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Article

Contact-Killing Antibacterial Polystyrene Polymerized Using a Quaternized Cationic Initiator

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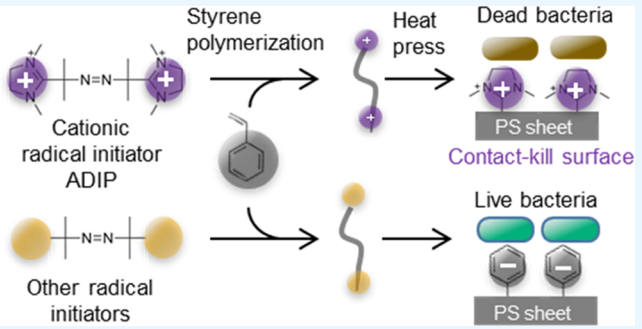
ABSTRACT: Contact-killing antibacterial materials are attracting attention owing to their ability for sustained antibacterial activity. However, contact-killing antibacterial polystyrene (PS) has not been extensively studied because its chemically stable structure impedes chemical modification. In this study, we developed an antibacterial PS sheet with a contact-killing surface using PS synthesized from 2,2′-azobis-[2-(1,3-dimethyl-4,5-dihydro-1*H*-imidazol-3-ium-2-yl)]propane triflate (ADIP) as a radical initiator with cationic moieties. The PS sheet synthesized with ADIP (ADIP-PS) exhibited antibacterial activity in contrast to PS synthesized with other azo radical initiators. Surface ζ-potential measurements revealed that only ADIP-PS had a cationic surface, which contributed to its contact-killing antibacterial activity. The ADIP-PS sheets also exhibited antibacterial activity after washing. In contrast, PS sheets containing silver, a typical leachable antibacterial agent, lost all antibacterial activity after the same washing treatment. The antibacterial ADIP-PS sheet demonstrated strong broad-spectrum activity against both Gram-positive and Gram-negative bacteria, including drug-resistant bacteria. Cytotoxicity tests using L929 cells showed that the ADIP-PS sheets were noncytotoxic. This contact-killing antibacterial PS synthesized with ADIP thus demonstrated good prospects as an easily producible antimicrobial material.



1. INTRODUCTION

The outbreak and spread of drug-resistant bacterial infections pose a serious challenge to healthcare. Antibacterial surface treatments may help prevent drug-resistant bacterial infection and disease outbreaks caused by contact with contaminated surfaces and medical equipment.[1−3] Antibacterial-treated surfaces can be classified into two categories based on their mechanisms of action, namely, contact-kill and leachable surfaces.[1,4,5] Contact-kill surfaces have specific biocides consisting of chemical structures or substances that are covalently fixed or physically attached and can damage microorganisms and directly kill them without being released into the environment. By contrast, leachable surfaces typically kill microorganisms by incorporating antibiotic compounds, such as nitric oxide[4] and silver nanoparticles,[6] into the material or surface during manufacture and releasing them into the environment by diffusion. To ensure that released antimicrobial agents do not contaminate the environment, antimicrobial and biodegradable polyester polyionenes with inserted ester functions have also been developed.[7]

Unlike leachable surfaces, contact-kill surfaces generally have long-term antibacterial effects,[8−11] eco-friendliness,[12,13] and low cytotoxicity.[11,14] Zander and Becker suggested that materials with contact-kill surfaces effectively reduce the occurrence of drug-resistant bacteria and have long-lasting antibacterial activities because the antibacterial substances remain in the material.[15] However, only a few general-purpose plastics, such as polystyrene (PS), polypropylene, and polyethylene, have been practically used as contact-killing antibacterial materials primarily because they have chemically stable structures, which impede chemical modification.

At present, contact-kill polymers are synthesized in two main steps, namely, polymerizing monomers with antibacterial functional groups[16] and modifying the prepared polymer with an antibacterial active group such as quaternary ammonium, chitosan, or silver.[6,17] Among these antibacterial compounds, quaternary ammonium groups are often used to synthesize contact-kill polymers[18] owing to their activities against a broad spectrum of bacteria, including *Escherichia coli*,[19,20] *Staphylococcus aureus*,[19,29] *Pseudomonas aeruginosa*,[19] and methicillin-resistant *S. aureus* (MRSA).[21] Notably,

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materials combining quaternary ammonium groups with general-purpose plastics, such as cationic PS,[22] poly(methyl methacrylate),[23] and polyurethane,[24] have been reported to exhibit antibacterial activity through contact-killing cationic surfaces. However, the synthesis of polymers with quaternary ammonium groups for general-purpose plastics is challenging. Generally, these polymers are synthesized through copolymerization with quaternary ammonium monomers or by quaternization of reactive amines in the polymer.[20] Quaternary ammonium monomers with antibacterial activity are not general-purpose monomers and often have specific structures,[25−27] which may make them expensive to produce at the industrial scale. Additionally, performing the quaternization reaction after polymerization increases the number of reaction steps and may necessitate the purification of unreacted agents.

In this study, we aimed to produce PS materials with contact-killing antibacterial surfaces via a facile one-pot polymer synthesis method. To prepare PS sheets with cationic surfaces, we polymerized PS with a cationic group at the end using a unique azo radical initiator with cationic moieties, namely, 2,2′-azobis-[2-(1,3-dimethyl-4,5-dihydro-1*H*-imidazol-3-ium-2-yl)]propane triflate (ADIP; Figure 1).[28−30] To clarify

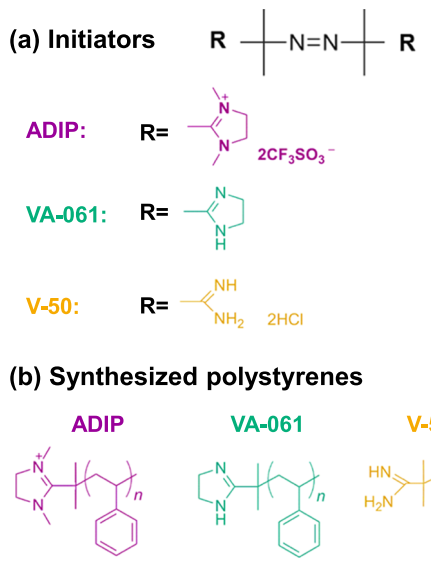

Figure 1. Chemical structures of (a) initiators and (b) synthesized polystyrenes (PSs) with 2,2′-azobis-[2-(1,3-dimethyl-4,5-dihydro-1*H*-imidazol-3-ium-2-yl)]propane triflate (ADIP), 2,2′-azobis[2-(2-imidazolin-2-yl)propane] (VA-061), and 2,2′-azobis(2-methylpropionamidine)dihydrochloride (V-50) attached to the ends of the PS chains.

the relationship between the end structure of PS and its antimicrobial activity, 2,2′-azobis[2-(2-imidazolin-2-yl)-propane] (VA-061, Figure 1) and 2,2′-azobis(2-methylpropionamidine)dihydrochloride (V-50; Figure 1) were used as amine-based water-soluble radical initiators to polymerize PS. The loss of antibacterial activity was compared upon washing PS synthesized with ADIP (ADIP-PS) and silver-containing PS (Ag-PS). In addition, the antibacterial spectrum of the ADIP-PS sheets was investigated.

2. EXPERIMENTAL SECTION

2.1. Materials. Ethanol, 2-propanol, V-50, VA-061, and soybean-casein digest broth containing lecithin and polysorbate 80 (SCDLP broth) were purchased from the FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Standard agar medium was purchased from EIKEN Chemical Co. Ltd. (Tokyo, Japan). Styrene and cethyltrimethylammonium chloride (CTAC) were purchased from the Tokyo Chemical Industry Corporation (Tokyo, Japan). Styrene monomers stabilized with *tert*-butylcatechol were passed through an inhibitor remover column (Sigma-Aldrich, St. Louis, MO) before use. 6-(*p*-Toluidino)-2-naphthalenesulfonic acid sodium salt (TNS) was also purchased from Sigma-Aldrich (St. Louis, MO). Streptomycin, penicillin, minimum essential medium (MEM), and fetal bovine serum (FBS) were purchased from Gibco (Waltham, MA). A Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Laboratories (Kumamoto, Japan). The cationic radical initiator ADIP was synthesized as described previously.[28] Specifically, it was synthesized through a three-step reaction using the well-established neutral radical initiator 2,2′-azobis-(isobutyronitrile) (AIBN). In the first step, the cyano groups of AIBN were converted to imidate groups under acidic conditions. Then, *N*-methylenediamine was attached to the imidate groups, followed by cyclization to form five-membered imidazoline rings. Finally, methylation was carried out using methyl trifluoromethanesulfonate under mild conditions to obtain ADIP. The overall yield of the three reactions was satisfactorily high (approximately 90%). Ultrapure water was prepared by using a Milli-Q water purification system (Q-POD1; LC-Pak Polisher, Millipore, Billerica, MA) and used in all experiments.

2.2. Synthesis of PS Using Various Azo Initiators. To compare the antibacterial activity of the initiator end in PS synthesized with different initiators, their number-average molecular weights (M_n) were aligned within a certain range. According to previous reports, approximately 1.8 initiator fragments can form chain ends in an average single PS chain.[31,32]

PS samples were synthesized with cationic azo initiators V-50, VA-061, and ADIP. PS was polymerized using ADIP using 2-propanol as the solvent, and ADIP was dissolved in pure water before being added to the reaction container. The detailed polymerization conditions are described in Table 1.

Table 1. Polymerization Reactions of PS with ADIP as an Initiator

sample	reaction temperature (°C)	reaction time (h)	styrene (M)	initiator concentration (mM)	water (vol %)
AD-2K	40	3	4.1	41	4.1
AD-4K	40	3	0.1	1	3.3
AD-6K	40	6	4.1	41	3.3
AD-8K	65	6	4.1	41	3.4

For samples AD-2K, AD-4K, and AD-6K, ADIP dissolved in water was added to styrene dissolved in 2-propanol. Each mixture was heated while being stirred with a magnetic stirrer or with stirring wings. For AD-8K, 70.5 mL of styrene and 53.5 mL of 2-propanol were added to a 300 mL separable flask and heated to 65 °C under stirring at 450 rpm with a mixing impeller with four leaf springs. When the temperature reached 65 °C, 3.7 g of ADIP was dissolved in 5 mL of water and 20 mL of 2-propanol. The ADIP-containing solution was added to a separable flask over 2 h using a peristaltic pump (Cat No. SJ-1211H; ATTO, Tokyo, Japan), after which the polymerization reaction was continued for 4 h. The obtained polymer was

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precipitated by pouring the solution into hexane. The liquid fraction was discarded by decantation, and methanol was added to the polymer precipitate, and the mixture was stirred using a magnetic stirrer. The polymer precipitate was collected by filtration and dried under reduced pressure to give a powder.

During emulsion polymerization using V-50, 400 mL of water, 11.5 mL of (200 mM) styrene, 271 mg of (2.0 mM) V-50, and 100 mL of ethanol were added to a 1 L separable flask and stirred with a mechanical stirrer under a nitrogen atmosphere at 70 °C for 7 h. After emulsion polymerization, the polymer sample was centrifuged at 40,000*g* for 2 h, and the pellet was washed with ethanol. The ethanol was removed by further centrifugation at 40,000*g* for 2 h, and the pellet was dissolved in chloroform and precipitated with hexane. The precipitates were washed with methanol, filtered, and dried under reduced pressure to obtain a PS powder.

During solution polymerization using VA-061, 14.1 mL of (4.1 M) styrene, 14.7 mL of (5.8 mM) 2-propanol, 306.4 mg of (40.7 mM) VA-061, and 1 mL of water were added to a 30 mL glass vial and stirred with a magnetic stirrer at 70 °C for 3 h. After solution polymerization, the separated polymer phase was reprecipitated with hexane. The precipitated polymer was washed with methanol, filtered, and dried under reduced pressure to obtain a PS powder. The yield was calculated as the ratio of the polymer weight obtained after purification to the total monomer weight input.

2.3. Characterization of the Synthesized PSs. The molecular weights of the synthesized PSs were determined by gel permeation chromatography (GPC) with a Shimadzu HPLC system (LC20AD, Shimadzu, Kyoto, Japan), a refractive index detector (RID-10A), and a Shodex KF-405LHQ column (Showa Denko, Tokyo, Japan) controlled at 40 °C. A calibration curve was obtained using PS standards (cat. no. 81434−1EA; Sigma-Aldrich, St. Louis) with chloroform as the eluent. Attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) was used to determine the characteristic functional groups of PS using a Nicolet IS50 FT-IR infrared spectrophotometer (Thermo Fisher Scientific) equipped with an ATR attachment with a diamond crystal plate as a reflector and a deuterated triglycine sulfate detector. The absorbance was measured in the range of 500−4000 cm^{-1} using 32 scans. The nitrogen content (%, N content) was measured by elemental analysis using a CHN coder MT-5 (Yanaco, Kyoto, Japan) analyzer. Based on previous reports,[31,32] assuming that 1.8 initiator termini are bound to PS, the ADIP residue content (mol %) can be calculated from the N content using eq 1

$$\text{ADIP residue content (mol \%)} = \frac{1.8 \times \text{molecular weight of ADIP residue}}{\text{molecular weight of ADIP-PS}} \quad (1)$$

where the molecular weight of the ADIP residue was taken to be 144.2, as shown in Figure 1b, and that of ADIP-PS was calculated as (1.8 × 28)/(N content).

2.4. Preparation of PS Sheets. PS sheets were pressed and molded from the synthesized PS powders or granules as follows. First, the PS powder was sandwiched between poly(ethylene terephthalate) (PET) sheets (Lumirror S10; Toray Industries, Tokyo, Japan), which were used as release agents and then sandwiched on the outside by stainless steel (SUS) plates. The two SUS plates were pressed at 150 °C for approximately 1 min per press. The heat-pressed PS sheet was peeled off from the PET sheets and cut into four sheets before being re-sandwiched between the PET sheets and SUS plates. The heat-pressing step was then repeated 10 times in the same manner. The diameter and thickness of the PS sheet were approximately 5 cm and 1 mm, respectively.

2.5. Preparation of Silver-Containing PS Sheets. To obtain Ag-PS, the base general-purpose PS (MW1D, Toyo Styrene, Japan; M_n = 1.1 × 10^5, M_w = 3.5 × 10^5), a pure PS pellet was melt-mixed with another PS pellet containing 5 wt % silver-exchanged zeolites in a twin-screw extruder (Xplore MC15HT; Xplore Instruments, Sittard, Netherlands) at 190 °C and 100 rpm for 3 min. The silver-containing PS pellet is commercially available and contains silver-exchanged zeolites, as confirmed by scanning electron microscopy−energy-dispersive X-ray spectroscopy (SEM-EDX) analysis. The resulting Ag-PS granules (1.5 g) were heat-pressed to prepare Ag-PS sheets with diameters and thicknesses of approximately 6 cm and 1 mm, respectively.

2.6. Evaluation of the Antibacterial Performance of the PS Sheets. *2.6.1. Antibacterial Activity Determination of the PS Sheets.* The antibacterial activity was evaluated based on ISO 22196:2011, which is the protocol for measuring the antibacterial activity of plastic materials against *S. aureus* (NBRC12732).[32] Glass plates or polyethylene sheets were used as references because they do not exhibit antibacterial activity. *S. aureus* levels increased similarly on the glass plates and polyethylene sheets. Therefore, the glass plates and polyethylene sheets were handled in the same way (Figure S1). For other experiments, MRSA (IID1677), vancomycin-resistant *Enterococcus faecium* (NCTC12204), *Listeria monocytogenes* (VTU206), and *P. aeruginosa* (NBRC 13275) were used for antibacterial evaluations. The different bacteria were precultured for 24 h on standard agar media (5 g/L meat extract, 10 g/L peptone, 5 g/L NaCl, 15 g/L agar). The colonies were then suspended in a diluted nutrient broth (NB), the concentration of which was changed, depending on the microorganisms used. Specifically, 1/100-, 1/200-, and 1/500-diluted NB medium was used for *E. faecium*, MRSA, and all other bacteria, respectively. The final bacterial concentration in each medium was 2.5 × 10^5 colony-forming units (CFU)/mL. The mixed solution was placed on the reference and test PS samples and incubated at 35 °C for 24 h under at least 90% humidity. The samples were then washed with 10 mL of the SCDLP medium. The washed-out SCDLP medium was serially diluted 10, 10^2, 10^3, and 10^4 times. Then, 1 mL of both the washout medium and each dilution was carefully dispensed into separate sterilized Petri dishes. Subsequently, 15−20 mL of melted standard agar medium was added to each Petri dish, ensuring thorough mixing. The Petri dishes were then covered and allowed to stand at room temperature until the agar medium solidified. When the agar solidified, the plates were incubated at 35 °C for 40−48 h. The colonies were counted, and log(CFU/cm^2) was calculated from the plate counts. If the number of colonies was zero, log(CFU) was set to 0.05 for convenience.

2.6.2. Assessment of Leaching of Antibacterial Components from ADIP-PS Sheets. The leaching of antibacterial components from the ADIP-PS sheets was assessed using zone inhibition tests. The standard agar media were inoculated with *S. aureus* bacteria at a level of 10^8 CFU/mL. The ADIP-PS sheets were placed on the agar plate. The cationic surfactant CTAC was applied as a control for the leachable antibacterial

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agent, and sterilized filter paper soaked with 10 μL of 0.4 mM CTAC solution and PBS was prepared and placed on the standard agar medium. After 24 h in a 35 °C incubator, the zone of inhibition around the sheet and filter papers was evaluated.

2.7. Measurement of the Surface ζ-Potential of the PS Sheets. The surface ζ-potential was measured using a Zetasizer Nano ZSP instrument with a surface ζ-potential accessory (Malvern Instruments, Malvern, U.K.). To measure the surface ζ-potential of the PS sheets, sheets with lengths, widths, and thicknesses of 5, 3.5, and 1.0 mm, respectively, were prepared. Anionic PS latex (Malvern, catalog no. DTS1235, −42 ± 4.2 mV) and aluminum oxide nanopowder (Sigma-Aldrich, catalog no. 544,833, >+30 mV) were used as tracer particles for anionic and cationic surface measurements, respectively. The suspension containing the cationic tracer particles was prepared by dispersing 0.1 g of aluminum oxide powder in 10 mL of water and leaving it overnight at room temperature (20−25 °C). After this period, the supernatant was used as the tracer particle suspension.

2.8. Confirmation of Cationic Groups on Sheet Surface by Anionic Dye. To confirm the presence of cationic groups on the surface of the ADIP-PS sheet, the surface adsorption of TNS, an anionic fluorescent dye, was qualitatively detected.[30] TNS was dissolved in ethanol to achieve a concentration of 25 μM and then diluted 10 times with water, resulting in a 2.5 μM TNS solution. To detect cationic groups on the surface, a 3 cm × 3 cm sheet consisting of ADIP-PS and general-purpose PS was loaded with 16 μL of the 2.5 μM TNS solution, and a circular glass cover with a diameter of 12 mm was placed on top. After the sample was left at room temperature for 1 min, the cover glass was removed, and 10 μL of the TNS solution was collected. To the collected liquid were added 90 μL of water and 400 μL of dioxane, and the fluorescence intensity at the maximum emission wavelength of 421 nm when excited at 370 nm was measured using a fluorescence spectrophotometer (JASCO Spectrofluorometer FP-8500). The concentration was determined from the calibration curve of the TNS, and the percentage of adsorption was then calculated.

2.9. Washing Procedure for PS Sheets. The ADIP-PS and Ag-PS sheets (approximately 1.5 g each) were each placed in a Petri dish and washed with 5 mL of water. Both sheets were washed at 25 °C for 8 days, and the water was replaced with 5 mL of new washing water every 24 h. Because PS sheets float in water owing to their density, the surface of the sheets that was always in contact with water was considered to be the washed surface and was used for further antibacterial tests.

2.10. Live/Dead Assay Method. A *S. aureus* suspension with a concentration of 1 × 10^7 CFU/mL (0.1 mL) was dropped onto an ADIP-PS sheet (2 cm × 2 cm). The sheet was incubated at 35 °C for 1 h under a humidity of at least 90% humidity. The *S. aureus* were stained using a LIVE/DEAD BacLight Bacterial Viability Kit (Cat. No. L13152, Thermo Fisher Scientific). One tube each of SYTO 9 and propidium iodide (PI) were dissolved in 1 mL of water to make a working solution. Then, the bacterial suspension on the ADIP-PS sheet was stained for 15 min after addition of 1 μL of the working solution (SYTO 9/PI = 1:1). The SYTO 9 dye fluoresces green for live bacteria, and PI fluoresces red to indicate cell-membrane-damaged bacteria. The stained samples were examined, and images were captured by a ZEISS LSM980 confocal microscope equipped with a Plan Apochromat 63× oil immersion objective with a 1.4 NA.

2.11. Cytotoxicity Testing of ADIP-PS. Cytotoxicity was assessed using a CCK-8 assay and the L929 mouse fibroblast cell line (ATCC CCL-1).[33,34] In this experiment, a PS dish for cell culture (Cat. No. 353,002, Falcon) was used as a positive control. Aliquots of 100 μL of the cell suspension (1 × 10^5 cells/mL in MEM with 10% FBS and 1% penicillin/streptomycin) were seeded into the ADIP-PS sheet and PS dish for cell culturing. Then, the plate was incubated at 37 °C under 5% CO_2 for 1 day. Subsequently, 100 μL of the culture medium was replaced with fresh MEM medium, and 10 μL of CCK-8 reagent was added to the medium, followed by incubation for 2 h. The absorbance at 450 nm of the medium was measured with a spectrophotometer.

3. RESULTS AND DISCUSSION

3.1. Preparation of PS Using Azo Radical Initiators with Different End Structures. We synthesized PS using three water-soluble azo initiators and verified whether antibacterial properties had been achieved. We obtained ADIP-PSs with different M_n values in the range of 2000−8000 by varying the temperature, reaction time, and molar ratio of ADIP to styrene for solution polymerization, as shown in Table 2. The PS with the VA-061-derived terminal group was synthesized in the same manner as the ADIP-PS, with approximately the same yield (Table 2, VA061−2K). For the PS with the V-50-derived terminal group, the low solubility of V-50 in 2-propanol caused extremely low reactivity between V-50 and styrene during solution polymerization. Therefore, emulsion polymerization was performed to obtain linear PS powder with a V-50-derived terminal group in a modest yield. The M_n values of the PSs synthesized with both VA-061 and V-50 were a few thousand, that is, within the molecular weight range of the ADIP-PSs synthesized in this study (Table 2). The successful synthesis of PS was confirmed using FTIR, as shown in Figure S2. Specifically, we observed the disappearance of the double-bond stretching vibration (1683 cm^{-1}) originating from the vinyl group of styrene. Additionally, strong absorption from the out-of-plane angles of CH in the benzene ring was also confirmed at approximately 750 and 700 cm^{-1}, which are characteristic of PS. Furthermore, absorption was observed only in the PS synthesized with ADIP near 1150 and 1270 cm^{-1}. These peaks are characteristic of the trifluoromethanesulfonic (triflate) ion and are presumably due to CF_3 stretching and SO_3 stretching modes, respectively.[35] These results suggest that the triflate ion was strongly coordinated to the terminal ADIP residue of ADIP-PS. The ADIP residue at the ends of the ADIP-PS polymer was

Table 2. Yield and Molecular Weight of the Obtained PSs with Different Azo Initiators

sample	initiator	M_n (×10^3)[a]	M_w (×10^3)[b]	M_w/M_n	yield (%)
AD-2K	ADIP	2.0	7.0	3.5	20
AD-4K	ADIP	4.7	20	4.3	42
AD-6K	ADIP	5.3	11	2.1	31
AD-8K	ADIP	8.0	15	1.9	37
VA061−2K	VA-061	2.1	8.1	3.9	37
V50−6K	V-50	6.6	96	14.5	13

[a]Number-average molecular weight (M_n) evaluated from GPC. [b]Weight-average molecular weight (M_w) evaluated from GPC.

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characterized by elemental analysis; because PS does not contain N atoms, the amount of ADIP was calculated by determining the nitrogen content ratio (Table 3). Nitrogen was not detected at any of the general-purpose PS, which does not contain nitrogen, but nitrogen was significantly detectable in ADIP-PS. The ADIP residue content was also confirmed to generally depend on the molecular weight.

Table 3. ADIP Residue Contents of ADIP-PS

sample	N content (wt %)	ADIP residue content (mol %)
AD-2K	1.12	5.8
AD-4K	0.70	3.6
AD-6K	0.66	3.4
AD-8K	0.65	3.3
GPPS[a]	0	

[a]General-purpose PS MW1D (Toyo Styrene; M_n = 1.1 × 10^5, M_w = 3.5 × 10^5).

3.2. Antibacterial Activity Evaluation of PS Sheets Synthesized with ADIP and Other Initiators. After obtaining the desired PSs with different end-group structures derived from the initiators, we evaluated the antibacterial activities of the PSs. The synthesized powders were first processed into sheets by heat-press molding. The PS sheets fabricated with the ADIP-PS had the characteristic transparency of PS but with a slightly yellow hue (Figure 2a). This yellow coloration was probably derived from the imidazolium group of ADIP on the surface of the PS sheet. The ISO 22196:2011 standard, which specifies a method for evaluating the antibacterial activity of antibacterial-treated plastics, was used with some modifications to evaluate the antibacterial activity,[36] and glass plates and polyethylene sheets were used as control samples. These samples did not inhibit bacterial growth and displayed almost the same degree of *S. aureus* growth after 24 h of incubation (Figure S1). By contrast, Figure 2b shows that the ADIP-PS sheets exhibited extremely high antibacterial activity at all tested average molecular weights (M_n = 2000−8000) and almost completely killed *S. aureus*. The proportion of *S. aureus* was reduced to less than 0.01% before incubation on the ADIP-PS sheets. However, the PS sheets synthesized with VA-061 and V-50 did not exhibit antibacterial activity. Although these radical polymerization initiators have cationic moieties similar to those of ADIP in water, ADIP differed from these initiators in that it conferred the PS with antibacterial properties.

3.3. Comparison of ADIP-PS with PS Synthesized with Other Initiators. We investigated the changes in the functions of the initiator-derived end groups on the sheet surfaces. Yamamoto et al. reported that PSs with a molecular weight of less than 1000 synthesized with V-50 and VA-044 (hydrochloride of VA-061) could function as leachable antibacterial agents.[37] However, because the polymers used in our study had molecular weights that exceeded 2000 (because low-molecular-weight fractions were removed by reprecipitation), we hypothesized that the antibacterial activity occurred through a contact-killing mechanism rather than through a leaching mechanism. A zone of inhibition test against *S. aureus* was performed using ADIP-PS sheets, as shown in Figure 3. No inhibition circles formed around the ADIP-PS sheets, whereas CTAC, a known leachable antibacterial agent,[38] clearly formed inhibition circles. This implies that the antibacterial component of the ADIP-PS sheet used in this study stays on the surface of the ADIP-PS sheet without diffusing. Table 4 shows the surface ζ-potentials of the

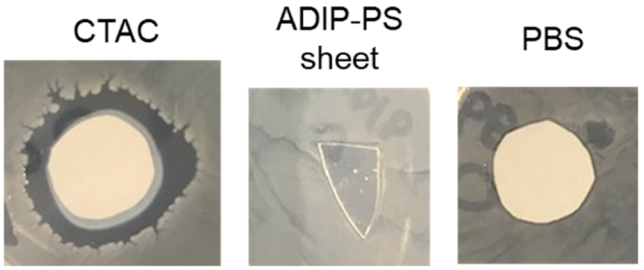

Figure 3. Inhibition zones against *S. aureus* on standard agar media with CTAC, the ADIP-PS sheet (AD-8K), and PBS.

Table 4. Surface ζ-Potentials of the PS Sheets

sample	initiator	ζ-potential (mV)
AD-5K	ADIP	36 ± 4.0[a]
VA061−2K	VA-061	−48 ± 10[b]
V50−6K	V-50	−64 ± 17[b]

[a]Mean ± SD (n = 4). [b]Mean ± SD (n = 5).

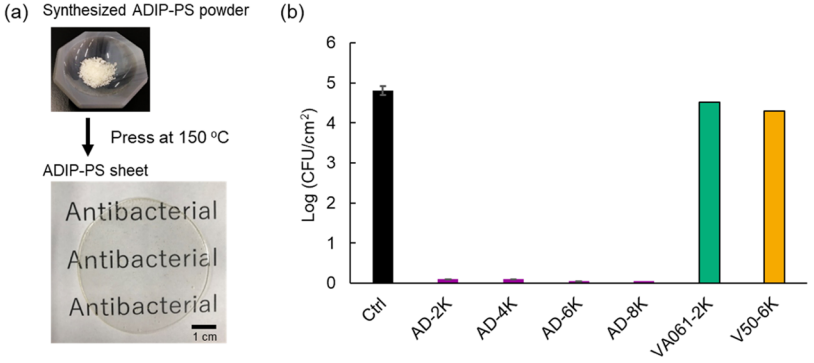

Figure 2. Antibacterial activity of ADIP-PS sheets. (a) Representative appearance of the synthesized ADIP-PS powder (top) and heat-pressed sheet (AD-8K, bottom). (b) Antibacterial activity of PS sheets synthesized with ADIP, VA-061, and V-50 against *S. aureus*. Error bars for the control (Ctrl) and ADIP-PSs represent the standard deviation (SD) with n = 5 and 3, respectively. The data for VA-061 and V-50 are the averages of two experimental results.

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PS sheets synthesized with VA-061, V-50, and ADIP. As expected, the surfaces of the PS sheets synthesized with VA-061 and V-50 were negatively charged, likely because of the π-electrons of the styrene. In contrast, the PS sheets synthesized with ADIP were positively charged. When the presence of cationic groups on the PS sheet surface was qualitatively evaluated using anionic fluorescent dye TNS (2.5 μM solution), the ADIP-PS sheet (AD-8K) exhibited 34% adsorption, while no adsorption was detected on the general-purpose PS (MW1D) sheet. This difference suggests that cationic functional groups existed on the surface at the molecular level. The ADIP residue of the PS end-group counteracted the negative charge of styrene and conferred the surface with a positive charge. Thus, PS surfaces with positive charges derived from active quaternary ammonium groups were fabricated from an ADIP-PS powder by a simple heat-pressing step. The PS sheets synthesized with V-50 and VA-061 exhibit anionic surfaces, possibly owing to the absence of proton donors such as water and acids during heat pressing. The cationic surface of the ADIP-PS sheet can be identified as a determinant of antibacterial activity during contact-killing.

We also investigated whether ADIP-PS sheets also damage the cell membranes of bacteria, as do other antibacterial materials containing quaternary ammonium salts.[39] Figure 4

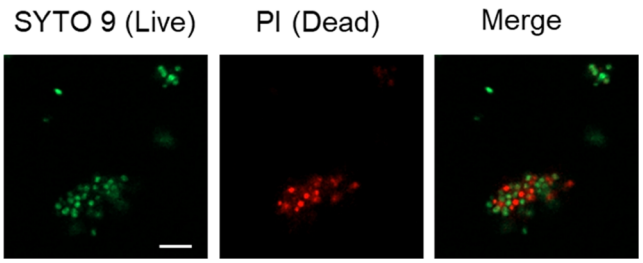

Figure 4. Representative images of *S. aureus* on an ADIP-PS (AD-8K) sheet were recorded by laser scanning confocal fluorescence microscopy. Merged images derived from dual labeling of bacteria with SYTO 9 (green) and PI stain (red). PI: Ex. 561 nm, Em. 565−727 nm, SYTO 9: Ex. 488 nm, Em. 380−548 nm. The white scale bar indicates 10 μm.

shows that PI, a nonmembrane permeable fluorescent dye, stained *S. aureus* after about 1 h contact with the ADIP-PS sheet. This suggests that the cationic surface of the ADIP-PS sheet, probably derived from the imidazolium group of ADIP, impairs the membrane of the bacteria and kills them. Jiang et al. reported that a dimethylethylammonium group grafted onto a PS resin did not exhibit antibacterial activity against *S. aureus* owing to its shorter alkyl chains.[40] Although ADIP has a quaternary ammonium structure that also consists of relatively short carbon chains, it demonstrated strong antibacterial activity, which suggests that the imidazolium group may have a higher antibacterial potential than the simple quaternary ammonium structure alone ($-NR_3^+$). Recently, an imidazolium-based zwitterionic polymer was reported to inactivate coronaviruses;[41] therefore, the imidazolium structure has attracted attention as a cationic group with high biological activity.

3.4. Comparison of ADIP-PS with PS Containing Silver. We selected silver as a leachable antibacterial agent and compared its antibacterial properties with those of ADIP-PS, as shown in Figure 5a. Silver-zeolite-containing PS (Ag-PS) significantly lost its antibacterial activity when the sample was washed with water. By contrast, the antibacterial activity of ADIP-PS sheets washed under the same conditions was unaffected (Figure 5b,c), suggesting that the ADIP-PS sheet had long-lasting antibacterial effects, unlike the Ag-PS sheet. Furthermore, the eluates from the washed ADIP-PS and Ag-PS sheets did not exhibit antibacterial activity (Figures 5d and S3), albeit for different reasons. The antibacterial component of ADIP-PS was not eluted. Instead, it remained on the surface of the ADIP-PS sheet, retaining the contact-killing antibacterial activity of the sheet. In contrast, we speculate that the 8-day washing procedure released so much silver from the zeolites on the Ag-PS surface that the antibacterial activity was lost from both the Ag-PS and its eluate. The washing conditions (8 days in 5 mL of water at 25 °C, replacing the water every 24 h) adopted in this study were milder than those used in research aimed toward medical applications such as dental materials, catheters, and wound healing materials[42−44] (e.g., immersing the materials in water at several tens of times this volume for months). For instance, silver nanoparticles designed to remain in prolonged contact with water do not lose their antimicrobial properties, even after several weeks of washing.[45,46] Generally, PS is used in food containers and consumer electronics casings, so we investigated and compared the antibacterial activity of commercially available silver-zeolite-based PS materials as a reference. In this study, we demonstrated changes in antibacterial activity under conditions where the materials were exposed to a certain amount of water for a week. Most antibacterial plastic materials with cationic surfaces reported thus far have exhibited very high antibacterial activity but were prepared by modification of the material surface.[47] No other contact-killing antibacterial polymer consisting only of monomers without antimicrobial activity and initiators has been reported, which is a unique feature of ADIP.

3.5. Antibacterial Activity of ADIP-PS against Various Bacteria. As the ADIP-PS sheets exhibited strong antibacterial activity against *S. aureus*, we evaluated their activity against other bacteria. As shown in Figure 6, the ADIP-PS sheet strongly inhibited the growth of MRSA, vancomycin-resistant *Enterococci* (VRE), and Gram-negative bacteria. Regarding the antibacterial activity of ADIP-PS against VRE, the degree of growth reduction of VRE was slightly lower than that of other bacteria. By the ISO standard, if the log of the reduction in bacterial growth is greater than 2.0, the sample is considered to exhibit antibacterial activity. Therefore, the ADIP-PS sheets exhibited antibacterial activity against VRE.

These results demonstrate the potential of using ADIP-PS in healthcare applications such as in medical devices and medical packaging of clinical or surgical equipment. However, there are two major hurdles to overcome before commercial use can be realized: the safety and durability of the PS material. If ADIP-PS sheets are to be applied to medical devices, further studies on their toxicity to human cells and biocompatibility in vivo are required.[8] Generally, pure PS is rather brittle and unsuitable for many applications. High-impact polystyrene (HIPS), which is PS-modified with rubber particles to improve toughness, has been used as a cost-effective material in the cosmetic, medical, and food-service packaging industries. Optimizing the mixing conditions of ADIP-PS with HIPS to effectively maintain the antibacterial activity of ADIP-PS will be the subject of future research.

3.6. Cytotoxicity of ADIP-PS Sheet. After verifying that the ADIP-PS sheet showed antibacterial activity, we investigated its cytotoxicity by performing a CCK-8 assay on L929

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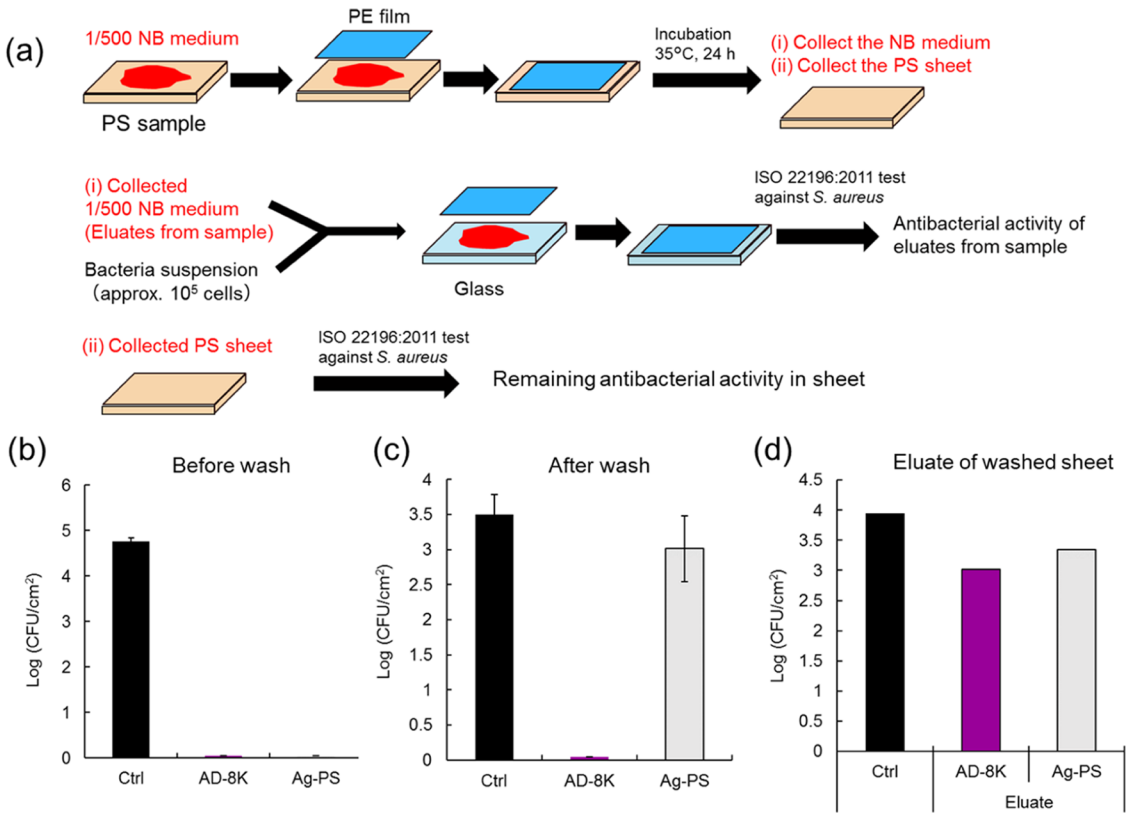

Figure 5. Comparison of the antibacterial properties of ADIP-PS and Ag-PS. (a) Experimental scheme for confirming the antibacterial activity of the sheets after elution and their elution products. (b) Antibacterial activity of ADIP-PS and Ag-PS before washing. Error bars represent the SD (Ctrl, 9; ADIP-PS, 3; Ag-PS, n = 3). (c) Antibacterial activity of ADIP-PS and Ag-PS after washing. Error bars represent the SD (Ctrl, n = 6; ADIP-PS, n = 3; Ag-PS, n = 3). (d) Antibacterial activity of the eluates from the PS sheets after 8 days of washing in water at 25 °C. Error bars for the Ctrl represent the SD (n = 3). The data for ADIP-PS and Ag-PS were obtained from a single experiment.

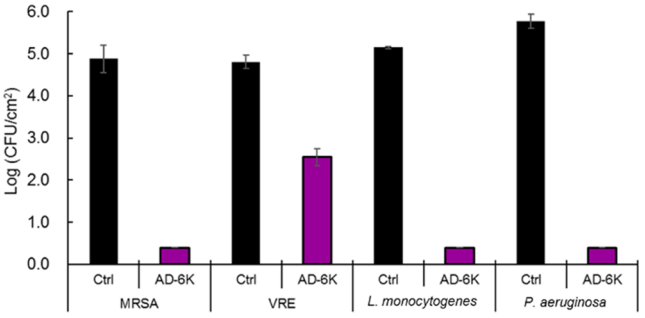

Figure 6. Antibacterial activity of the ADIP-PS sheets against drug-resistant (MRSA and VRE) and Gram-negative (*L. monocytogenes* and *P. aeruginosa*) bacteria. The log CFU values are the averages obtained by using three samples (mean ± SD).

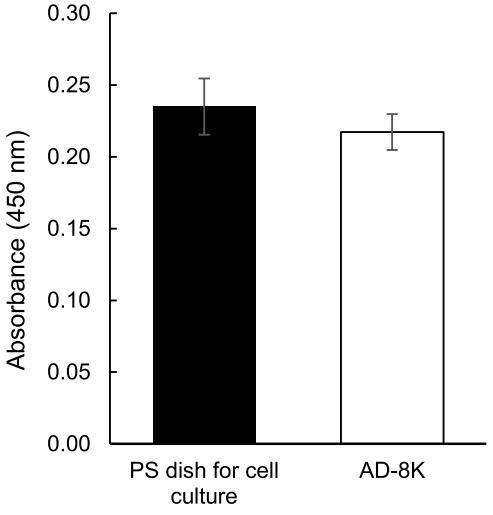

Figure 7. Cell viability and proliferation. The CCK-8 assay determined the proliferation of L929 cells after incubation for 1 day on a PS cell culture dish and an ADIP-PS sheet (AD-8K). The absorbance values are the averages obtained using three samples. The error bars represent the SD (n = 3).

cells. As shown in Figure 7, a t test ($p < 0.05$) between the commercially available PS dish for cell culture and the ADIP-PS sheet showed no significant differences in cell proliferation, suggesting the negligible influence of the cationic antibacterial surface of ADIP on cell growth.

4. CONCLUSIONS

In this study, we synthesized antibacterial PS using ADIP as a radical initiator by using a simple method that did not involve the grafting of biocides or the production of antibacterial monomers. To fabricate general-purpose antibacterial PS resins, antibacterial agents, such as silver, are often kneaded into the resin, but the antibacterial components can elute from the materials with use. We determined that styrene polymerization by ADIP enabled the production of a highly durable antibacterial PS with a contact-killing surface without the use of additional antibacterial agents. ADIP-PSs with an M_n of 2,000−8,000 also exhibited antibacterial activity against *S.*

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aureus. These ADIP-PS samples also exhibited antibacterial activity against MRSA, VRE, and Gram-negative bacteria. The ADIP-PS samples retained their antibacterial activity even after washing, demonstrating their potential as long-term antimicrobial materials. ADIP-PS was also found to be highly antibacterial but not cytotoxic. However, it is important to note that this method is limited to PS and may not be applicable to other materials. Despite this possible material constraint, this method remains a convenient and effective choice for creating antibacterial materials, specifically with PS. Our findings suggest that the initiator could significantly alter the polymer function. In addition to its antibacterial activity, ADIP may be used to introduce new polymer functions.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c10233.

S. aureus growth on glass plates and polyethylene sheets (Figure S1); FTIR spectra of synthesized PSs with different azo initiators (Figure S2); and antimicrobial activity of eluates from Ag-PS sheets before washing (Figure S3) (PDF)

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Notes

The authors declare the following competing financial interest(s): Akiko Jitsuhiro, Akiko Ogawa, Sayuri Yamada, Yuki Konoeda, Hiroki Maruyama and Toshikazu Tsuji were employed by Kirin Holdings Company Ltd.

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■ ABBREVIATIONS

PS, polystyrene; ADIP, 2,2′-azobis-[2-(1,3-dimethyl-4,5-dihydro-1*H*-imidazol-3-ium-2-yl)]propane triflate; MRSA, methicillin-resistant *Staphylococcus aureus*; VRE, vancomycin-resistant *Enterococci*; VA-061, 2,2′-azobis[2-(2-imidazolin-2-yl)-propane]; V-50, 2,2′-azobis(2-methylpropionamidine)dihydrochloride; SUS, stainless steel; M_n, number-average molecular weight; M_w, weight-average molecular weight; HIPS, high-impact polystyrene

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