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Preparation of Novel Synthetic Cryoprotectants

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We developed various novel synthetic polyampholytes *via* reversible addition fragmentation chain transfer (RAFT) polymerization for cryopreservation of cells. The polymers, in spite of being structurally analogous with each other, showed very different cryoprotective properties. Copolymer of methacrylic acid (MAA) and N, N-dimethylaminoethyl methacrylate (DMAEMA) showed excellent cryoprotective property and the hydrophobic modification of the copolymer enhanced the cell viability significantly. On the other hand, another polymer with similar structure, poly-carboxymethyl betaine (poly-CMB), a zwitterion-type polyampholyte, exhibited no cryoprotective property and the addition of hydrophobicity did not have much effect on the cell viability, whereas a similar zwitterion type polyampholyte, poly-sulfobetaine (poly-SPB), exhibited intermediate cryoprotective property. These findings suggest that cryoprotective property depends to a great extent on the polymer structure and the structural differences affect the interaction among polymer chains in solution.

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INTRODUCTION

Cryopreservation refers to the process, in which cells, organs, tissues, etc. are stored at very low temperature, and can be recovered back to its original state at any time. The first ever reported cryopreservation process was carried out by Polge et al.[1] for the preservation of sperm cells using glycerol. Years later, another group[2] reported the use of dimethyl sulfoxide (DMSO) for the cryopreservation of red blood cells. DMSO shows high toxicity[3] and affects the differentiation of various types of cells. These inadequacies lead to the development of more efficient cryoprotectants.

Previously, our group reported that carboxylated poly-L-lysine (COOH-PLL), a polyampholyte, showed excellent cryoprotective properties[4] and did not require the addition of any other low-molecular-weight cryoprotectant or protein. A Few years later, synthetic polyampholyte synthesized *via* reversible addition fragmentation chain transfer (RAFT) polymerization also displayed excellent cryoprotective properties and its properties could be tuned very easily by modifying parameters like hydrophobicity and molecular weight. This polymer was synthesized from copolymerization of methacrylic acid (MAA) and N, N-dimethylaminoethyl methacrylate (DMAEMA)[5].

RAFT Polymerization was employed for the synthesis of the polyampholytes because of its well-documented versatility such as its application to a wide range of functional and nonfunctional monomers under an array of reaction conditions and solvents[6]. The first report about the likelihood of

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performing controlled radical polymerization using dithiocarbonyl compounds surfaced in 1998[7]. The report described that polymers with pre-determined molecular mass and structure can be procured easily.

In the present study, propensity of polyampholytes to cryopreserve cells was examined by preparing structurally analogous synthetic polyampholytes. Herein, we used poly-CMB and poly-SPB with the aim to develop new cryoprotective agents as well as to check their ability to cryopreserve cells, irrespective of its structure.

MATERIALS AND METHODS

Synthesis of poly-(MAA-DMAEMA)

Poly-(MAA-DMAEMA) was prepared as described in our previous study[5]. Briefly, DMAEMA, MAA (Wako Pure Chem. Ind. Ltd., Osaka, Japan), 2-(dodecylthiocarbonothioylthio) -2-methylpropionic acid (RAFT agent, Sigma–Aldrich), and V-501 (initiator, TCI, Tokyo, Japan) were added to a reaction vial, and 20 mL of water–methanol mixture (1:1 [v/v]) was then added (Fig. 1a). The solution was purged with nitrogen gas for 1 hour and stirred at 70°C. After 24 hours, the reaction mixture was precipitated using 2-propanol (nacalai tesque, Inc., Kyoto, Japan), the precipitates were collected by centrifugation, and the compound was dried over vacuum.

Synthesis of poly-CMB

Carboxymethyl betaine (CMB) monomer (Osaka Organic Chem. Ind. Ltd., Osaka, Japan), 2-(dodecylthiocarbonothioylthio)-2-methylpropionic acid, azobisisobutyronitrile (AIBN) (initiator, Wako Pure Chem. Ind. Ltd., Osaka, Japan) were dissolved in ethanol (nacalai tesque Inc., Kyoto, Japan). The solution was purged with nitrogen gas for 1 hour and stirred at 70°C (Fig. 1b). After 48 hours, the reaction mixture was precipitated using 2-propanol, the precipitates were collected by centrifugation, and the compound was dried over vacuum.

Synthesis of poly-SPB

Sulfobetaine (SPB) monomer (Osaka Organic Chem. Ind. Ltd., Osaka, Japan), 2-(dodecylthiocarbonothioylthio)-2-methylpropionic acid, AIBN were dissolved in methanol-water mixture (3:1 v/v %). The solution was then purged with nitrogen gas for 1 hour and stirred at 70°C (Fig. 1c). After 6 hours, the reaction mixture was dialyzed against methanol and water successively for 24 hours each with constant change of solvent. The polymer was then obtained after lyophilization.

Introduction of hydrophobicity

To introduce hydrophobic moieties to the polyampholytes, 1–10% of the total monomer amount of n-butyl methacrylate (Bu-MA) or n-octyl methacrylate (Oc-MA) was added in the reaction mixture. After the reaction, samples were removed periodically (25 μL), and the conversion at each reaction time was obtained by ^{1}H NMR (400MHz, Bruker).

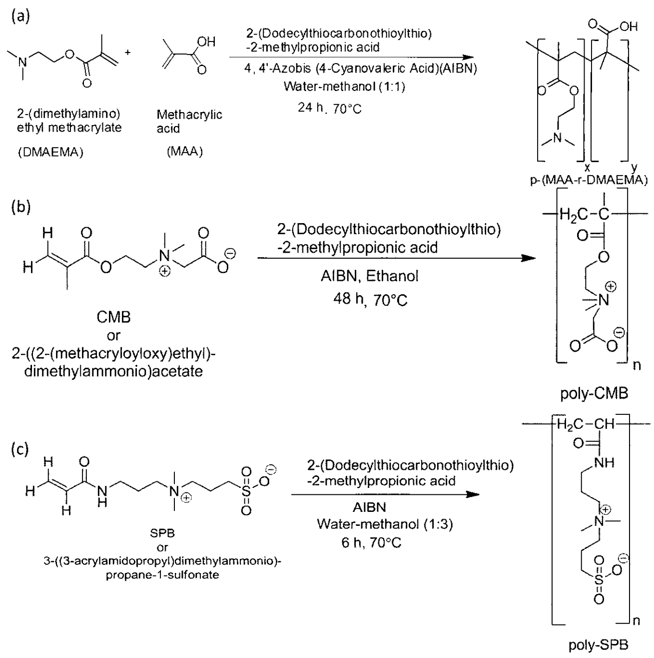

Fig. 1. Schematic illustration of the living radical (RAFT) polymerization of (a) MAA and DMAEMA, (b) CMB, (c) SPB.

Cryopreservation of cells

Polyampholyte solutions were prepared in DMEM without FBS at 5–15% concentrations. The pH was adjusted to 7.4 by using HCl or NaOH, and the osmotic pressure was adjusted to 500 mmol/kg by the addition of sodium chloride and measured using a vapor pressure Osmometer (VAPRO Model 5660, WESCOR Biomedical Systems, UT, USA). The solutions were filter sterilized using a MILLEX GP Filter Unit 0.22 μm (Millipore Corp., Billerica, MA, USA) and one million L929 cells were suspended in 1 mL of this solution and stored at -80°C without controlling the cooling rate. Each cryo-vials were thawed in a water bath at 37°C with gentle shaking, followed by 10-fold dilution with DMEM followed by centrifugation at 1000 rpm for 5 minutes. The cells were centrifuged, and the supernatant was removed; the cell pellet was then resuspended in 5 mL of medium. The supernatant was discarded, and fresh DMEM was added. The cells were centrifuged again, and the cell pellet was suspended in a small amount of fresh DMEM. A portion of the suspension was subsequently removed to determine cell viability, which was determined by staining with trypan blue.

RESULTS AND DISCUSSION

Characterization of polyampholytes

Polymerization of the monomers was investigated and their conversion to the respective polymers was determined using ^{1}H-NMR. Time dependent NMR study of poly-SPB showed that the polymerization gets completed within 6 hours as indicated by the disappearance of vinyl protons (5.7 and 6.2 ppm). Poly-CMB on the other hand does not reach completion after 48 hours of the reaction. Even after prolonged reaction for more than 60 hours, a maximum of around 98% monomer gets converted to the polymer. Kinetic plots of the polymers indicated that living polymerization was successfully carried out as displayed by the linear relationship between the conversion with the reaction time (Fig. 2), indicating that it follows first order kinetics[8]. In case of poly-(MAA-DMAEMA), 80% of the monomers were converted within the first 6 hours, whereas poly-CMB and poly-SPB exhibited 80% conversion within 2 hours of the reaction. Also molecular weight of each polymer showed almost 4000-5000 and the molecular weight distribution (M_w/M_n) showed between 1.2 and 1.5 by gel permeation chromatography, indicating that the living polymerization was successfully performed.

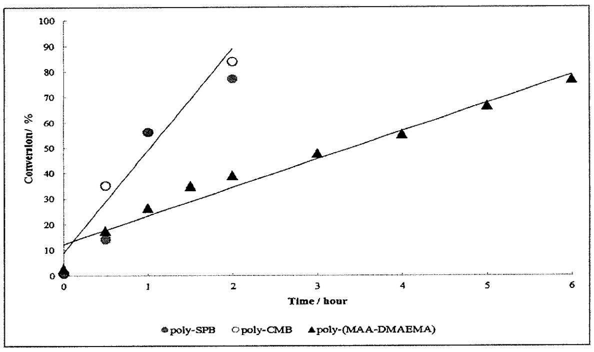

Fig. 2. Kinetic plot for the conversion vs. time of poly-SPB (closed circle), poly-CMB (open circle) and poly-(MAA-DMAEMA) (closed triangle).

Cryopreservation properties of polyampholytes

From the result of Fig. 3, Poly-(MAA-DMAEMA) with a 1:1 ratio of MAA and DMAEMA showed the highest cell viability after thawing. It exhibited excellent cell viability of over 90% at 15% polymer concentration. In the case of poly-CMB, almost all the cells were found to be dead after cryopreserving for 24 hours. This

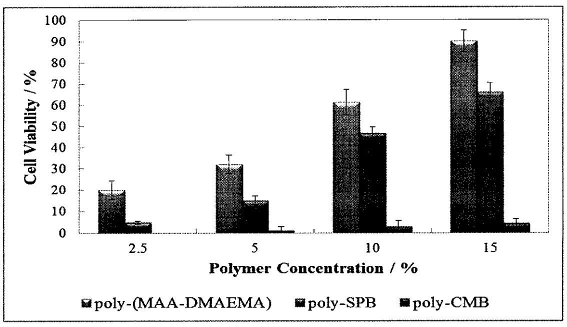

Fig. 3. Cryoprotective properties of polymers at different polymer concentration with L929 cells. Data are expressed as the mean ± SD for 3 independent experiments (5 samples each).

may be due to the tendency of polymer chains to undergo association in solution[9]. According to a previous report[10], zwitterion polymers fold intramolecularly into a loop conformation in which positively charged group interacts with the negatively charged group. Previous studies revealed that polyampholytes interact with the cell membrane during freezing and protects it from damage. Another study on the polyampholytes reported that polyampholytes act as a cryoprotective agent by protecting cells from stresses such as drastic changes in soluble space size and osmotic pressure[11]. The presence of charged moieties is required to trap water and salt. Apparently, these intramolecular interactions render the polymer unable to interact with the cell membrane electrostatically and thus becoming inadequate to protect the cell membrane. The increase in polymer concentration did not seem to have any significant effect on cell viability. Poly-SPB on the other hand showed intermediate cryoprotective properties between poly-(MAA-DMAEMA) and poly-CMB. It showed around 65% cell viability at 15% polymer concentration. This may probably be due to less intramolecular association as a result of longer distance between the positively and the negatively charged groups.

Effect of hydrophobicity

In our previous study, the introduction of monomers like Bu-MA and Oc-MA to the polyampholyte significantly improved cell viability[5]. Previously it was reported that, in amphiphilic polymers such as polyethylene glycol and polyvinyl alcohol, introduction of hydrophobicity showed enhanced cell membrane attachment via hydrophobic interactions between membrane lipids and the alkyl chain[12]. Another report on lysine based surfactants/gelators suggested that long alkyl chains are crucial for ice recrystallization inhibitory action (IRI)[13]. When cells are cryopreserved, freezing-induced damage

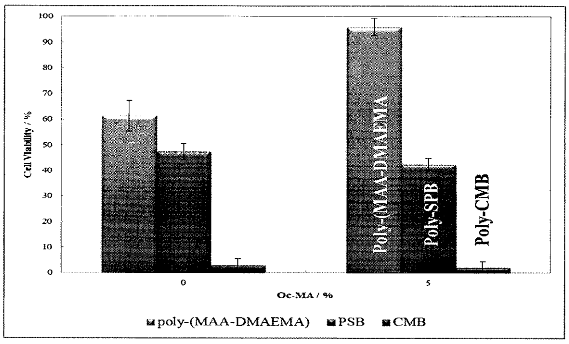

Fig. 4. Effects of hydrophobicity of polyampholytes on cryopreservation. L929 cells were cryopreserved without (0% Oc-MA) and with 5% Oc-MA (at 10% polymer concentration). Data are expressed as the mean ± SD for 3 independent experiments (5 samples each).

occurs because of intracellular ice formation, and its growth through the process of ice recrystallization leads to cell death[14]. IRI activity was found to enhance the cryopreservation of sheep and human red blood cells[15]. In the case of poly-CMB, introduction did not affect cell viability (Fig. 4). Therefore, it can be concluded that poly-CMB does not show any cryoprotective property in the presence or absence of hydrophobic moiety. On the other hand, introduction of hydrophobicity in poly-SPB did not enhance cell viability. In fact, the cell viability decreased slightly on introduction of hydrophobicity. In future, we should investigate the interaction of the polyampholytes (with and without the hydrophobic monomers) as well as their localization and orientation around the cell membrane to completely understand the mechanism. Although further study is required to find out to completely understand the molecular mechanism of cryopreservation by polyampholytes, we reported in this study that novel synthetic polyampholytes showed high cryoprotective properties against mammalian cell line.

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