Antimicrobial activities of low molecular weight polymers synthesized through soap-free emulsion polymerization

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Abstract

To evaluate the influence of low molecular weight polymers synthesized through soap-free emulsion polymerization on the indicator microorganism, *Micrococcus luteus*, methyl methacrylate (MMA) and styrene were polymerized by using various initiators. The MMA polymer colloid was not toxic to *M. luteus*. High volumes of polystyrene were continuously synthesized, and low-molecular weight polystyrenes were removed from the suspension to the supernatant. During the bioassay test, as the molecular weight of the cationic polystyrene in the supernatant decreased, the inhibition zone against *M. luteus* grew larger. Low molecular weight cationic polystyrene was toxic to *M. luteus*. Hence, the supernatant containing low molecular weight cationic polystyrenes (particularly those with molecular weights below 1000 g/mol) obtained from soap-free emulsion polymerization should be treated carefully prior to its discharge to the environment. Moreover, such supernatants should be treated with more care if they are to be discharged into seas or rivers. One solution to reduce the toxicity of the supernatant against *M. luteus* is the addition of *N*-vinylacetamide monomers during polymerization.

Keywords: Low molecular weight polystyrene, Antimicrobial activity, Indicator microorganism

1. Introduction

Polymeric particulate materials are used by many industries in products, such as toner, cosmetics, and medicines [1]. These materials are synthesized through emulsion polymerization with surfactants or stabilizers [2-4]. Such polymerization processes require the use of surfactants and therefore their toxicity against microbes has been studied [5, 6]. Furthermore, we should consider the environmental impacts of the surfactants present in the wastewater discharged from these processes [7]. Therefore, soap-free emulsion polymerization, which is believed to have insignificant environmental impacts since it does not use surfactants, is generally conducted by using an ionic radical initiator to synthesize polymer particles in water [8-11]. However, in this process, low molecular weight polymers were continuously synthesized in water even at a conversion efficiency of 1.0 [12]. Furthermore, these polymers could be treated as surfactants as they are composed of hydrophobic polymer chains and hydrophilic groups that originated from the ionic initiator. Although the antimicrobial activities of the polymers was investigated [13-15], the environmental impacts of polymers formed through soap-free emulsion polymerization have not been evaluated. From a drug delivery point of view, recent studies have reported that smaller polymer particles can penetrate the lipid bilayer of cells easily [16-18]. Therefore, this study investigates the environmental impacts of low molecular weight polymers, including polymeric nanoparticles, synthesized through soap-free emulsion polymerization by using *Micrococcus luteus* as the indicator microorganism. This microbe is commonly detected in soil and water [19-21].

This study has two objectives: 1) to investigate the effects of methyl methacrylate (MMA) or styrene polymers synthesized through soap-free emulsion polymerization on microbes and evaluate their potential for environmental pollution and 2) to determine the molecular weights of the polymers in the supernatant of polymer colloids synthesized through soap-free emulsion polymerization.

2. Experimental

2.1 Materials used for soap-free emulsion polymerization

The water used in the soap-free emulsion polymerization was purified by using a purification system (Auto Still WG250, Yamato), after which it was bubbled with nitrogen gas to remove any dissolved oxygen. We selected two typical monomers, styrene and MMA, for use in the polymerization. The styrene monomers (Tokyo Chemical Industry) were washed with a 10% sodium hydroxide solution four times to remove any polymerization inhibitors. Subsequently, these monomers were purified by distillation under reduced pressure. MMA (Tokyo Chemical Industry) was used, as received, as a monomer for soap-free emulsion polymerization. Water-soluble initiators, 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (V-50, Wako Pure Chemical), 2,2'-Azobis[2-(2-imidazolin-2-yl)propane] dihydrochloride (VA-044, Wako Pure Chemical), and potassium persulfate (KPS, Sigma Aldrich), and an oil-soluble initiator, 2,2'-Azobis(2-methylpropionitrile) (AIBN, Sigma Aldrich), were used as radical initiators without further purification. AIBN is not as water-soluble as the other initiators. However, we recently found that it could be used as an initiator for soap-free emulsion polymerization of styrene to

synthesize polystyrene colloids with negative charges, and works like KPS [22, 23]. The synthesized polymers were positively charged by V-50 and VA-044. The charge of the initiators in water is important for investigating the effects of the synthesized polymers on the microbes. Therefore, these four initiators were used as radical initiators for the polymerization.

2.2 Synthesis of polymer colloids through soap-free emulsion polymerization

The polymerization reaction to synthesize polymer colloids was conducted in a 30-mL round-bottom reactor. The temperature and rotation speed of the impeller in the reactor were controlled by using a magnetic stirrer with heating (EYELA, RCH-20L). The conditions for polymerization are listed in **Table 1**. A reaction time of 6 h was selected because previous studies reported that almost complete polymerization could be achieved within this time frame [23-25]. The centrifugal separator (3700, KUBOTA) was operated at 15,000 rpm for 3 h to separate the polymer colloid into polymer materials and supernatant containing low molecular weight polymers.

Table 1. Experimental conditions of soap-free emulsion polymerization.

Water [g]	15
Monomer [mM]	64 ~ 128
Initiator [mM]	2.03 ~ 20.3
Temperature [°C]	70

2.3 Bioassay test

The antibacterial activities of the polymer samples against the indicator microorganism, *Micrococcus luteus,* were evaluated by agar well diffusion method [26].

The component of bioassay plate was described by Arakawa et al. [27]. The bioassay plate had two layers; the bottom layer consisted of tryptic soy broth (TSB; Difco Laboratories) with 1.5% agar, while the top layer consisted of TSB with 0.8% agar supplemented with a 2% *M. luteus* fill-growth suspension. Then, a hole with a diameter of 8 mm was punched with a sterile cork borer on the bioassay agar plate with *M. luteus*. Polymer samples (80 μl) were placed into these agar wells, and were then incubated at 28 °C for 48 h. The antibacterial activities were evaluated by measuring the diameter of the inhibition zone surrounding the polymer sample.

2.4 Analysis of polymers

The size of the particles synthesized through polymerization was measured by scanning electron microscopy (FE-SEM; JSM-7500FA, JEOL). SEM samples were prepared as follows: a small amount of the solution was sampled from the reactor, a drop of it was placed on a freshly cleaved mica plate, the specimen was dried, and then coated with a thin osmium film by chemical vapor deposition (CVD; Osmium Plasma Coater OPC60A, Filgen). The average particle size was calculated by averaging the particle sizes of over 200 particles identified in the SEM images. The zeta potential of the polymer colloid was measured by using a Zetasizer Nano ZS (Malvern Co., Ltd.) after diluting the sample slurry with deionized water. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

(MALDI-TOF-MS) (AXINA-CFR+, SHIMADZU) was used to determine the molecular weight of polystyrene, especially to detect the lower molecular weights of the polymers dissolved in the supernatant of the polymer colloid. The polymer samples were prepared using dithranol (Sigma Aldrich) and subsequently freeze-dried (EYELA, FDU-1200). The details of this method are available in a previous study [28].

3. Results and Discussion

3.1 Effect of polymer colloids on Micrococcus luteus

Polymer colloids were synthesized through soap-free emulsion polymerization of MMA or styrene under a constant initiator concentration of 2.03 mM (or 4.06 mM when KPS was used). The effect of each polymer colloid was examined via bioassays; the results obtained are presented in **Fig. 1**. Polystyrene latex (PSL) colloids prepared by using V-50 had zeta potentials of +31.1 mV and created an inhibition zone, as shown in **Fig. 1b**. However, the impact of poly methyl methacrylate (PMMA) on *M. luteus* was less. Furthermore, to investigate the effects of the charge of the initiator on the inhibition zone, PSL colloids prepared using VA-044, AIBN, and KPS were studied. The size of the inhibition zone of the PSL colloid prepared with VA-044 was the largest, as shown in **Fig. 1c**. However, no inhibition zone was formed when AIBN and KPS were used, as shown in **Figs. 1d** and **1e** respectively. From these results, negative charged polystyrene using KPS and AIBN would more suitable when soap-free emulsion polymerization of styrene, so they could minimize environmental pollution. Furthermore, cationic polystyrenes synthesized using V-50 and VA-044 were toxic to *M. luteus*. Recent studies reported that positively charged PSL with a

size of 100 nm was toxic to *Lactococcus lactis* and *Pseudomonas fluorescens*; however, negatively charged PSL was not [29, 30]. The results of the present study are in agreement with those of recent studies. The effect of polystyrene synthesized using V-50 and VA-044 on *M. luteus* is further discussed in the following sections.

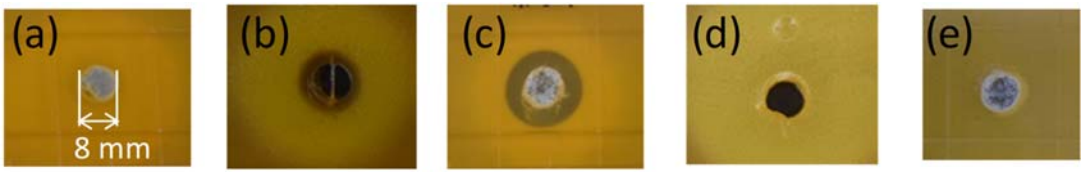

Fig. 1 Bioassay of polymer colloids prepared using the following monomers and initiators: (a) MMA and V-50; (b) Styrene and V-50; (c) Styrene and VA-044; (d) Styrene and AIBN; (e) Styrene and KPS. The white particles seen at the centers of the images are the polymeric particle sediments.

3.2 Effects of the polymer materials and supernatant of polymer colloid on the size of the inhibition zone

The cationic PSL colloids were toxic to *M. luteus*. The low and high molecular weight polystyrenes were classified by using a centrifugal separator and the effect of the molecular weight of polystyrene on the size of the inhibition zone was investigated. The polymer materials were obtained in the solid state by using a centrifugal separator and re-dispersed in pure water to prepare PSL colloids by following an ultrasonic procedure (US-5KS, SND). No inhibition zone was observed when the colloids of polymer materials synthesized by using VA-044 and V-50 were used, as shown in **Figs. 2a** and **2c** respectively. However, as shown in **Figs. 2b** and **2d**, the supernatant produced noticeable inhibition zones; the inhibition zone of the supernatant obtained by using VA-044 was larger than those of the supernatants obtained by using the other initiators. Although the supernatants were transparent, they were

toxic to *M. luteus.* To study the supernatant, a drop of the solution that was used for the bioassay test shown in **Fig. 2d** was placed on a mica surface and dried so that it could be observed by FE-SEM (shown in **Fig. 3).** Positively charged spherical adsorbates smaller than 30 nm, which originated from VA-044, were adsorbed onto the negatively charged mica surface [31], and were present in the supernatant. The zeta potential of the supernatant obtained by using V-50 was +16.9 mV. These results indicate that low molecular weight polystyrene would be present in the supernatant and toxic to *M. luteus*. The polymer molecules were comprised of polystyrene and a portion decomposed from the initiator. To

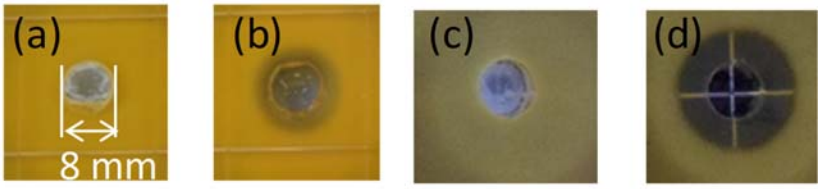

Fig. 2 Results of the bioassays of particles or supernatant prepared using the following monomers and initiators: (a) particles with size of 180 nm synthesized using styrene and V-50; (b) supernatant obtained using styrene and V-50; (c) particles with size of 108 nm synthesized using styrene and VA-044; (d) supernatant obtained using styrene and VA-044.

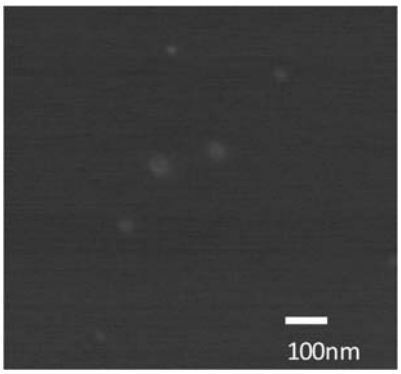

Fig. 3 SEM image of the supernatant polymers, obtained through soap-free emulsion polymerization of styrene with 2.03 mM VA-044, adsorbed electrostatically on the mica surface.

determine the effect of the initiator alone on the inhibition zone, 2.03 mM VA-044 solutions with and without heating at 70 °C for 6 h were prepared. Heating enabled the initiator to decompose and form radical segments. Neither of the initiators exhibited toxicity against *M. luteus* in the bioassay tests. Thus, the initiators and initiator radicals were not involved in the antimicrobial activity against *M. luteus*. Furthermore, the toxicity of pure water saturated with styrene monomers, which have been reported to be toxic to other microbes [32], was investigated. No inhibition zone against *M. luteus* was observed. Additionally, no inhibition zone was observed when water was used as the solvent in the bioassays. Therefore, cationic initiator, styrene monomer, and water were not toxic to *M. luteus*. However, the low molecular weight polystyrene particles in the supernatant contributed to the formation of inhibition zones. This relationship between particle size and inhibition zones agreed with the results of a previous study on nanoparticles, which reported that small particles were cytotoxic [33]. The molecular weights at which polystyrene was toxic to *M. luteus* were subsequently analyzed.

3.3 Effect of the molecular weights of the polymers in the supernatant on the inhibition zone

To determine the effect of the molecular weights of the polymers in the supernatant on the inhibition zone, soap-free emulsion polymerizations of styrene were conducted using VA-044 under two different conditions. Under the first condition, the concentrations of the initiators and monomers were 20.3 mM and 64 mM, respectively, while the other conditions were the same as those listed in **Table 1**. Under the second condition, the concentrations of the initiators and monomers were 2.03 mM and 128 mM, respectively. The polymers in the

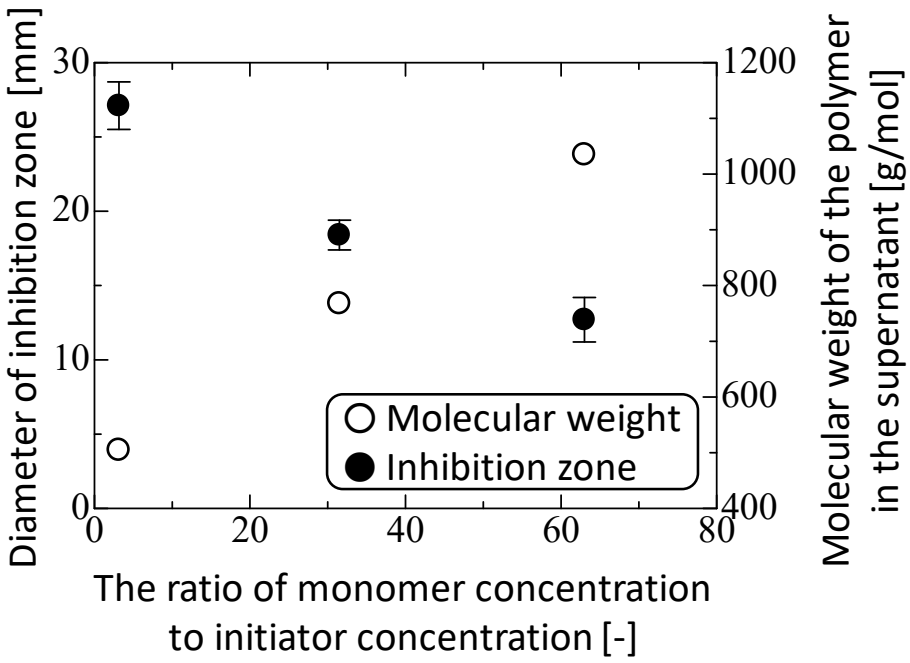

Fig. 4 Influence of the ratio of monomer concentration to initiator concentration on the molecular weight of the synthesized polymer dissolved in the supernatant, inhibition zone examined via a bioassay test.

supernatant obtained by using the centrifugal separator were characterized by following the MALDI-TOF-MS method. The toxicity of each supernatant against *M. luteus* was investigated by measuring the size of the inhibition zone.

The relationships between the ratio of monomer concentration to initiator concentration in the conditions and molecular weight of the synthesized polymer dissolved in the supernatant, the diameter of the inhibition zone are presented in **Fig. 4**. The lowest molecular weight detected by MALDI-TOF-MS was plotted as the vertical axis because low molecular weight polystyrene particles were significantly correlated with the inhibition zone diameter. As the concentration ratio of monomer and initiator decreased, molecular weight was also decreased. Although the concentrations of the polymers in the supernatants differed,

the inhibition zone diameter increased as the molecular weight decreased. Low molecular weight polystyrene particles were more harmful to *M. luteus*. The three samples in Fig. 4 exhibited antimicrobial activities equivalent to that of 4-28 μg/mL of ampicillin (beta-lactam antibiotic). Concretely, inhibition zone of 12.7 mm was worth of antimicrobial activity of 4 μg/mL of ampicillin, on the other hand, that of 27.1 mm was equivalent with the 28 μg/mL of ampicillin

3.4 Examination of the validity of the present method for direct evaluation of the antibacterial properties of the polymer particles and polymers dissolved in the supernatant

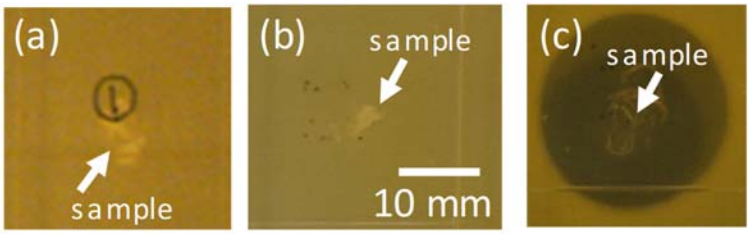

Fig. 5 Results of the bioassays of polystyrene plates prepared with: (a) no polymer; (b) polymer materials; (c) polymers remaining in the supernatant.

To evaluate the direct toxicity of the solid polymers against *M. luteus*, the polystyrene plates were electrostatically modified with the polymers that were synthesized through soap-free emulsion polymerization of styrene (64 mM) and VA-044 (2.03 mM) in water. These plates were dried to create samples for the bioassay test with *M. luteus* as the indicator microorganism. The polystyrene plates were placed directly on the agar surface and bioassay tests were conducted. The results are shown in **Fig. 5**. The unmodified polystyrene plate and the polystyrene materials, obtained through centrifugal separation, adsorbed on the plate did

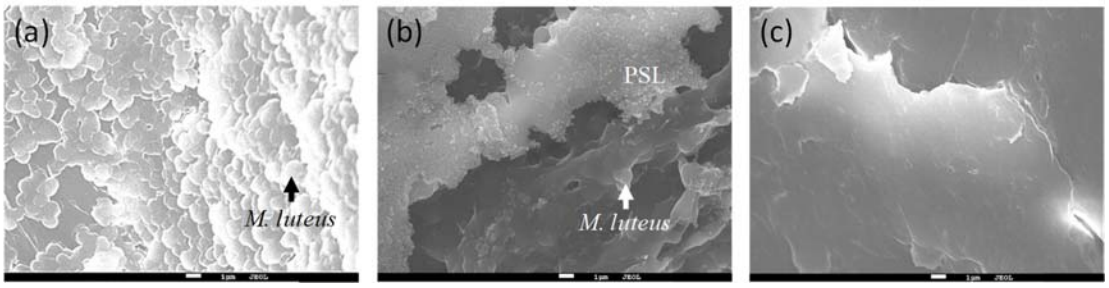

Fig. 6 SEM images of the polystyrene plates modified with: (a) no polymer; (b) polymer materials obtained through centrifugal separation; (c) polymers remaining in the supernatant after the bioassay. The scale bars correspond to 1 μm.

not exhibit antibacterial activity against *M. luteus* **(Figs. 5a** and **5b)**. However, a clear inhibition zone was formed around the plate modified with the polymers remaining in the supernatant after centrifugal separation (**Fig. 5c**). After the bioassay tests, each polystyrene plate was washed with pure water and examined by FE-SEM, as shown in **Fig. 6**. **Fig. 6a** shows that the unmodified polystyrene plate was fully covered with *M. luteus*. This indicates that the polystyrene plate was not toxic to *M. luteus* and could therefore be used to evaluate the antibacterial properties of polystyrene synthesized through soap-free emulsion polymerization with VA-044. Furthermore, as shown in **Fig. 6b**, the polystyrene plate modified with polystyrene materials was covered with *M. luteus*. However, the low molecular weight polymers remaining in the supernatant were toxic to *M. luteus* because *M. luteus* was not present throughout the polystyrene plate modified with these polymers, as shown in **Fig. 6c**. This modification technique demonstrates potential as a method for the development of antibacterial plastics. The results obtained agreed well with each other and indicate that low molecular weight cationic polystyrene was toxic to *M. luteus*. Furthermore, these results demonstrated the validity of the bioassay tests conducted using the liquid samples, such as

polymer colloid and supernatant, directly for the evaluation of the antibacterial properties of the various samples as described in the experimental section.

Some studies have reported that polycyclic aromatic hydrocarbons and surfactants are harmful to the environment and human health [7, 34-37]. Low molecular weight polystyrenes, particularly those with molecular weights below 1000, could be regarded as surfactants because they are comprised of hydrophobic chains of styrene monomers and hydrophilic groups that originated from the ionic initiator. Furthermore, polystyrene is similar to polycyclic aromatic hydrocarbons because it has a similar number of phenyl rings. No inhibition zone was observed for the PMMA colloid, as shown in **Fig. 1a**, because PMMA did not have phenyl rings. Recent molecular dynamic simulations have demonstrated that smaller cationic particles can penetrate the lipid bilayer of cells more easily than negatively charged nanoparticles [16, 38]. Hence, it is possible that the low molecular weight cationic polystyrene particles remaining in the supernatant after centrifugal separation penetrated the *M. luteus* cells easily. The phenyl rings of these polymers may have been responsible for the toxic effects [39, 40] against *M. luteus,* due to which an inhibition zone was formed in the bioassay test.

Furthermore, to control the antibacterial activities of the low molecular weight cationic polystyrene in the supernatant, *N*-vinylacetamide monomers (NVA) were added during the polymerization of styrene with V-50. NVA is a nonionic and amphiphilic monomer. The synthesized polystyrenes were coated with NVA monomers and polymers to decrease their zeta potentials [41]. **Fig. 7** shows the influence of the NVA concentration used in the polymerization on the inhibition zone evaluated via bioassay tests using the supernatant

obtained through centrifugal separation. The size of the inhibition zone decreased as the concentration of NVA used in the polymerization increased. This suggests that the addition of NVA during the polymerization of styrene with cationic initiators helped control the antimicrobial activities of molecular cationic polystyrene against *M. luteus*.

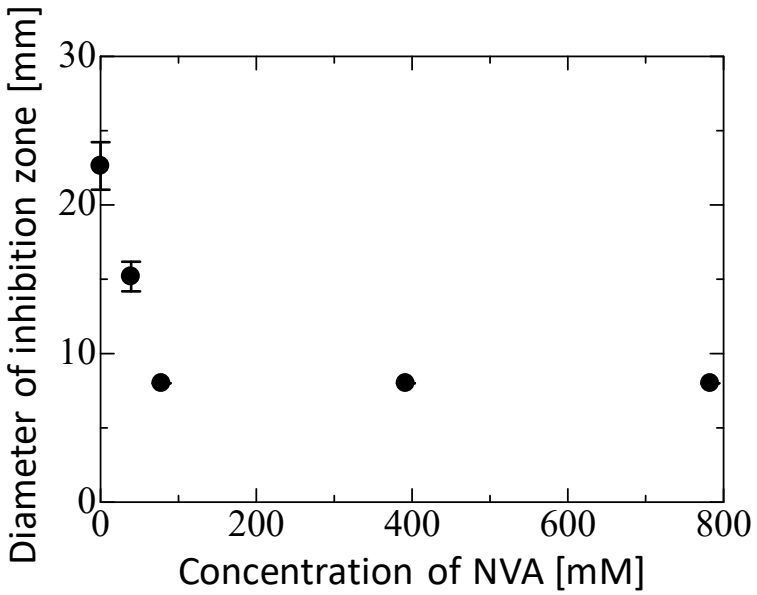

Fig. 7 Influence of the NVA concentration used in the polymerization of styrene with V-50 on the inhibition zone examined by a bioassay test using the supernatant. The 8 mm diameter dots indicate the diameter of the inhibition zone for the present sample.

4. Conclusions

This study focuses on low molecular weight polymers synthesized through soap-free emulsion polymerization of MMA or styrene by using initiators. Furthermore, the effects of these polymers on *Micrococcus luteus* were investigated. The bioassay tests provided the following observations:

• PMMA colloid synthesized by using V-50 was not toxic to *M. luteus*. However, PSL colloid synthesized by using V-50 created an inhibition zone against *M. luteus.*

• KPS and AIBN were more suitable than cationic initiators for soap-free emulsion polymerization of styrene because they were less harmful to *M. luteus*.

• Polystyrenes were consistently synthesized throughout the reaction. Furthermore, low molecular weight polystyrenes remained in the supernatant after centrifugal separation of the polystyrene colloid.

• Cationic initiator, styrene monomer, and water were not toxic to *M. luteus.*

• As the molecular weight of the polystyrenes remaining in the supernatant, of the polymer colloids synthesized through soap-free emulsion polymerization with VA-044, decreased, their toxicity against *M. luteus* increased.

Based on these observations, it can be concluded that low molecular weight polystyrene synthesized using cationic initiators was toxic to *M. luteus*. Furthermore, the supernatant containing polystyrene with molecular weights lower than 1000 could potentially pollute the environment. Therefore, supernatant obtained from soap-free emulsion polymerization of styrene by using cationic initiators should be treated more carefully if it is to be discharged into seas or rivers.

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