-MAA) solution including various concentrations of Bu-MA was enhanced. A similar effect was observed in cells frozen in 7.5% polymer with 5% Oc-MA (Figure 3b), which showed almost double the viability of cells frozen in the polyampholyte without the hydrophobic moiety. Figure 3c shows the cell viability after cryopreservation with hydrophilic and hydrophobic polyampholyte solutions at the same polymer concentration (10%). Under these conditions, and with the same ratio of hydrophilic and hydrophobic moieties, the cryoprotective properties of the solution were strongly correlated with hydrophobicity.

### 3.2.3 Effects of Molecular Weight

Next, we examined variations in the viability of L929 cells after cryopreservation with 1:1 copolymer solutions having different molecular weights. As shown in Figure 4a, cell viability increased with increasing molecular weight of the copolymer. One more copolymer, i.e., 1:1 copolymer with 8% Bu-MA, was also synthesized at different molecular weights, and its effects on cell viability were also examined in L929 cells. Again, cell viability increased when the molecular weight of the copolymer was increased (Figure 4b). A previous study also demonstrated that high-molecular-weight

HES was more effective as a cryoprotectant than low-molecular-weight HES in Chinese hamster cells [30]. In general, the cryoprotective effects of polymers such as HES depend on its ability to absorb water molecules and keep these thermally inert in a glassy state without experiencing any phase transitions during cooling. Absorption of water molecules depends on the molecular weight and concentration of the molecule. Although further research should be conducted, our current data strongly support the hypothesis that the protective properties of these cryoprotectants may be related to water absorption, which is dependent on the molecular weight of the compound. Thus, careful control of molecular weight using RAFT polymerization may be effective in the development of polyampholyte cryoprotectants.

### 3.3 Leakage Experiment

We have previously used a soluble fluorescent dye (CF) to investigate the protective effect that polyampholytes provide against leakage during freezing and thawing [31]. In the present study, we used CF again to analyze leakage in L929 cells. When no polyampholyte was added to L929 cells before cryopreservation, fluorescent leakage was maximal (Figure 5). However, when the amount of polyampholyte solution was increased, leakage began to decrease, indicating that liposomes were protected when the polyampholyte solution was added. This trend was again observed when a small amount of hydrophobic monomer (Bu-MA) was introduced into the 1:1 copolymer; the

fluorescent intensity decreased compared to the intensity when no hydrophobic monomer was present. Introduction of Oc-MA in particular yielded the highest liposome protection properties, and less than 10% leakage was seen with a 10% polymer solution, in accordance with the results of cell viability after cryopreservation. These results indicated that hydrophobicity provided more efficient protection of the cell membrane during cryopreservation. Since freezing is a biologically relevant stress factor that involves a dramatic decrease in water activity, as shown in many previous publications [32], freezing of liposomes without a protectant results in complete lysis of the vesicles. Thus, our experiment using freeze/thaw cycles directly showed that polyampholytes are able to stabilize phospholipid bilayers under freezing stress conditions.

These results strongly supported our hypothesis that polyampholytes protect the cell membrane during freezing. In the literature, many proteins that protect cells from the damage induced during freezing have been reported, especially in freezing-tolerant plants, anhydrobiotic invertebrates, and fungi [33]. Interestingly, these proteins share many common characteristics, including highly charged polyampholytes with hydrophobic moieties [34,35]. According to a report by Tolleter et al., [31] polyampholitic protein, which is classified as late embryogenesis abundant (LEA) protein, protects the liposome from desiccation and freezing damage with membrane

attachments via electrostatic interactions between LEA protein molecules, which have many positive and negative charges, and phospholipid molecules. Although the mechanisms of protection are still unknown, a relationship may exist between these proteins and our synthesized polyampholytes.

### 3.4 Biocompatibility of the novel Polyampholytes

The novel polyampholytes had a much higher $IC_{50}$ in L929 cells than DMSO ($IC_{50}$ = 2.519%) at 48 h after thawing (Figure 6a), indicating that these synthetic polyampholytes were much less cytotoxic than DMSO. This result agrees with our previous report, which concluded that polyampholytes whose positive and negative charges are balanced have low cytotoxicity [7]. Moreover, introduction of a small amount of hydrophobic moiety to the completely synthetic polyampholyte further increased biocompatibility over that of DMSO.

When L929 cells were cryopreserved with the polyampholytes and subsequently cultured in DMEM supplemented with 10% FBS, the cells proliferated well after 7 days (Figure 6b). After 7 days of culture, cell numbers for cryopreserved cells were not significantly reduced compared to those of the unfrozen control. These data suggested that the novel polyampholytes may be useful for cell cryopreservation in research and clinical applications due to its lower cytotoxicity and negligible effects on cell

proliferation after thawing. The advantages of the polyampholytes synthesized by RAFT polymerization in terms of cryoprotective properties were clearly indicated, and we expect that the control of dispersity, composition, and precise polymerization, such as block copolymerization, may enable researchers to prepare cryoprotective agents with high cell viability and to elucidate the mechanisms of cryoprotection of polyampholytes.

## 4. Conclusion

We have successfully demonstrated that synthetic polyampholytes made of methacrylic acid and 2-(dimethylamino)ethyl methacrylate can efficiently cryopreserve various types of cells without the requirement for any other cryoprotectants. Additionally, introduction of hydrophobicity and an increase in molecular weight promoted cell viability after thawing. Leakage experiments suggested that polyampholytes protected the cell membrane during cryopreservation, and this effect was enhanced by increased hydrophobicity. Moreover, due to low cytotoxicity, these polyampholytes have the potential to replace the conventionally used cryoprotective agent DMSO. The present study is the first to show that we can design a polymeric cryoprotectant that will protect the cell membrane during freezing using appropriate polymerization techniques.

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**Disclosures**

The authors have no conflicts of interest to declare.

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**Table 1**

Characteristics of various polyampholytes prepared via RAFT polymerization.

| Entry | | Composition | | | | | Molar ratio[b)] | $Mn \times 10^{-3}$[,c)] | Mw/Mn[c)] |
|---|---|---|---|---|---|---|---|---|---|
| | | DMAEMA | MAA | Bu-MA | Oc-MA | HEMA | | | |
| 1 | In feed | 50 | 50 | 0 | 0 | 0 | 100:1:5 | 4.9 | 1.21 |
| | In polymer[a)] | 50.3 | 49.7 | 0 | 0 | 0 | | | |
| 2 | In feed | 50 | 50 | 0 | 0 | 0 | 250:1:5 | 14.7 | 1.49 |
| | In polymer[a)] | 50.4 | 49.6 | 0 | 0 | 0 | | | |
| 3 | In feed | 66 | 33 | 0 | 0 | 0 | 125:1:5 | 7.12 | 1.57 |
| | In polymer[a)] | 67.5 | 32.5 | 0 | 0 | 0 | | | |
| 4 | In feed | 33 | 66 | 0 | 0 | 0 | 125:1:5 | 4.2 | 1.20 |
| | In polymer[a)] | 32.2 | 67.8 | 0 | 0 | 0 | | | |
| 5 | In feed | 49 | 49 | 2 | 0 | 0 | 102:1:5 | 4.9 | 1.32 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | In polymer[a)] | 49.3 | 48.7 | 2.0 | 0 | 0 | | | |
| 6 | In feed | 47.5 | 47.5 | 5 | 0 | 0 | 105:1:5 | 5.05 | 1.36 |
| | In polymer[a)] | 47.7 | 47.3 | 5.0 | 0 | 0 | | | |
| 7 | In feed | 46 | 46 | 8 | 0 | 0 | 54:1:5 | 2.3 | 1.19 |
| | In polymer[a)] | 46.1 | 45.5 | 8.4 | 0 | 0 | | | |
| 8 | In feed | 46 | 46 | 8 | 0 | 0 | 108:1:5 | 4.9 | 1.28 |
| | In polymer[a)] | 46.6 | 45.2 | 8.2 | 0 | 0 | | | |
| 9 | In feed | 49 | 49 | 0 | 2 | 0 | 102:1:5 | 4.95 | 1.23 |
| | In polymer[a)] | 49.4 | 48.6 | 0 | 2.0 | 0 | | | |
| 10 | In feed | 48.5 | 48.5 | 0 | 3 | 0 | 103:1:5 | 5.03 | 1.14 |
| | In polymer[a)] | 48.2 | 48.3 | 0 | 3.5 | 0 | | | |
| 11 | In feed | 48 | 48 | 0 | 4 | 0 | 104:1:5 | 5.15 | 1.25 |
| | In | 48.5 | 47.6 | 0 | 3.9 | 0 | | | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | polymer[a)] | | | | | | | | |
| 12 | In feed | 47.5 | 47.5 | 0 | 5 | 0 | 105:1:5 | 5.36 | 1.32 |
| | In polymer[a)] | 47.6 | 47.5 | 0 | 4.9 | 0 | | | |
| 13 | In feed | 47.5 | 47.5 | 0 | 0 | 5 | 105:1:5 | 4.3 | 1.31 |
| | In polymer[a)] | 48.1 | 46.5 | 0 | 0 | 5.4 | | | |

a) Determined by $^{1}$H-NMR, b) [monomer]:[initiator]:[RAFT agent] in molar ratio.

c) Determined by GPC.

**Figure Captions**

**Figure 1.** Characterization of RAFT polymerization products. (a) Kinetic plot for the conversion vs. time of the 1:1 MAA-DMAEMA copolymer. (b) Plots of time vs. Mw/Mn and time vs. Mn for the polymerization of the 1:1 MAA-DMAEMA copolymer. (c) Plots of the concentration of RAFT agent used vs. the degree of polymerization and the concentration of RAFT agent used vs. the molecular weight.

**Figure 2.** Cryoprotective properties of copolymers with different copolymer ratios. L929 cells were cryopreserved with MAA-DMAEMA copolymers synthesized using different ratios of MAA and DMAEMA (15% polymer concentration).

**Figure 3.** Effects of hydrophobicity of polyampholytes on cryopreservation. (a) L929 cells were cryopreserved with polyampholytes synthesized with a 1:1 ratio of MA and DMAEMA and different concentrations of Bu-MA (15% polymer concentration). (b) L929 cells were cryopreserved with polyampholytes synthesized with a 1:1 ratio of MA

and DMAEMA and different concentrations of Oc-MA at various polymer concentrations. (c) L929 cells were cryopreserved with different polymers (10% polymer concentration) and 5% Bu-MA or Oc-MA. A comparison with a polymer containing the hydrophilic monomer HEMA is shown. Data are expressed as the mean ± SD of 3 independent experiments (5 samples each). *** $P < 0.001$.

**Figure 4.** Effects of the molecular weight of polyampholytes on cryopreservation. (a) L929 cells were cryopreserved with the 1:1 MAA-DMAEMA copolymer (10% polymer concentration) at different molecular weights. (b) L929 cells were cryopreserved with different concentrations of polyampholytes synthesized with a 1:1 ratio of MA and DMAEMA and 8% Bu-MA having 2 different molecular weights. Data are expressed as the mean ± SD for 3 independent experiments (5 samples each). *** $P < 0.001$, ** $P < 0.05$.

**Figure 5.** Protection of liposomes during freezing by polyampholytes. CF leakage from liposomes cryopreserved with various polyampholytes solutions at different polymer concentrations. Data are expressed as the mean ± SD for 3 independent experiments (5 samples each).

**Figure 6.** Cytocompatibilities of polyampholytes. (a) Cytotoxicity of DMSO (open circles), 1:1 MAA-DMAEMA copolymer (closed circles), 1:1 MAA-DMAEMA copolymer with 5% Oc-MA (open triangles), and 1:1 MAA-DMAEMA copolymer with 5% Bu-MA (closed triangles). L929 cells were incubated with the indicated concentration of each compound for 48 h, followed by CCK assay. Data are described as the percentage of viable cells as compared to the number of untreated cells. Mean values and standard deviations for independent triplicate experiments (8 samples each) are shown. (b) Growth curves of L929 cells, unfrozen control (open circles), cryopreserved with 1:1 MAA-DMAEMA copolymer (closed circles), 1:1 MAA-DMAEMA copolymer with 5% Oc-MA (open triangles), and 1:1 MAA-DMAEMA copolymer with 5% Bu-MA (closed triangles) for 7 days. Data are expressed as the mean ± SD of 3 independent experiments.

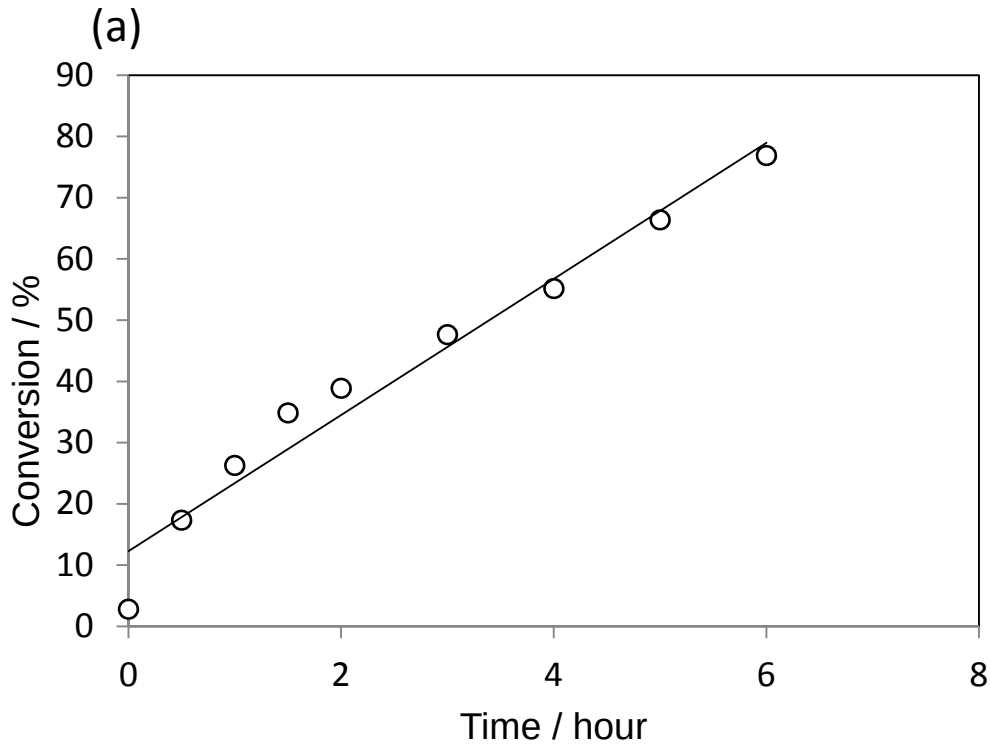


Fig1a

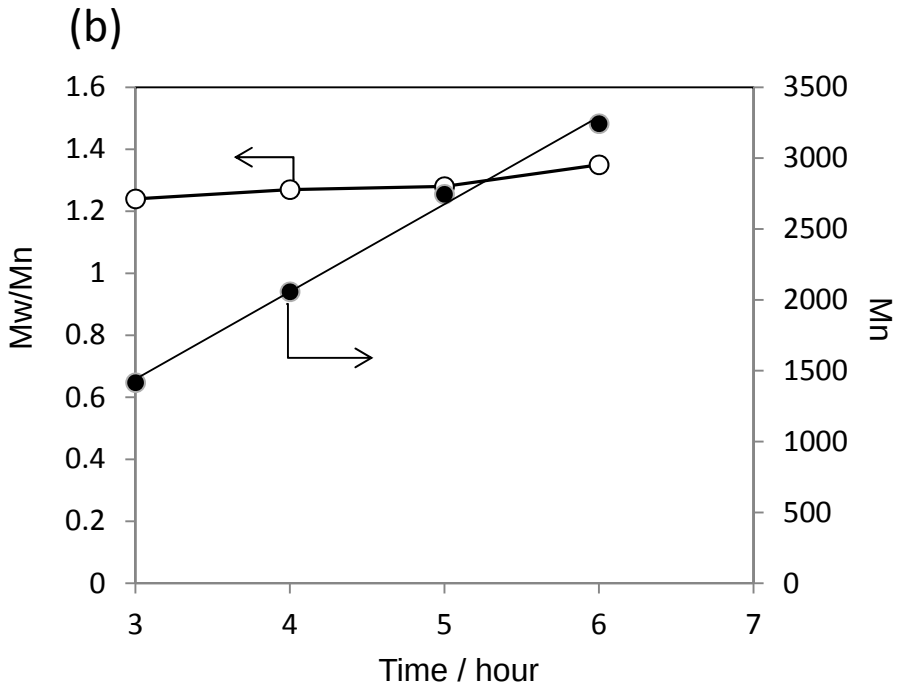
(b)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Mw/Mn
3500
3000
2500
2000
1500
1000
500
0
Mn
3
4
5
6
7
Time / hour

Fig1b

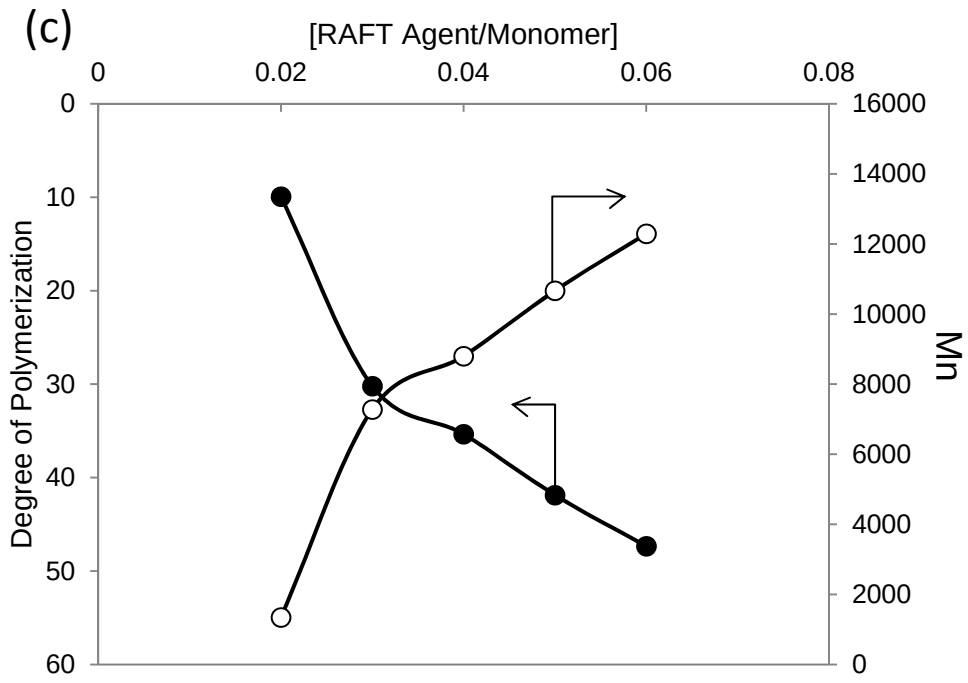
(c)
[RAFT Agent/Monomer]
0
0.02
0.04
0.06
0.08
Degree of Polymerization
0
10
20
30
40
50
60
Mn
16000
14000
12000
10000
8000
6000
4000
2000
0

Fig1c

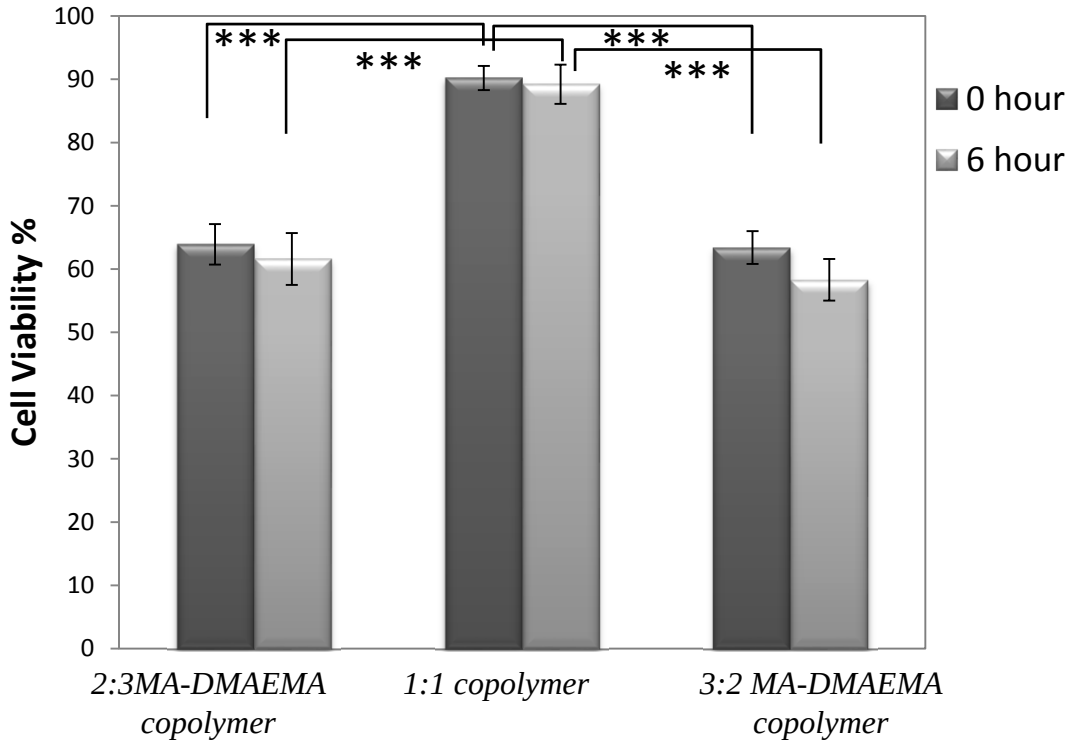
100
90
80
70
60
50
40
30
20
10
0
Cell Viability %
***
***
***
***
0 hour
6 hour
2:3MA-DMAEMA copolymer
1:1 copolymer
3:2 MA-DMAEMA copolymer

Fig2

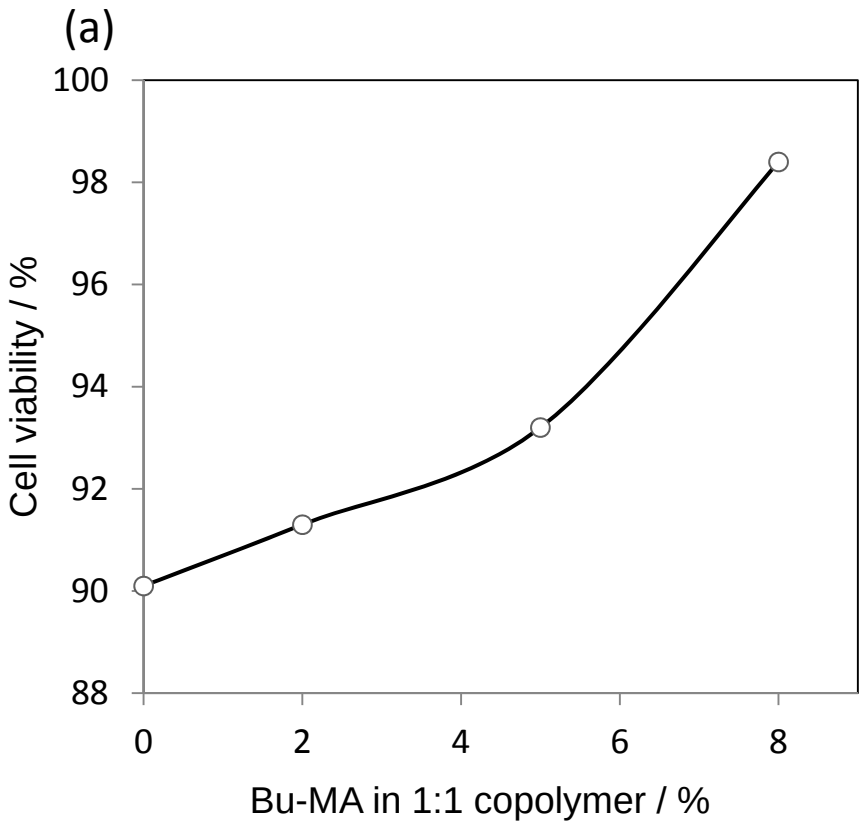
(a)
100
98
96
94
92
90
88
Cell viability / %
0
2
4
6
8
Bu-MA in 1:1 copolymer / %

Fig3(a)

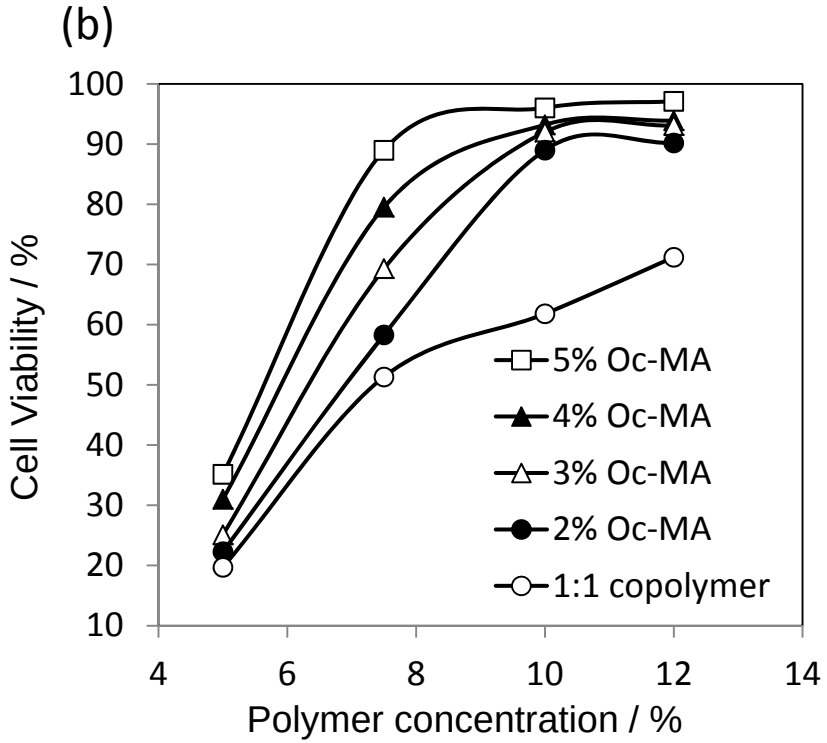
(b)
Cell Viability / %
100
90
80
70
60
50
40
30
20
10
4
6
8
10
12
14
Polymer concentration / %
5% Oc-MA
4% Oc-MA
3% Oc-MA
2% Oc-MA
1:1 copolymer

Fig3(b)

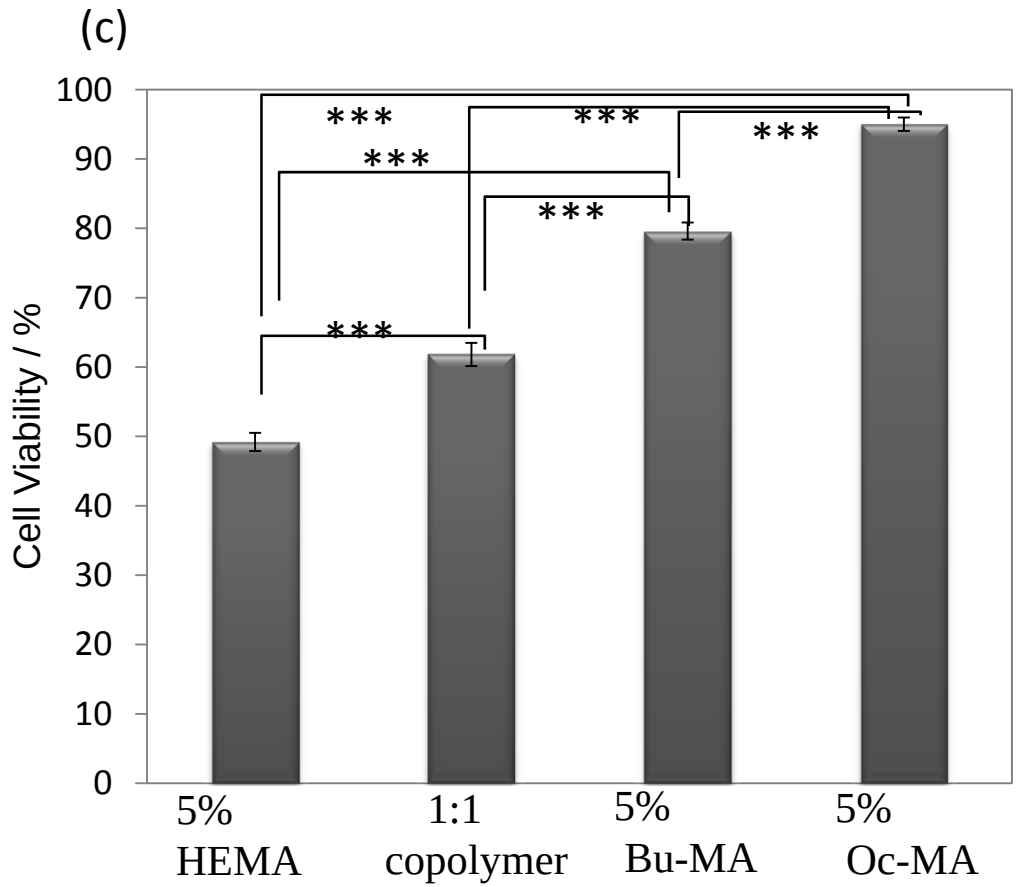
(c)
Cell Viability / %
100
90
80
70
60
50
40
30
20
10
0
***
***
***
***
***
5% HEMA
1:1 copolymer
5% Bu-MA
5% Oc-MA

Fig3(c)

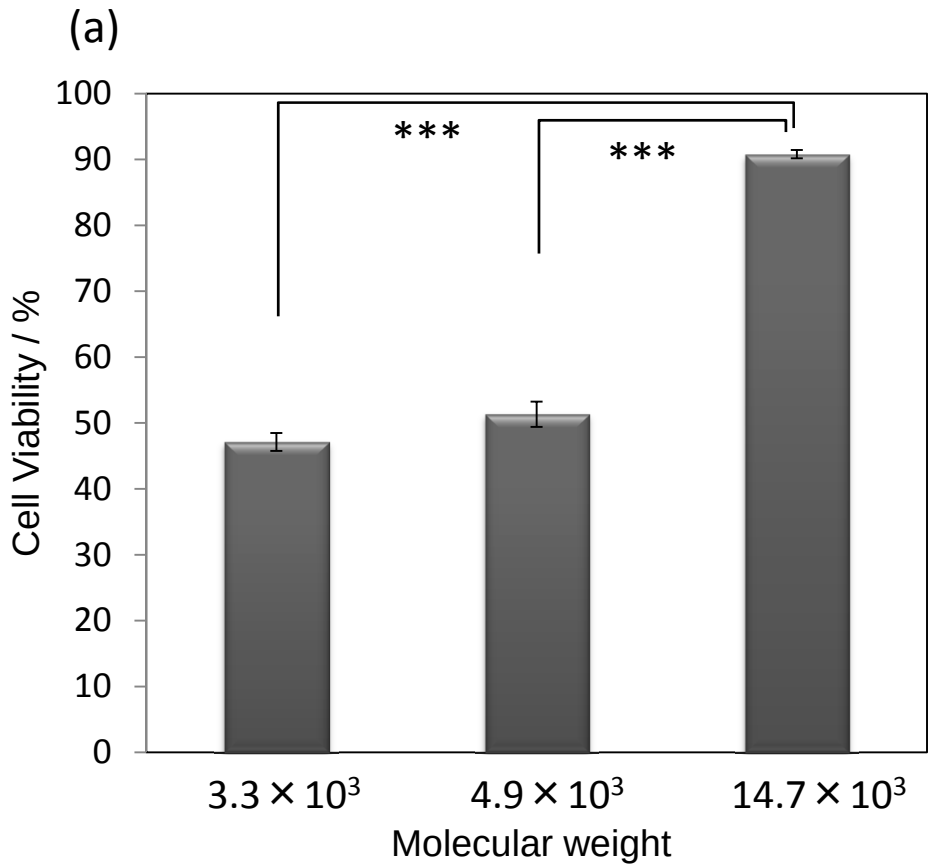
(a)
100
90
80
70
60
50
40
30
20
10
0
Cell Viability / %
***
***
3.3 × 10³
4.9 × 10³
14.7 × 10³
Molecular weight

Fig.4(a)

(b)

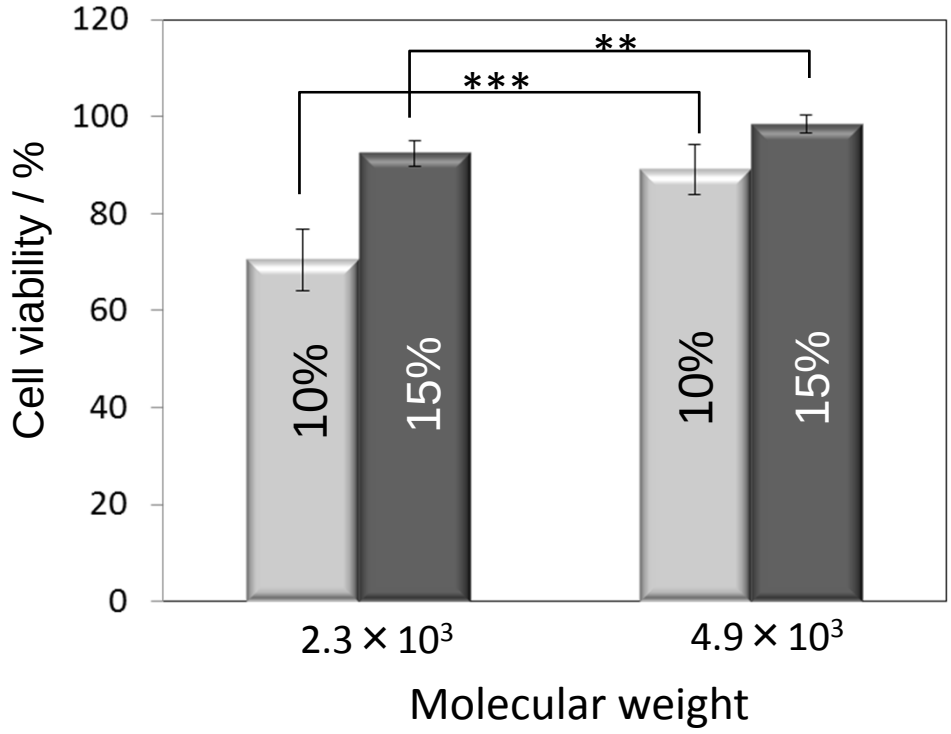
120
100
80
60
40
20
0
Cell viability / %
**
***
10%
15%
10%
15%
2.3 × 10³
4.9 × 10³
Molecular weight

Fig.4(b)

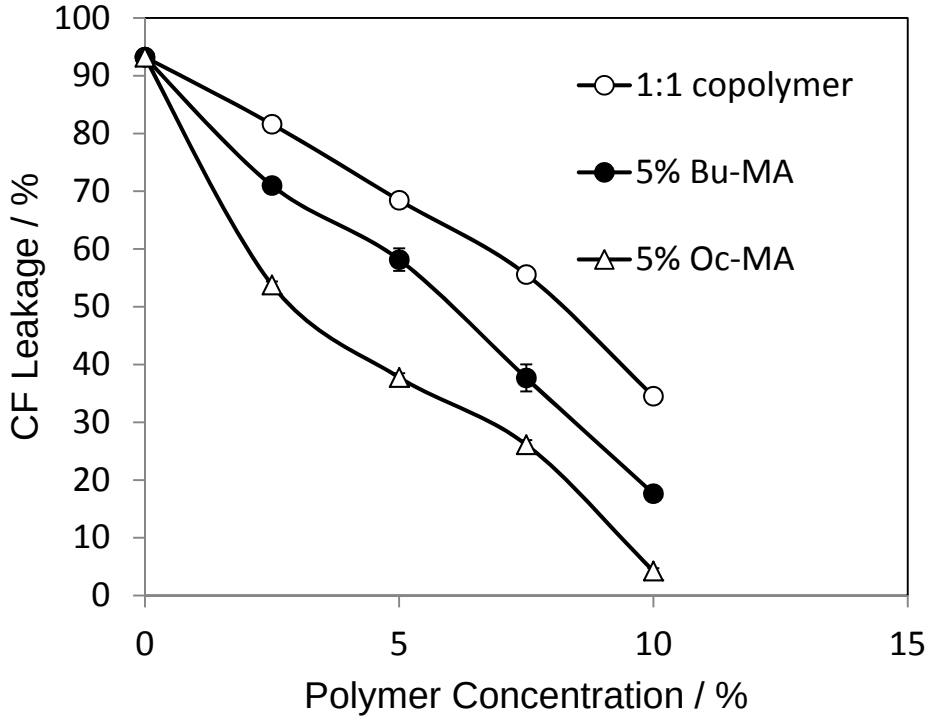
100
90
80
70
60
50
40
30
20
10
0
CF Leakage / %
0
5
10
15
Polymer Concentration / %
1:1 copolymer
5% Bu-MA
5% Oc-MA

Fig.5

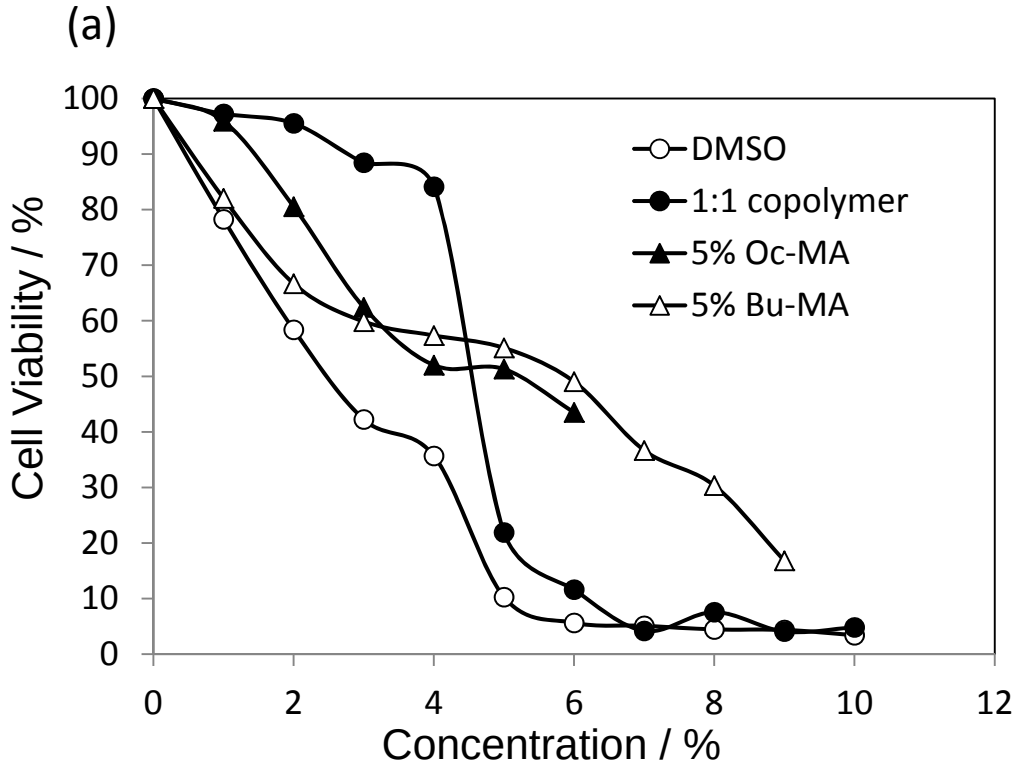
(a)
Cell Viability / %
Concentration / %
DMSO
1:1 copolymer
5% Oc-MA
5% Bu-MA
0
10
20
30
40
50
60
70
80
90
100
0
2
4
6
8
10
12

Fig.6(a)

(b)

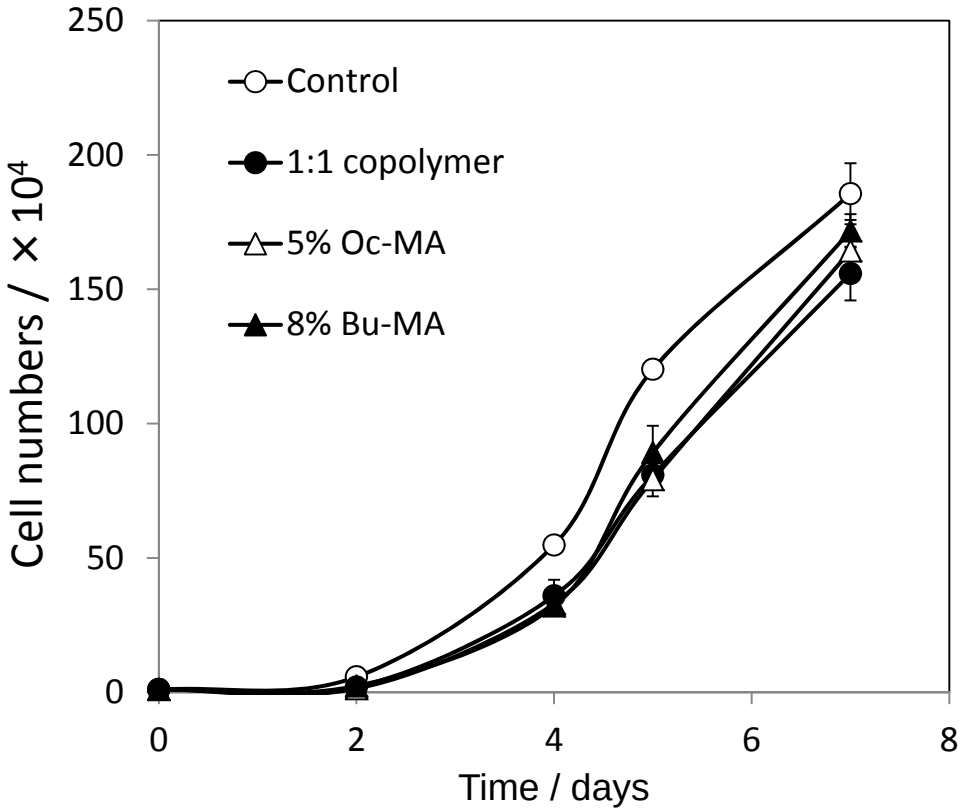
250
200
150
100
50
0
Cell numbers / × 10⁴
Control
1:1 copolymer
5% Oc-MA
8% Bu-MA
0
2
4
6
8
Time / days

Fig.6(b)