Relationship between dispersion-forming capability of poly(4-vinylaniline) colloids and antimicrobial activity

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Abstract

Cationic polymers synthesized from aromatic monomers such as styrene or 4-vinylaniline, and with molecular weight less than 1,000 Da display antibacterial activities against *Micrococcus luteus*. We prepared polymer colloids exhibiting different tendencies to form stable dispersions in order to clarify the influence of this property on their antimicrobial activity against *M. luteus*. 4-Vinylaniline was polymerized by a soap-free emulsion procedure using cationic and anionic initiators to prepare polymer colloids with different capacities for forming dispersions. All synthesized colloids displayed positive zeta potentials due to the presence of amino groups from the monomers and/or initiators and included polymers with molecular weights < 1,000 Da. However, only colloids prepared using a cationic initiator (VA-044 or V-50) and capable of forming stable dispersions were antimicrobially active. Colloids synthesized using an anionic initiator (KPS or V-501), which aggregate easily due to electro-attractive forces between functional groups, were not antimicrobial. Thus, the antimicrobial activity of a polymer colloid is closely associated with its ability to form a stable dispersion.

Keywords: Soap-free emulsion polymerization, Dispersion stability, Antimicrobial activity, 4-Vinylaniline

1. Introduction

Polymer colloids are useful materials in many fields including paints, pharmaceuticals, and manufacturing [1-5]. Among various synthetic approaches, emulsion polymerization is used to prepare polymer colloids less than 200 nm in size [6-8]. Soap-free emulsion polymerization, which is performed without a surfactant, is a well-known, simple, and environmentally friendly preparation method [9-11]. Surfactants and ionic radical initiators promote good colloidal stability during preparation [12-14]. Radical initiators dissolved in an aqueous phase facilitate the radical polymerization that generates ionic polymers in the bulk. The resulting polymers carry positive or negative charges on functional groups derived from the aqueous initiators [15-18]. For example, initiators bearing amino groups generate positively charged polymers that nucleate particle growth during soap-free emulsion polymerization [19]. Radical polymerization continues in bulk during particle growth, producing low-molecular-weight ionic polymers [15, 18].

The toxicities of nanomaterials have been evaluated in various ways [20-24]. We previously reported that < 1 kDa cationic polymers synthesized from aromatic monomers such as styrene or 4-vinylaniline exhibit antibacterial activities against *Micrococcus luteus*, which is commonly found in soil and water [25-27] and showed sensitive to our synthesized polymers compared with *Escherichia coli*, because the structures of the polymers are similar to those of surfactants [28-31]. Toxicity against *M. luteus* decreases with increasing molecular weight of the polystyrene unit in the polymer colloid [29]. Therefore, we have focused on cationic low molecular-weight polymers because the monomer,

radical initiator, initiator radical, and negative polymer are not antimicrobially active [29]. Cationic polymer colloids with various dispersion-forming capabilities were recently prepared using 4-vinylaniline and ionic initiators, but the effect of colloid stability on antimicrobial activity has not been investigated [32].

We investigated colloidal stability [33] and antimicrobial activity [29] separately. So far, it was not clear whether the antimicrobial activity was influenced by colloidal stability. Therefore, in this study, we seek to clarify the relationship between antimicrobial activity and stabilities of the colloids via the soap-free emulsion polymerization of 4-vinylaniline using cationic and anionic initiators. The stabilities of the polymeric colloids were evaluated by macroscopic and microscopic techniques, after which they were bioassayed against *M. luteus* to determine their antimicrobial activities.

2. Experimental

2.1 Materials

The water used in soap-free emulsion polymerization was purified with a Yamamoto Scientific WG250 system and sparged with nitrogen gas to remove dissolved oxygen. Styrene and 4-vinylaniline (Tokyo Chemical Industry) were employed as monomers in the soap-free emulsion polymerization. Cationic and anionic initiators were used to study the influence of charge on the dispersion capability of the synthesized particles. The cationic initiators 2,2'-azobis[2-(2-imidazolin-2-yl)propane] dihydrochloride (VA-044, Fujifilm Wako Pure Chemical Corporation) [33] and 2,2'-azobis(2-

methylpropionamidine) dihydrochloride (V-50, Fujifilm Wako Pure Chemical Corporation) [34], and anionic initiators potassium persulfate (KPS, Sigma Aldrich) [35] and 4,4'-azobis(4-cyanovaleric acid) (V-501, Fujifilm Wako Pure Chemical Corporation) [36], were used without further purification. Their chemical structures are shown in **Fig. 1**.

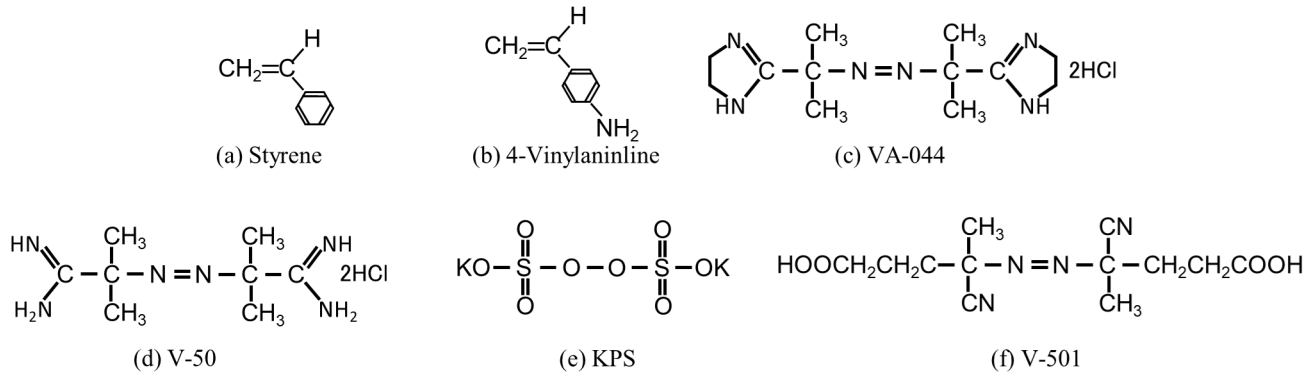


Fig. 1 Chemical structures of (a) styrene, (b) 4-vinylaniline, (c) 2,2'-azobis[2-(2-imidazolin-2-yl)propane] dihydrochloride (VA-044), (d) 2,2'-azobis(2-methylpropionamidine) dihydrochloride (VA-50), (e) potassium persulfate (KPS), and (f) 4,4'-azobis(4-cyanovaleric acid) (V-501).

2.2 Soap-free emulsion polymerization

Polymerization was conducted in a 30-mL round-bottom reactor. The temperature and rotation speed of the impeller were controlled using a heated magnetic stirrer (RCH-20L, EYELA). Polymerization conditions are listed in **Table 1**. A 6-h reaction time was employed, because previous studies report that almost complete polymerization can be achieved within this interval [37].

Table 1. Experimental soap-free emulsion polymerization conditions.

Water [g]	Monomer [mmol/L]	Initiator [mmol/L]	Temperature [°C]
15.0	64.0	20.3	80

2.3 Bioassaying

The antibacterial activities of polymer samples against the indicator microorganism, *M. luteus,* were evaluated following the bioassay protocol in Arakawa *et al.* [38] with some modifications. An 8-mm diameter hole was punched in an *M. luteus* bioassay agar plate with a sterile cork-borer. Polymer samples (80 μL) were placed on the *M. luteus* bioassay plate and incubated at 28 °C for 48 h. Antibacterial activity was evaluated by observing the inhibition zone surrounding the polymer sample. The bioassay plate contained two layers: a bottom layer consisting of tryptic soy broth (TSB, Difco Laboratories) with 1.5% agar and a top layer consisting of TSB with 0.8% agar supplemented with a 2% *M. luteus* fill-growth suspension. Antibacterial activity was evaluated by measuring the diameter of the inhibition zone surrounding the polymer sample.

2.4 Polymer analysis

The morphologies of the particles synthesized via soap-free emulsion polymerization were examined by scanning electron microscopy (SEM; JSM-7500FA, Jeol). SEM samples were prepared using the following procedure. A drop taken from a small aliquot of reactor solution was placed on a

freshly cleaved mica plate. The specimen was dried and coated with 10-nm-thick osmium film by chemical vapor deposition (CVD; Osmium Plasma Coater OPC60A, Filgen). The zeta potential of the polymer colloid was measured using a Zetasizer Nano ZS instrument (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 weights of the polymers, with specific focus on the low-molecular-weight polymers dissolved in the polymer colloid [39]. Polymer samples were prepared using dithranol (Sigma-Aldrich) and freeze-dried (FDU-1200, Eyela) as detailed in a previous report [40].

3. Results and Discussion

Fig. 2 shows the relationship between the VA-044 initiator concentration used to prepare a polystyrene colloid and the size of the inhibition zone. The weight-average molecular weight (M_w) of the polystyrene determined by size-exclusion chromatography (SEC; CO-2065, JASCO) ranged from 80k to 2,100k Da, and their standard deviations were 2.22 ~ 3.34. The inhibition zone increased in size with increasing initiator concentration, probably because more low molecular-weight polystyrene is produced under these conditions. Notably, greater antimicrobial activity is observed when the molecular weight of the cationic polystyrene is less than 1,000 Da, as observed in our previous study, probably because smaller cationic polymers can penetrate the lipid bilayer of cells more easily and the phenyl rings of these polymers may have been responsible for the toxic effects [29]. An inhibition zone

with a diameter of 12.7 mm corresponds to an antimicrobial activity of 4 μg/mL of ampicillin, whereas a 27.1-mm diameter zone is equivalent to 28 μg/mL of ampicillin [29]. The soap-free emulsion polymerization of 4-vinylaniline was conducted at an initiator concentration of 20.3 mM.

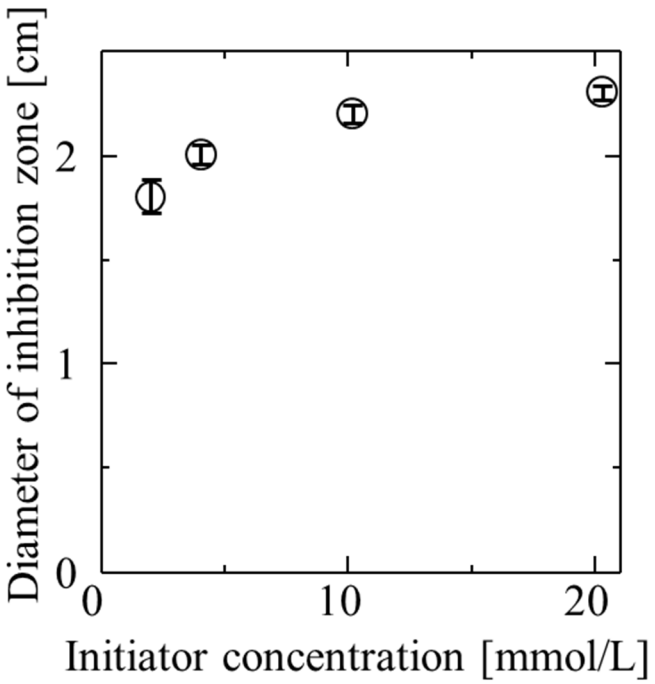
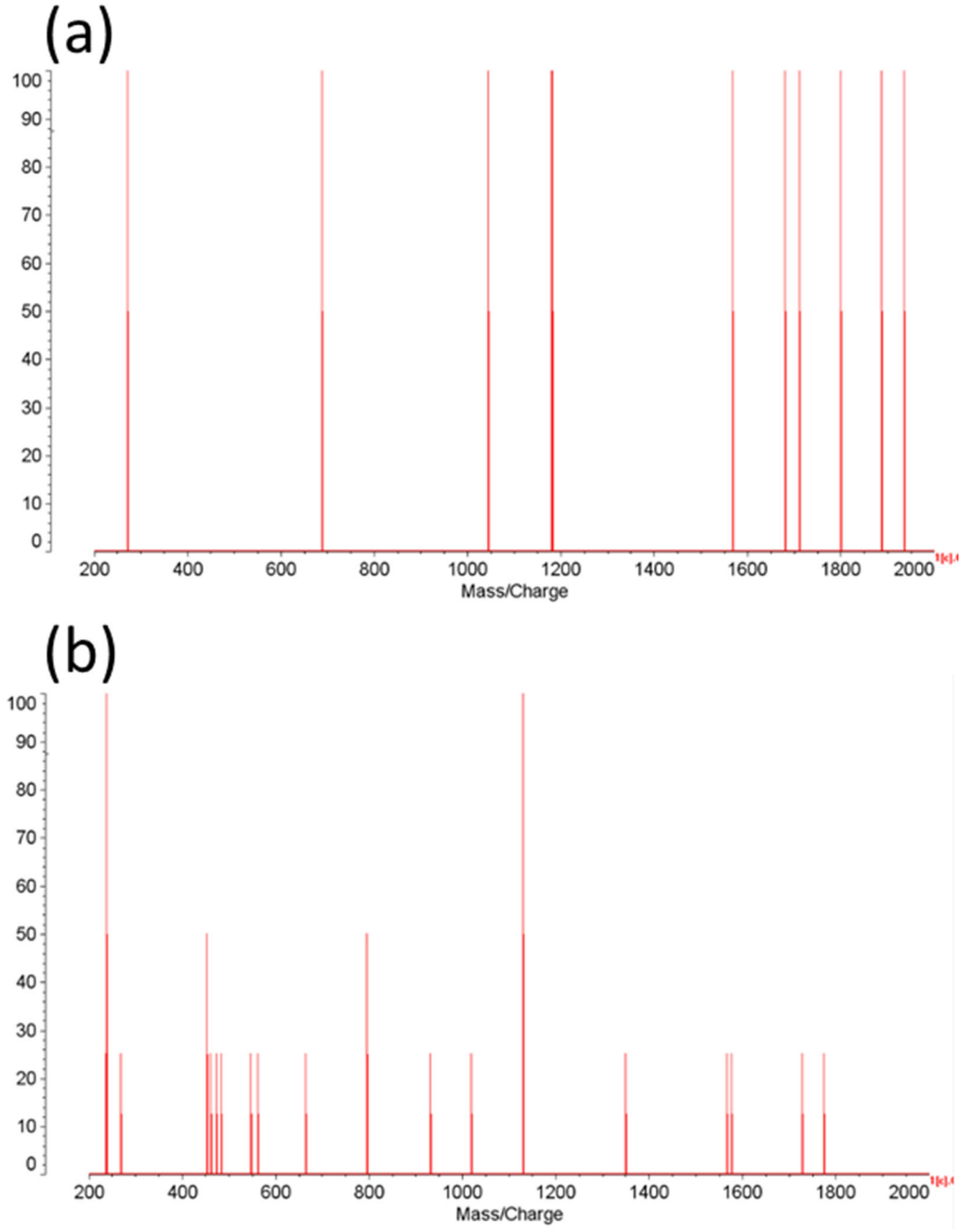


Fig. 2 Inhibition-zone diameter as a function of VA-044 concentration used in the soap-free emulsion polymerization of styrene.

Because our attention was paid to the low-molecular-weight polymers, the MALDI-TOF-MS molecular weights of the polymers prepared using 4-vinylaniline and the four initiators are shown in **Fig. 3**. All polymer colloids contain poly-4-vinylaniline units with a molecular weight < 1,000 Da. The recently determined zeta potentials of the polymer colloids [32] are listed in **Table 2**. The colloids are positively charged even when an anionic initiator (KPS or V-501) is used for polymerization. We recently reported that positively charged, benzene-ring containing polymers synthesized by soap-free emulsion polymerization with a molecular weight < 1,000 Da are antimicrobially active against *M.*

luteus [28, 29]. Therefore, we used exactly the same method as in Yamamoto *et al*. [28] to bioassay the four polymer colloids.



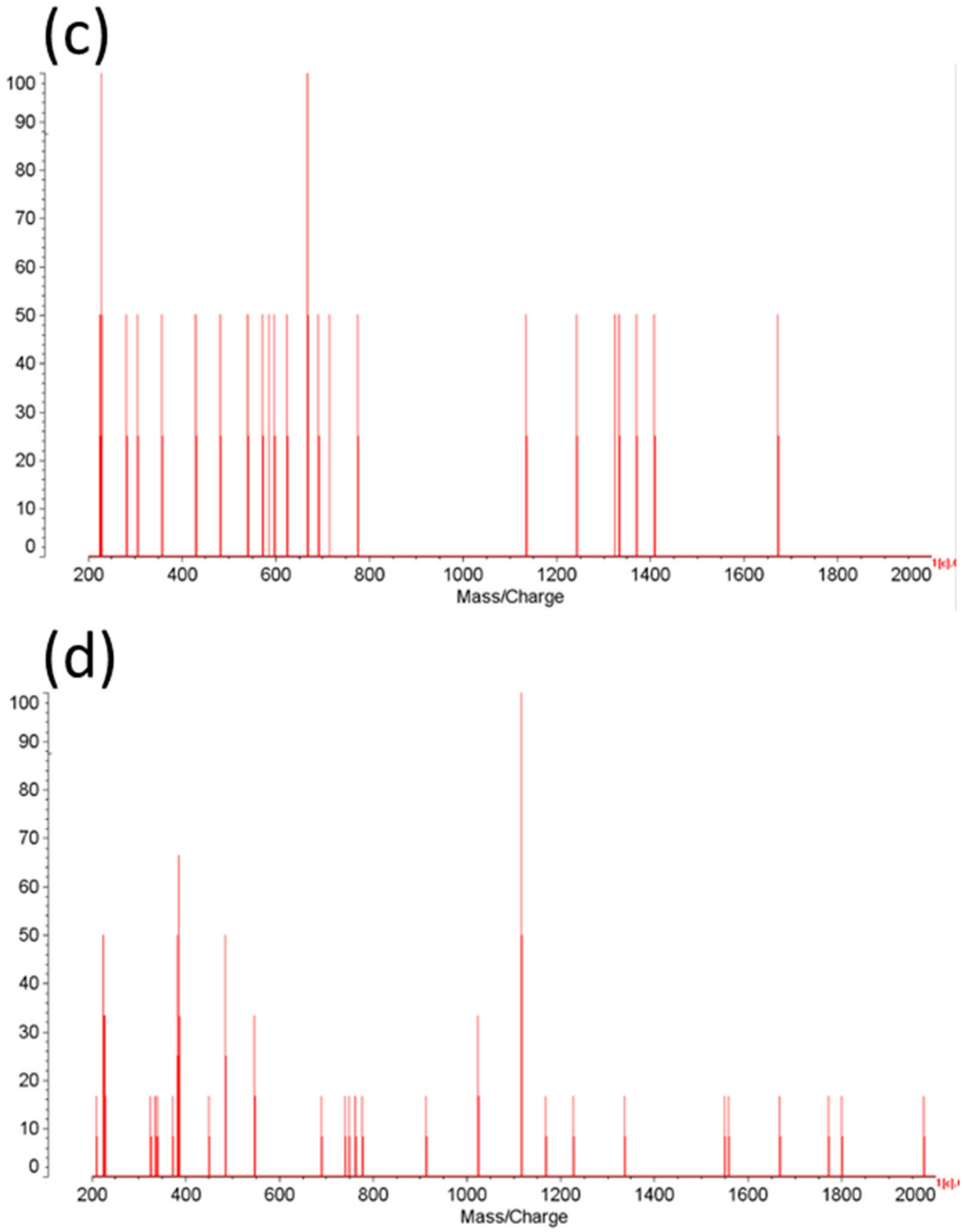


Fig. 3 MALDI-TOF mass spectra of polymer colloids prepared via the soap-free emulsion polymerization of 4-vinylaniline with (a) VA-044, (b) V-50, (c) KPS, and (d) V-501 as initiators.

Table 2. Zeta potentials [mV] of polymer colloids prepared using four kinds of initiators [32].

VA-044	V-50	KPS	V-501
49±4	25±6	23±3	10±4

Figs. 4a and **4b** show that polymer colloids prepared using cationic initiators (VA-044 and V-50) produce clear inhibition zones against *M. luteus*. These results agreed well with our previous study [29]. However, inhibition zones are not observed in **Figs. 4c** and **4d**, which indicates that polymers prepared using anionic initiators are not antimicrobial despite their positive zeta potentials.

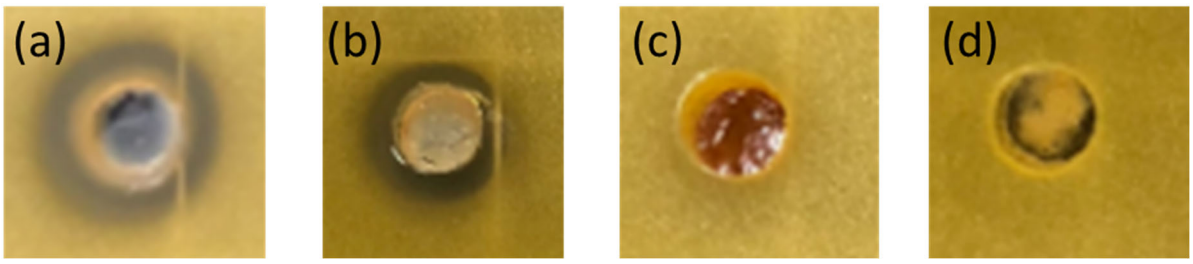


Fig. 4 Bioassay images of polymer colloids prepared via the soap-free emulsion polymerization of 4-vinylaniline using (a) VA-044, (b) V-50, (c) KPS, and (d) V-501 as initiators. Each central hole was 0.8 cm in diameter.

To identify the reasons for the absence of antimicrobial activity in some positively charged polymers, colloids synthesized using the four initiators were examined visually and using SEM. The macroscopic visualizations are presented photographically in **Fig. 5**. Dispersions stable for at least 24 h were obtained when cationic initiators (VA-044 and V-50) were used to polymerize 4-vinylstyrene,

because the positively charged amino groups derived from the monomer and initiator repel each other. However, polymer colloids prepared using KPS and V-501 yielded precipitates.

Polymer colloids were also were examined via SEM. The VA-044- and V-50-initiated polymer colloids exhibited monodispersed particles with good dispersion stability, as previously observed [32]. However, **Fig. 6** shows that colloids synthesized using KPS and V-501 include many coagulated particles because the cationic and anionic functional groups of the monomers and initiators attract one another [32]. Coagulation likely occurs between potentially antimicrobial polymers with molecular weights less than 1,000 Da. As polymer size is a primary determinant of antimicrobial activity [41], coagulation prevents these polymers from attaining this property.

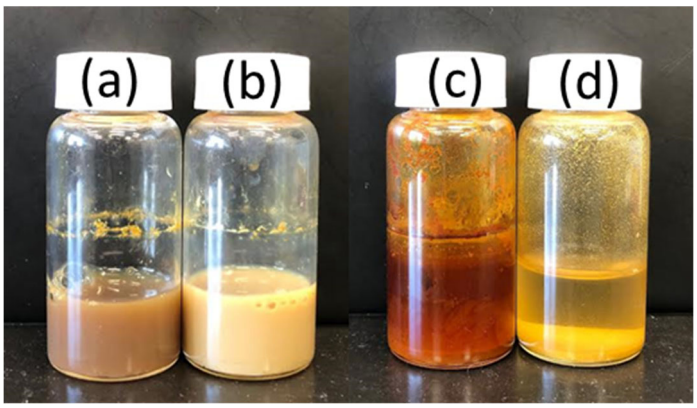


Fig. 5 Dispersion stabilities of polymer colloids prepared via soap-free emulsion polymerization of 4-vinylaniline using (a) VA-044, (b) V-50, (c) KPS, and (d) V-501 as initiators.

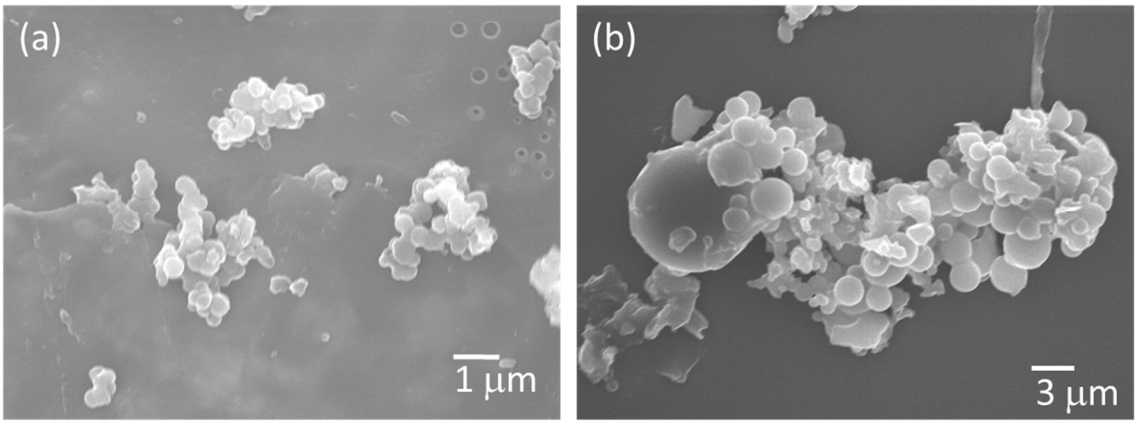


Fig. 6 SEM images of polymer colloids prepared by soap-free emulsion polymerization of 4-vinylaniline with (a) KPS and (b) V-501 as initiators.

In a final experiment, the coagulated polymers synthesized in water using 4-vinylaniline and KPS were removed from the aqueous phase by centrifugation and dissolved in toluene (FUJIFILM Wako Pure Chemical); they did not exhibit antimicrobial activity against *M. luteus* [42], as shown in **Fig. S1** in the supporting information. The bioassay of the toluene solution shown in **Fig. 7** reveals the formation of a distinct inhibition zone that occurred because the coagulated polymer formed smaller particles when dissolved in toluene. We conclude that the antimicrobial activities of polymer colloids correlates closely with their ability to exist as stable dispersions.

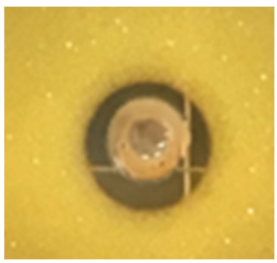


Fig. 7 Bioassay image of a toluene solution of the polymer prepared by the soap-free emulsion polymerization of 4-vinylaniline with KPS.

4. Conclusions

Polymer colloids synthesized by polymerization of 4-vinylaniline in water with cationic (VA-044, V-50) and anionic (KPS, V-501) initiators exhibited different tendencies to form stable dispersions. The relationship between dispersion formation and antimicrobial activity was investigated. All colloids included positively charged polymers with molecular weights < 1 kDa. These properties satisfied the conditions for the polymer colloids to show antimicrobial activities from our previous research. Materials synthesized by polymerization with cationic initiators, such as VA-044 and V-50, exhibited antimicrobial activities against *Micrococcus luteus* because the dispersion stabilities of the polymers were good enough to keep the nano size of the polymer much more stable in the aqueous phase due to the electro repulsive force between the functional groups of the monomer and initiator. However, materials synthesized by polymerization with anionic initiators, such as KPS and V-501, did not create an inhibition zone against *M. luteus* because the low-molecular-weight polymers coagulate into larger particles due to the electrostatic attractive forces between the cationic functional group from the monomer and the anionic functional group from initiator. However, when dissolved in toluene to form a stable dispersion, the coagulated KPS-prepared polymer did display antimicrobial activity because the sizes of the polymers became smaller. Thus, a polymer colloid capable of forming a stable dispersion is the potentially antimicrobially active regardless of its method of preparation.

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