Influence of the size of polystyrene synthesized through soap-free emulsion polymerization on antimicrobial activity

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Abstract

Low-molecular-weight polymers formed by soap-free emulsion polymerization using a cationic initiator, styrene, and water are considered to be surfactants and consequently harmful to *Micrococcus luteus*. However, the effect of high-molecular-weight polymers, which form particulate materials in water, on *M. luteus* has not been elucidated. Herein, the influence of polymer morphology on toxicity against the indicator microorganism *M. luteus* was evaluated using polystyrene prepared by soap-free emulsion polymerization using 2,2'-azobis[2-(2-imidazolin-2-yl)propane] dihydrochloride (VA-044), potassium persulfate (KPS), or 2,2'-azobis(2-methylpropionitrile) (AIBN) as the initiator. Low-molecular-weight polymers were removed from the suspension by centrifugation to ensure that only high-molecular-weight polymers were investigated in this study. Although particulate materials were formed in water, they were dissolved in toluene, which induced changes in morphology and size. The toluene solution of high-molecular-weight polystyrenes prepared using either VA-044 or KPS created an inhibition zone against *M. luteus* in the bioassay test. These results indicate that the morphology and size of the higher-molecular-weight polymer are closely related to antimicrobial activity. Particulate materials larger than 150 nm did not create inhibition zones in the bioassay test, although their toluene solutions were toxic to *M. luteus*. Therefore, the particulate or coagulated state in solution that avoids the formation of aggregates smaller than 20 nm is considered to be the environmentally safe polymer morphology.

Keywords: High-molecular-weight polystyrene, Indicator microorganism, Morphology

1. Introduction

Although novel polymer and composite polymer materials provide convenience and functionality in daily life [1-4], their pollution, such as microplastic pollution, is a major environmental and social problem [5, 6]. They exist as particulate materials in products, such as toners, cosmetics, and medicines [7], and are thus synthesized based on the desired sizes or surface properties [1]. Since the effects of surfactants and stabilizers can be neglected in fundamental research, sub-micrometer-sized particles with high monodispersities are prepared generally by soap-free emulsion polymerization in aqueous media [8-12]. The environmental load of this process is lower than that of emulsion polymerization using surfactants [8, 10, 13, 14]. Actually, cationic surfactants [15] used in emulsion polymerization, such as hexadecyltrimethylammonium bromide [16], showed antimicrobial activities against the indicator microorganism *Micrococcus luteus*. However, soap-free emulsion polymerization in the aqueous phase continuously generates low-molecular-weight polymers, which are considered surfactants, until polymerization is complete [17]. Hence, the environmental impact of polymers formed by soap-free emulsion polymerization was evaluated in our recent research. Low-molecular-weight cationic polystyrenes that remained in the bulk were detected by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry [18, 19] and were found to be toxic to *M. luteus* [20], which is commonly detected in soil and water [21-23]. These polymers formed inhibition zones in the bioassay

test, and their toxicities against *M. luteus* were attributed to their phenyl rings [20, 24-26]. Polystyrenes with weight-average molecular weights (M_w) below 1,000 are unlikely to form particulate materials even in a poor solvent like water because their hydrophilic functional groups that originate from the ionic initiator significantly enhance their dispersion stabilities. Moreover, particulate polystyrenes larger than 150 nm were not toxic to *M. luteus* [20]. These bioassay tests suggest that the morphology or size of polystyrene is closely related to the toxic effects on *M. luteus*.

Therefore, as a fundamental study, herein we investigated the effects of the morphologies of higher-molecular-weight polystyrenes that form particulate materials in water, synthesized by soap-free emulsion polymerization, on their antimicrobial activities against *M. luteus*. The M_w and size of the high-molecular-weight polymers synthesized using a cationic, anionic, or non-ionic initiator were first evaluated. Bioassay tests were then performed using toluene solutions of the polymers because toluene is well known as a good solvent for polystyrene [27] that hinders the formation of particulate materials.

2. Experimental

2.1 Materials

The water used in the soap-free emulsion polymerization was purified using an Auto Still WG250 purification system (Yamato Scientific) and de-oxygenated by bubbling nitrogen gas. Styrene (Tokyo Chemical Industry) was washed four times with 10% sodium hydroxide solution to remove any polymerization inhibitors and subsequently purified by distillation under reduced pressure. 2,2'-Azobis(2-(2-imidazolin-2-yl)propane)

dihydrochloride (VA-044; FUJIFILM Wako Pure Chemical), potassium persulfate (KPS; Sigma-Aldrich), and 2,2'-azobis(2-methylpropionitrile) (AIBN; Sigma-Aldrich) were used as radical initiators without further purification. In water, VA-044 or KPS [28] render the synthesized polystyrenes positively or negatively charged, respectively, through their ionic functional groups [29]. On the other hand, AIBN does not have any ionic functional groups [30] that contribute to the stabilities of dispersions of synthesized polymer colloids in water. The chemical structures of the monomer and initiators are shown in **Fig. 1**.

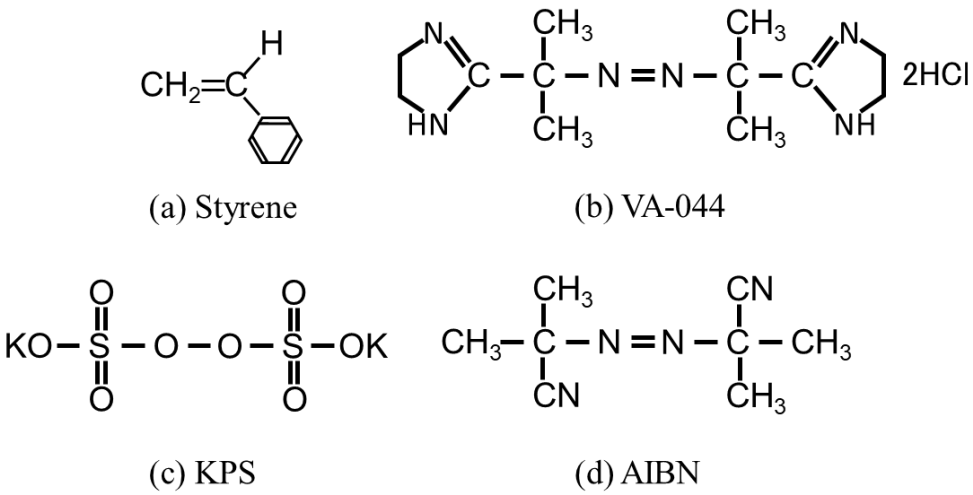

Fig. 1 Chemical structures of (a) styrene, (b) VA-044, (c) KPS, and (d) AIBN.

2.2 Synthesis of polystyrene through soap-free emulsion polymerization

The polymerization reaction used to produce polystyrene colloids was conducted in a 30-mL round-bottom reactor. The temperature and rotation speed of the impeller in the reactor were controlled by a RCH-20L magnetic stirrer with heater (EYELA World). The conditions for polymerization are listed in **Table 1**. A reaction time of 24 h was selected

because previous studies indicated that polymerization was almost complete within 6 h and the polymer with higher molecular weight was treated in the bioassay test [31-33]. Only higher-molecular-weight polymers were investigated in this study; hence, the supernatant containing low-molecular-weight polymers was removed after triple centrifugation using a centrifugal separator (Model 3700, KUBOTA) operated at 15,000 rpm for 30 min per run. The polymer solutions for the bioassay tests were prepared by dissolving the solid polymers in toluene (FUJIFILM Wako Pure Chemical).

Table 1. Experimental soap-free emulsion polymerization conditions.

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

2.3 Analysis of polymers

Particle sizes were measured by field-emission scanning electron microscopy (FE-SEM; JSM-7500FA, JEOL). SEM samples were prepared by sampling a small amount of each polymer solution from the reactor, placing a drop of the solution on a freshly cleaved mica plate, drying the specimen, and then coating it with a thin Os film by chemical vapor deposition (OPC60A osmium plasma coater, Filgen). The average particle size was calculated by averaging the particle sizes of over 200 particles identified in the SEM images. The M_w of the synthesized polystyrene dissolved in *N*,*N*-dimethylformamide (DMF;

FUJIFILM Wako Pure Chemical) was obtained by size-exclusion chromatography (SEC; CO-2065, JASCO). The sizes of the polymers in toluene were estimated using a dynamic light scattering (Zetasizer Nano ZS, Malvern Panalytical).

2.4 Bioassays

The antibacterial activities of the polymer samples against *M. luteus* were evaluated by the agar well diffusion method [34]. The composition of the bioassay plate has been described by Arakawa et al. [35]. The bioassay plate had two layers: a bottom layer, consisting of a tryptic soy broth (TSB; Difco Laboratories) with 1.5% agar, and a top layer, consisting of TSB with 0.8% agar supplemented with 2% *M. luteus*-filled growth suspension. Subsequently, a hole with a diameter of 8 mm was bored in each agar plate using a sterile cork borer. Polymer samples (8 μL) were placed in the agar wells and then incubated at 28 °C for 48 h. The antibacterial activity was determined by measuring the diameter of the inhibition zone surrounding the polymer sample [20, 26].

3. Results and Discussion

3.1 Characterizing the polystyrene synthesized using VA-044

Polystyrene colloids were synthesized by soap-free emulsion polymerization using VA-044 at different concentrations (2.03, 4.06, 10.2, and 20.3 mM). **Fig. 2** shows the SEM images of the synthesized polystyrene colloids after the low-molecular-weight polystyrene was removed by centrifugation. The average size of the monodispersed particles is greater than 150 nm. The particle size increased as the initiator concentration was increased from

2.03 to 10.2 mM because of the increased amount of small particles, which, as our previous research showed, are generated secondarily and contribute to the growth process through the hetero coagulation [36]. On the other hand, the particle size decreased at a concentration of 20.3 mM, as per the LaMer diagram [37, 38]. Our recent research showed that polystyrene particles of these sizes do not create inhibition zones against *M. luteus* [20]. The M_w of the polystyrenes in DMF, as measured by SEC, was over 80,000. These high-molecular-weight polystyrenes dissolved completely in toluene to show transparent and their good dispersion stabilities, and the resulting polymer solutions were used in the bioassay.

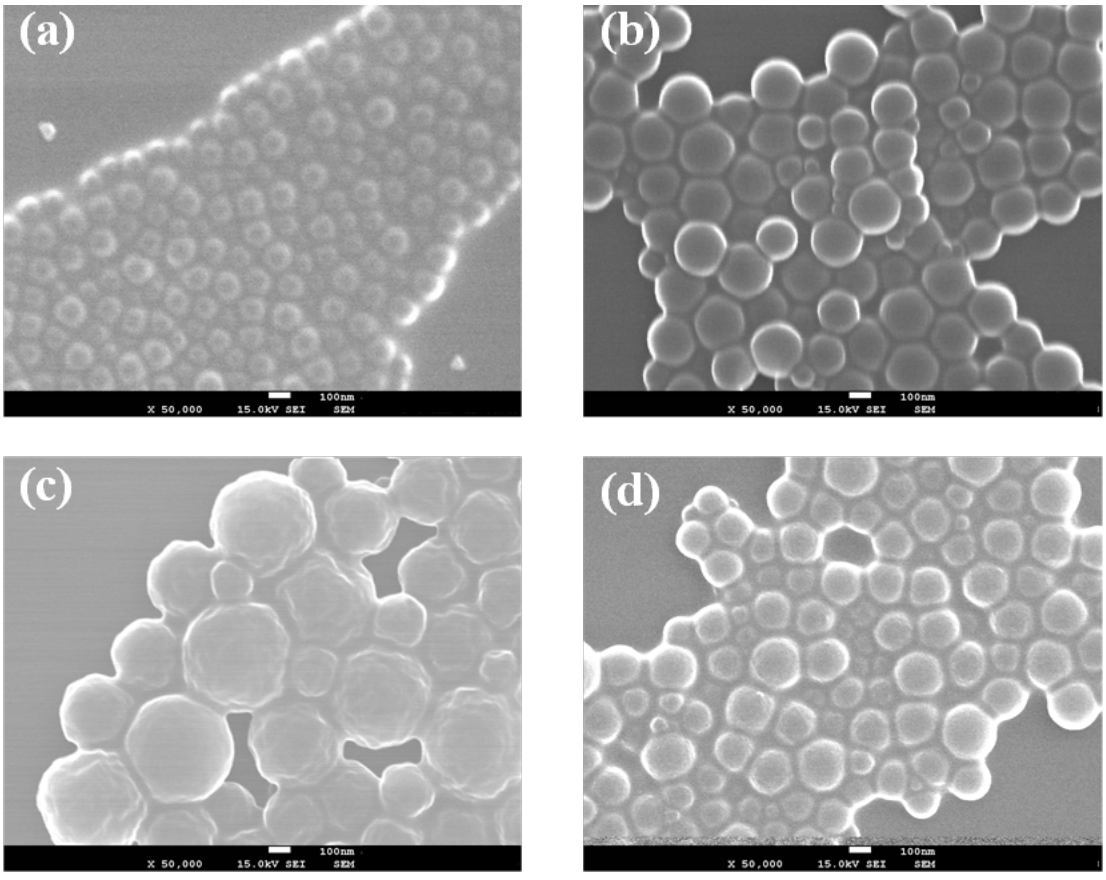

Fig. 2 FE-SEM images of polystyrene particles synthesized using 2,2'-azobis[2-(2-imidazolin-2-yl)propane] dihydrochloride (VA-044) at different concentrations: (a) 2.03, (b) 4.06, (c) 10.2, and (d) 20.3 mM. The scale bars indicated 100 nm.

3.2 Effect of the morphology of the high-molecular-weight polystyrene prepared using VA-044 on antimicrobial activity

The high-molecular-weight polystyrene synthesized using VA-044 and purified by centrifugation formed particulate materials in water because water is a poor solvent for this polymer. Herein we focused on the effects of the morphologies of such polystyrenes dissolved in toluene on the inhibition zone formed in a bioassay test.

First, we confirmed that toluene on its own is not toxic toward *M. luteus*, indicating that the solvent is not involved in antimicrobial activity [39]. In addition, no inhibition zone was observed when polystyrene colloids produced in water were tested. **Fig. 3** shows the influence of toluene on *M. luteus* by FE-SEM, where the FE-SEM samples were prepared as described in section 2.3. Toluene treatment, in which toluene is added to *M. luteus* on a freshly cleaved mica plate until it evaporated, removes water components inside *M. luteus* or agar to show the wrinkles on their surfaces; however, *M. luteus* does not disappear, as shown in Fig. 3b. In addition, it has been reported that some bacteria are attracted to toluene during bioassay testing [39]. These results also support the non-toxicity of toluene toward *M. luteus*.

Subsequently, bioassay tests were conducted using toluene solutions with a constant polymer weight concentration of 223 mg/mol toluene, and the results are shown in **Fig. 4**. Although the particulate materials do not create inhibition zones, the polymers dissolved in toluene are toxic to *M. luteus*. With increasing initiator concentration, the inhibition zone

increases slightly, probably because the increased density of ionic functional groups against polymer length enables the polystyrenes to penetrate the cell membranes more easily [20, 40]. Thus, the polymer morphology is closely associated with its antimicrobial activity. Finally, the influence of the polymer weight concentration on the inhibition zone using polystyrene with M_w = 210,000 was investigated, as shown in **Fig. 5**. There is no dependence on the polymer concentration in toluene because toluene does not contribute to the diffusion of the polystyrenes in agar containing water.

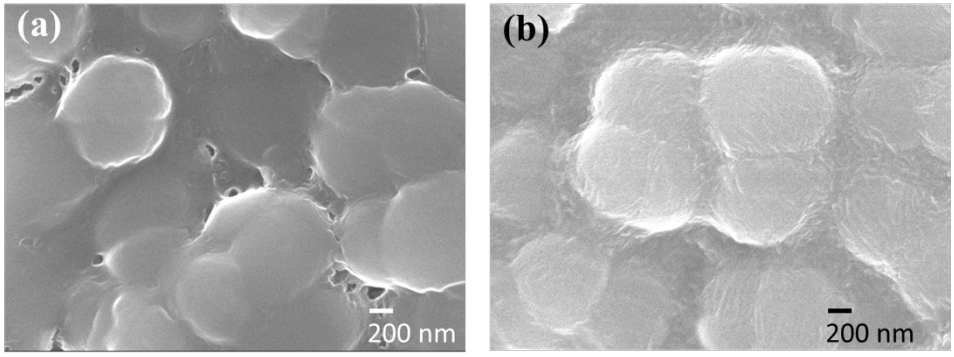

Fig. 3 Influence of toluene treatment on *M. luteus*, as observed by FE-SEM: (a) before treatment; (b) after toluene treatment.

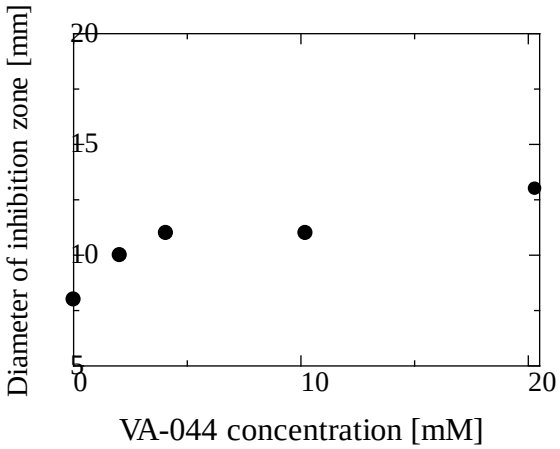

Fig. 4 Effect of VA-044 concentration on the inhibition zone formed in the bioassay test.

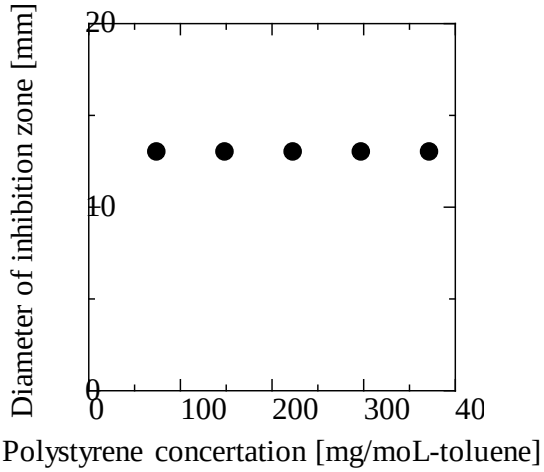

Fig. 5 Effect of polystyrene concentration in toluene on the inhibition zone formed in the bioassay test.

3.3 Effect of higher-molecular-weight polymers synthesized using KPS or AIBN on antimicrobial activity

Furthermore, the effects of the initiator on the inhibition zone were investigated using styrene as the monomer and KPS and AIBN as the initiators in the polymerization reaction. In our recent study, polystyrene colloids prepared in water using KPS showed no inhibition zone in the bioassay test because they were negatively charged and existed as particulate matter in water [20]. Herein, they were dissolved in toluene to independently break the assembled particulate polystyrene and obtain high-molecular-weight polystyrene chains.

The soap-free emulsion polymerization of styrene was conducted using KPS at various concentrations. The high-molecular-weight polystyrenes obtained using the centrifugal separator were dissolved in toluene to prepare polymer solutions with weight concentrations of 223 mg/mol toluene. **Fig. 6** clearly shows that the high-molecular-weight

polystyrenes prepared using KPS also create inhibition zones, and are therefore harmful to *M. luteus*.

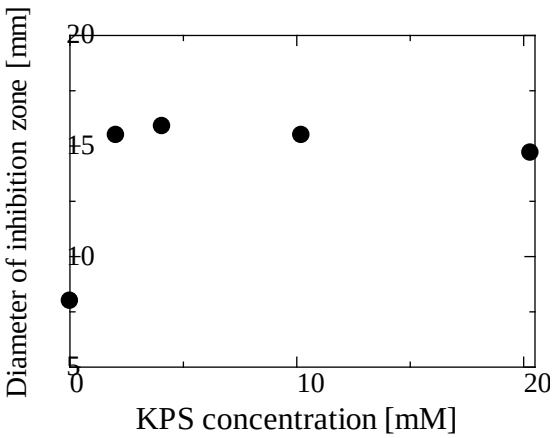

Fig. 6 Effect of KPS concentration on the inhibition zone formed in the bioassay test.

Bioassay tests were performed using toluene solutions of polystyrene prepared by polymerizing 0.1 g styrene with 2.03 mM of the nonionic initiator, AIBN, in the absence of water at 70 °C for 12 h. The results obtained thus far show that polystyrenes with ionic functional groups that originate from the initiator are toxic to *M. luteus*; however, toluene solutions of polystyrene prepared using AIBN did not create any inhibition zones. These results indicate that the ionic functional groups of polystyrene are hydrophilic and interact with the hydrophilic surfaces of *M. luteus* as a consequence.

Moreover, polystyrene does not have a particulate morphology; however, its size in toluene allows it to exhibit antimicrobial activity. Hence, **Fig. 7** shows the sizes of the polystyrenes prepared using the different initiators, VA-044, KPS, and AIBN, in toluene. A comparison with **Fig. 2** shows that the sizes changed from > 150 nm to < 20 nm. Within this

size range [40], the polystyrene are toxic to *M. luteus* [20]. However, since the differences between the sizes are negligible, the ionic functional groups of polystyrene originating from the initiators are closely associated with the toxicity.

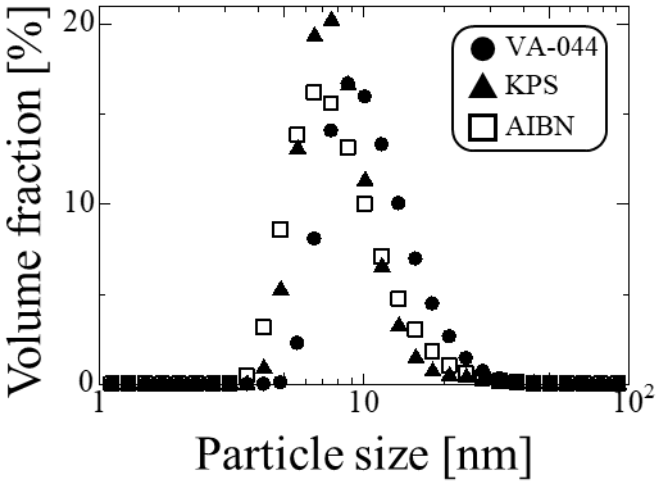

Fig. 7 Size distributions of the polystyrenes synthesized using VA-044, KPS, and AIBN in toluene.

4. Conclusions

Herein we focused on the morphology and size of high-molecular-weight polystyrene synthesized by soap-free emulsion polymerization using ionic or non-ionic initiators. The effects of these polymers on *M. luteus* were investigated through bioassays, and the following observations were made:

• Even when larger polystyrenes were synthesized using VA-044 or KPS, their toluene solutions were toxic to *M. luteus* because the morphology of polystyrene in toluene changed from particulates larger than 150 nm to ones smaller than 20 nm.

・Polystyrene synthesized by the nonionic initiator, AIBN, did not show any antimicrobial activity, indicating that the ionic functional groups originating from the initiator are closely related to toxicity.

From these observations, we conclude that high-molecular-weight polystyrene with hydrophilic groups, prepared using ionic initiators, exists as particulate material in water but dissolves in toluene to form smaller particles, which are toxic to *M. luteus*.

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