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Development of Antimicrobial Thermoplastic Material from Archaeal Poly-γ-L-Glutamate and Its Nanofabrication

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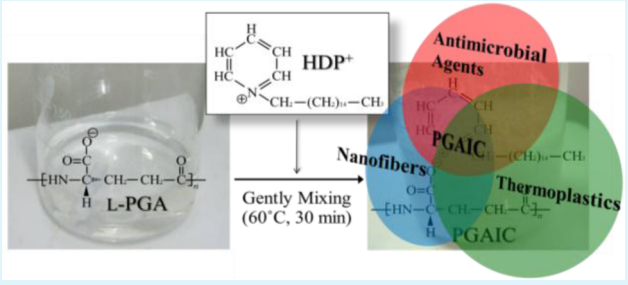
Supporting Information

ABSTRACT: Here we describe a stoichiometric ion-complex of archaeal poly-γ-L-glutamate (L-PGA) and hexadecylpyridinium cation (HDP^+), called PGAIC, which shows remarkable chemical resistance and potential as a novel functional thermoplastic. PGAIC films suppressed the proliferation of prokaryotic (*Escherichia coli*, *Bacillus subtilis*, *Salmonella typhimurium*, and *Staphylococcus aureus*) and eukaryotic (*Saccharomyces cerevisiae*) microorganisms. Moreover, its antifungal activity was demonstrated against a prevalent species of Candida (*Candida albicans*) and a filamentous fungus (*Aspergillus niger*). The minimal inhibitory concentrations were estimated as 0.25 mg mL^{-1}, and zones of growth inhibition appeared when PGAIC-coated polyethylene terephthalate (PET) films were placed in culture plates, whereas PET had very little effect on fungal growth. Soluble PGAIC thus shows promises as an antimicrobial and as a coating substrate. We also succeeded in synthesizing an L-PGA-based nanofiber using an ethanol solution of PGAIC.



KEYWORDS: *poly-γ-glutamate, antimicrobial, thermoplastic, nanofiber, archaeal polymer, cationic surfactant*

INTRODUCTION

As increasing concern for microbial infections,[1] the development of high-performance plastic materials possessing antimicrobial activity as well as biodegradability (or biocompatibility) has become a requisite in the food packaging industries[2] and for producing advanced pharmaceuticals.[1,3] Although elemental silver and its formulation as nanoparticles are the most effective antimicrobial agents,[1] there are concerns regarding its toxicity for humans and environmental impacts due to the uncontrolled release of silver cations.[3] Material chemists have also paid great attention to the contact-active antimicrobial activities of quaternary ammonium compounds,[4−6] as alternatives for silver composites employing time-limited antimicrobial performance.[7,8] In fact, such organic agents are loaded into commercially available acrylic cement[9] that secures the prosthesis to bone and teeth. Petroleum-derived acrylic materials (e.g., polymethylmethacrylate) serve as a basal polymer in cement,[10] whereas current information on the safety and renewability of biobased polymers recommends them as replacements for petroleum-derived polymers.

Here we focused on poly-γ-glutamate (PGA), a biopolymer that is structurally similar to polyacrylate. PGA is attracting interest from commercial sources,[11] and its practical uses depend on its high solubility in water. Attempts to use it for industrial purposes for producing important water-insoluble materials, such as plastics, fibers, and films, have been largely unsuccessful because of its hygroscopic nature. In fact, if stereoregular L-PGA, isolated from a halophilic archaeon *Natrialba aegyptiaca*, is lyophilized so that its moisture content is <5% of the total weight, it exhibits the properties of a thermoplastic (T_m = 149 °C, T_d = 237 °C). Unfortunately, it is not thermoplastic under ambient humidity. In course of our most recent investigations on reforming functional PGA, we identified a compound used in toothpaste, called hexadecylpyridinium cation (HDP^+), as a potent candidate to suppress the extreme hydrophilicity of PGA.

Here we show that L-PGA is easily transformed from a highly water-soluble adhesive to a stable thermoplastic material. We also report the antimicrobial properties of the plasticized L-PGA and techniques for its nanofabrication.

EXPERIMENTAL SECTION

Materials. L-PGA was isolated from the culture media of *N. aegyptiaca* according to published procedures.[12] The bromide and

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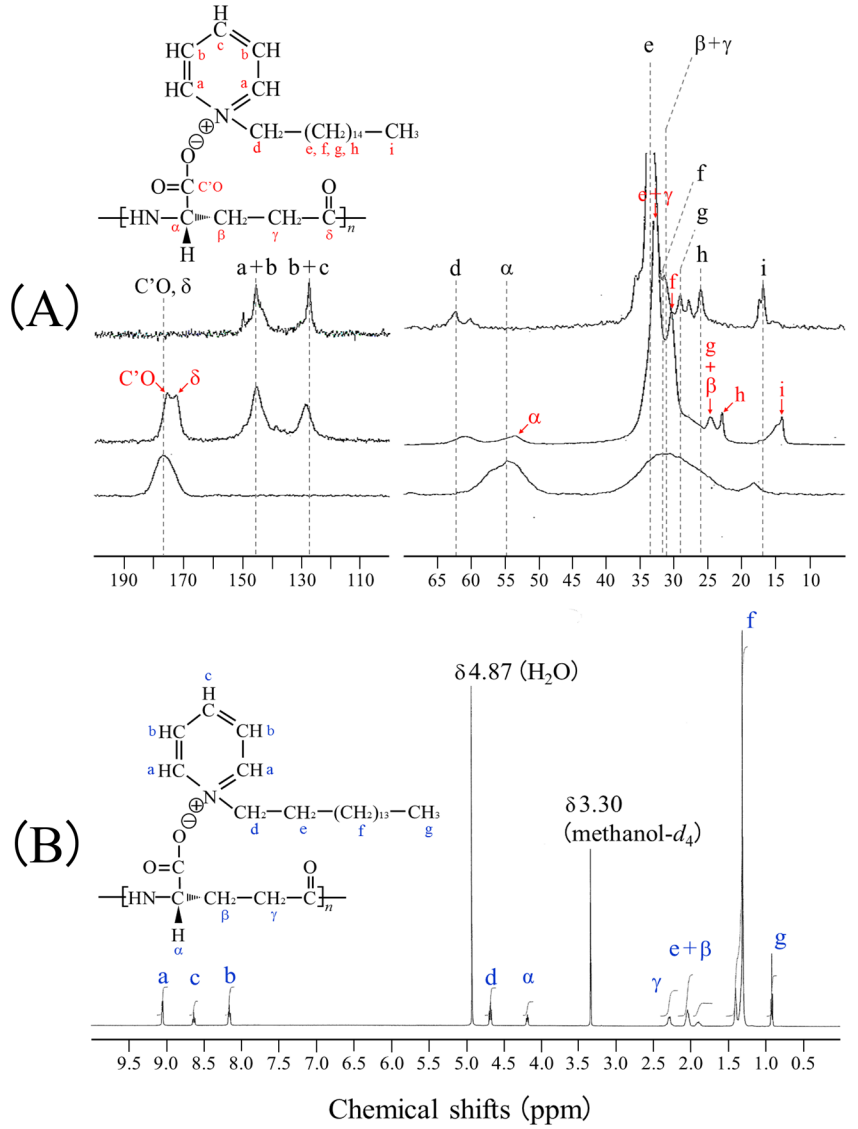

Figure 1. Spectra of (A) ^{13}C NMR of HDP$^+$ (upper graph), PGAIC (middle), and L-PGA (lower) and (B) ^{1}H NMR of PGAIC. The insets illustrate a predicted PGAIC structure. In part A, the chemical shifts are summarized in more detail (see Table S1 in the Supporting Information), and the corresponding signals of the C′O and δ carbons were finally assigned by ^{1}H−^{13}C heteronuclear shift correlation spectroscopy (HETCOR; see Figure S1 in the Supporting Information).

chloride salts of HDP$^+$ were purchased from Sigma-Aldrich (St. Louis, MO) to synthesize PGAIC. All other chemicals were of analytical grade.

Synthesis of PGAIC. Water-insoluble PGAIC was readily formed by mixing a 2 wt % solution of L-PGA with HDP$^+$ solution (approximately 120 mol % of the carboxyl groups contributed by L-PGA molecules). The pellets were collected by centrifugation, washed with distilled water at 60 °C, and lyophilized.

Nuclear Magnetic Resonance (NMR) Spectroscopy. *(a) Solid-State Analysis.* Carbon thirteen cross-polarization and magic angle spinning (^{13}C CP-MAS) NMR spectroscopy of lyophilized samples (50 mg each) was performed using a Bruker DSX300 spectrometer operating at 75.5 MHz. We scanned 256, 4096, and 2048 times at 8 kHz for HDP$^+$, PGAIC, and L-PGA, respectively.

(b) Liquid-State Analysis. A lyophilized PGAIC sample (6 mg) was dissolved in 0.6 mL of tetradeuterated methanol (methanol-d_4) and analyzed using a Bruker AVANCE500 spectrometer operating at 500 MHz. NMR signals were assigned by analyzing the chemical shifts (ppm), and their relative intensities and coupling patterns in addition to comparisons with the signals of the L-PGA and HDP$^+$ starting materials.

Calorimetric Measurements. Differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA) of lyophilized PGAIC (~10 mg) were performed at a heating rate of 10 °C min^{-1} under a nitrogen atmosphere using a Seiko DSC EXTRA6000 and a SSC5200 (Chiba, Japan), respectively.

Preparation of L-PGA-Based Cast Films. A 400 μL aliquot of 5 wt % L-PGA solution (weight, 20 mg) was added dropwise onto a water-repellent paper disk (ca. 13 mm dia). The disk was then dried at 100 °C on a Corning PC-400D hot plate (Tewksbury, MA) to strip an L-PGA-based cast film from the surface.

Preparation of PGAIC-Based Thermoplastic Films. A standard thermoprocessing strategy was utilized for the preparation of PGAIC films. A pellet of PGAIC (~30 mg) was first placed between two glass plates on a hot plate and heated at 100 °C until it became soft and its transparency increased (typically for 5 min). The softened pellet was pressed and easily thinned, and the thickness of the resulting film was measured using a Teclock contact-pressure thickness gauge PF-02J (Nagano, Japan).

Preparation of PGAIC-Coated Plastic Films. A concentrated solution of PGAIC (30 wt %) was prepared using ethanol (>99.5 wt %) and applied to the surface of thin films (50 μm thick) of

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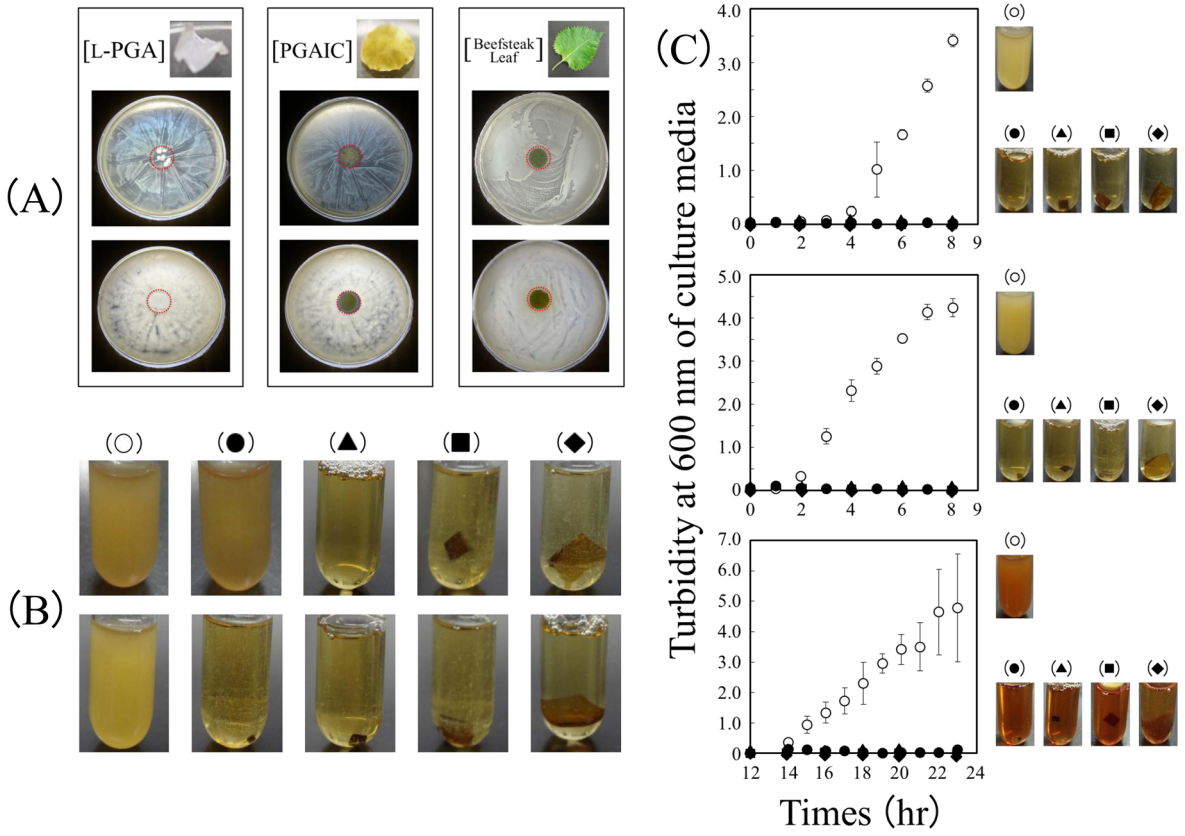

Figure 2. Antimicrobial performance of PGAIC: (A) effects in solid and (B) liquid culture conditions. (A left) Use of L-PGA-based cast films; (middle) PGAIC-based thermoplastic films; and (right) disklike pieces of a beefsteak leaf cut. These sizes are ca. 13 mm in diameter and 0.25 mm in the thickness of PGAIC films (weight, 20 mg). Photos: (upper) *E. coli*; and (lower) *B. subtilis*. (C) Growth suppression against *S. aureus* (upper graph and photos), *S. typhimurium* (middle), and *S. cerevisiae* (lower). In parts B and C, microorganisms were tested in the absence (open circles) and the presence of PGAIC films (closed symbols: circles, 0.13 cm × 0.13 cm × 0.25 mm, 0.38 mg; triangles, 0.25 cm × 0.25 cm × 0.25 mm, 1.5 mg; squares, 0.5 cm × 0.5 cm × 0.25 mm, 6 mg; diamonds, 1 cm × 1 cm × 0.25 mm, 24 mg) (n = 3). The images of the cultures were acquired at the end of cultivation.

polyethylene terephthalate (PET) (Toyobo, Shiga, Japan) using a Baker-type Applicator (Tester Sangyo, Saitama, Japan). The resulting films were dried at 100 °C, cut into circle, and used as PGAIC-coated PET films.

Cultivation of Microorganisms. Luria−Bertani (LB)[13] and YPD[14] media were used for the cultivation of prokaryotic (*E. coli*, *B. subtilis*, *S. typhimurium*, and *S. aureus*) and eukaryotic (*S. cerevisiae*) microorganisms, respectively. Under solid-culture conditions, cultures of *E. coli* and *B. subtilis* (~5 × 10^4 CFU each) were spread on LB agar plates and incubated at 37 °C for 24 h. For liquid culture, the microorganisms (~5 × 10^5 CFU each) were inoculated into 5 mL of the indicated broths; bacteria and yeasts were cultured at 37 °C for 24 h and at 30 °C for 48 h, respectively. The net growth rates of *S. aureus*, *S. typhimurium*, and *S. cerevisiae* were estimated by monitoring culture turbidity at 600 nm using a spectrophotometer.

Assays for Antifungal Activity. Cultures of *Candida albicans* NBRC1594 and *Aspergillus niger* NBRC945 (~10^7 CFU mL^{-1} each) were diluted 16-fold using the standard medium for antifungal susceptibility testing.[15] Warm (~45 °C) but still melting agar (the final concentration 2%) and various concentrations of PGAIC were added to the resulting suspensions, which were incubated at 28 °C for 5 days. The minimal inhibitory concentration (MIC) was determined according to the guidelines of the Clinical and Laboratory Standards Institute (formerly called the National Committee for Clinical Laboratory Standards).[15] The antifungal activities of the PGAIC-coated films were qualitatively detected using the halo assay described in the Japan Industrial Standards (JIS L 1902).[16]

Electrospinning. Lyophilized L-PGA and PGAIC were dissolved in 15 M acetic acid or ethanol, respectively. These resulting solutions were processed using a Kato-Tech Nanofiber Electrospinning Unit instrument (Kyoto, Japan) equipped with a stainless steel needle (inner diameter 0.8 mm) under the conditions as follows: flow rate, 16 mL min^{-1}; applied voltage 8.0 kV (for PGAIC solutions) or 29 kV (for L-PGA solutions); collection distance, 10 cm; and ambient humidity, <60%.

Scanning Electron Microscopy (SEM). The morphology of electrospun nanofibers was observed using a Hitachi Field Emission Scanning Electron Microscope S-4500 (Tokyo, Japan) with an acceleration voltage of 5 kV. To determine mean fiber diameters (MFD), five fibrils were randomly selected from an SEM image (n = 3).

RESULTS

Structural Features of PGAIC. After the synthesis of PGAIC, little or no L-PGA (<1 wt % of the initial amounts; n = 5) was detected in the aqueous phase of the reaction mixtures using published methods,[17] indicating that L-PGA was almost completely converted to a water-insoluble form by interacting with HDP^+ to form ionic complexes (PGAIC). Specifically, the NMR signals of the C′O (COO; carboxyl) and δ (CONH) carbons from stereoregular L-PGA (Figure 1A, lower graph) overlap, whereas the C′O peak of PGAIC can be distinguished from that of the corresponding δ carbon (middle). These observations of its solid form imply the presence of molecules that associate with the carboxyl groups of PGA. Furthermore, the proton signals found in Figure 1B were assigned as follows:

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pyridinium ring (chemical shifts *a*, *b*, and *c*, 9.01, 8.61, and 8.13; relative intensities, 2.00, 1.00, and 2.00), pyridinyl CH_2 (d, 4.64; 1.95), αCH−PGA (α, 4.15; 0.96), γCH_2−PGA (γ, 2.26; 1.81), pyridinyl methylene and βCH_2−PGA (e + β, 2.10−1.81; total 3.83), pyridinyl alkane (f, 1.40−1.26; total 26.3), and pyridinyl CH_3 (g, 0.89; 2.89). The data indicate that PGAIC is a stoichiometric ion complex, containing equally the carboxyl groups of PGA and HDP^+.

Thermal Plasticity of PGAIC. As shown in Figure 2A (small photograph inserted in the left column), the shape of the L-PGA-based cast films changed immediately in situ. In contrast, the calorimetric measurements of PGAIC indicated that its melting point (T_m) was 60 °C and its decomposition (T_d) started at 210 °C (see Figure S2 in the Supporting Information). PGAIC thus possesses the potential to form a thermoplastic. Thin films were obtained from PGAIC pellets via a simple pressurization (Figure 2A, small photograph inserted in the middle column). As shown in Figure 2B, PGAIC films would be easily processed in the shapes and sizes.

Antimicrobial Performance of PGAIC. We found that PGAIC suppressed microbial proliferation on the surface of media, in this case a piece of beefsteak leaf, only in contact with its film (Figure 2A, area circled by dotted red lines). This activity was observed in liquid culture as well (Figure 2B), though it was apparently more effective for a Gram-positive (*B. subtilis*) than for a Gram-negative bacterium (*E. coli*). We also demonstrated growth inhibition of two pathogenic bacterial species (*S. aureus* and *S. typhimurium*) and *S. cerevisiae* (Figure 2C).

Chemical Stability of PGAIC. Culture media for microorganisms generally contain a considerable amount of salts required to support growth. Because of this, we were surprised that the PGAIC films remained square during the antimicrobial tests (Figure 2). Therefore, we focused on the chemical stability of PGAIC and found that the films were resistant to salt and acid (Figure 3). We were also surprised that the square did not change shape after soaking in alkaline solutions (see Figure S3 in the Supporting Information). There may be a driving force for the solidification of PGAIC other than typical ionic interaction, for example, a hydrophobic interaction with the long aliphatic chain of HDP^+. We examined the solubility of PGAIC in organic solvents, along with HDP^+, anionic L-PGA (the predominant form at neutral pH), and protonated L-PGA (L-PGAH) (Table 1). Because many PGA-based materials developed to date as well as PGA are unlikely to dissolve to a significant extent in any organic solvent,[12] it is noteworthy that PGAIC exhibited good solubility in alcohols. These properties must be taken into account when processing PGA for diverse applications.

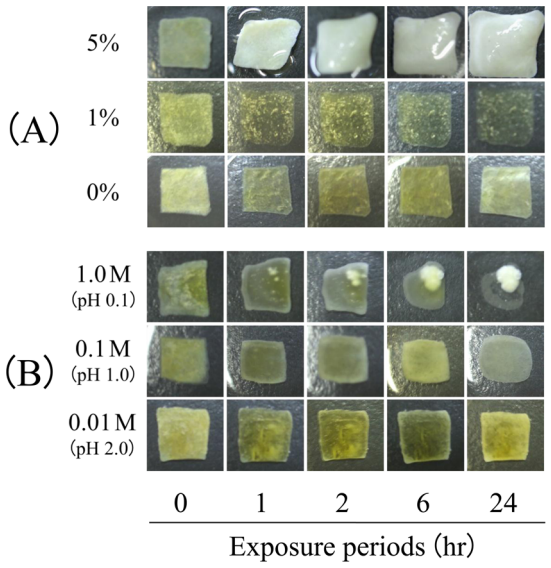

Figure 3. Durability of PGAIC films. The square films (1 cm × 1 cm × 0.25 mm) were exposed to the concentrations of NaCl (A) and sulfuric acid (B) for the indicated times and little or no changes of the shape were observed under conditions near to those of saline (~0.8%) and stomach acid (~pH 0.1).

Table 1. Solubility of PGAIC and Its Precursors for Organic Solvents[a,b]

	organic solvents						
	MeOH	EtOH	BuOH	DMSO	NMPy	acetone	hexane
HDP^+	+	+	+	+	−	+	−
L-PGA	−	−	−	−	−	−	−
L-PGAH	−	−	−	+	G	−	−
PGAIC	+	+	+	−	−	−	−

[a]Abbreviations: MeOH, methanol; EtOH, ethanol; BuOH, butanol; DMSO, dimethyl sulfoxide; NMPy, *N*-methyl-2-pyrolidone. [b]PGAIC and its precursors were added at theoretical concentration of 1 wt %. Symbols: +, soluble; −, insoluble; G, gelation.

Applicability of PGAIC as Antifungal Materials. Because antibiotics continue to be used inappropriately, the emergence of drug-resistant strains of fungal pathogens is increasing. *Candida* species, in particular, cause serious health problems and are often associated with life-threating mycoses.[18,19] Therefore, developing antifungal agents to address this issue is urgently required. Here we examined the inhibitory effects of PGAIC on the proliferation of the highly prevalent *C. albicans* and a filamentous fungus (*A. niger*). The MIC values for both organisms were 0.25 mg mL^{-1}, indicating that PGAIC is classified into potent anti-Candida agents.[20]

Because PGA and its derivatives possess potential for use as surface-contact adhesives,[12] we coated a plastic surface with PGAIC dissolved in ethanol. The original and PGAIC-coated PET disks (6.0 mm dia) were placed on a bed of *C. albicans* in an agar plate. The antifungal activities were based on the diameter of the zone of inhibition and these values for *C. albicans* are as follows: PET = 0 mm and PGAIC-coated PET = 7.0 mm. The zone of inhibition observed for *A. niger* = 6.7 mm. In contrast, PET had little effect on the growth of *A. niger*.

Nanofabrication of PGAIC. It is well-known that beefsteak leaves serve as a good preservative for perishable foods, because they transiently suppress the proliferation of microbial contaminants. However, they are more difficult to produce and to process than thermoplastics. To examine the advantages of PGAIC in processing materials, we attempted its nanofabrication using electrospinning.[21] It is usually necessary to covalently cross-link nanofibers (where PGA serves as the basal polymer) to retain their shape;[21] nevertheless, we succeeded in synthesizing an L-PGA-based nanofiber without such covalent cross-linking, but by using an ethanol solution of PGAIC. This generated a stable fibro-structure (Figure 4, images c and d), though the nanofiber synthesized from the L-PGA solution was comparatively unstable (images a and b).

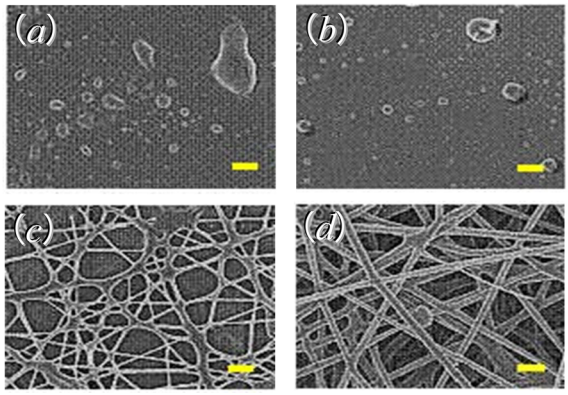

Figure 4. SEM images of nanofibers synthesized from PGAIC. The length of the yellow bar is 3 mm. Data are obtained using (a) 5 wt % L-PGA solution, (b) 10 wt % L-PGA solution, (c) 5 wt % PGAIC solution (MFD, 303 nm), and (d) 10 wt % PGAIC solution (MFD, 1040 nm).

DISCUSSION

Although PGA shows great promise for pharmaceutical and environmental applications,[11,12,22] its hygroscopic nature has been an obstacle to its plasticization and fabrication. In the present study, we demonstrated the ability of HDP^+ to transform PGA into a functional thermoplastic material, called PGAIC. HDP^+ is a potent and broadly acting microbicidal agent against bacteria to yeasts; here, we also verified that it inhibits the growth of the filamentous fungus *A. niger*. The structure of HDP^+ comprises a hydrophobic chain (aliphatic alkane) and a hydrophilic ring (pyridinium cation). The hydrophobic chain serves primarily to make initial contact with a cell and attach subsequently to membranes, while the hydrophilic ring increases the permeability of the membrane causing the cytoplasmic contents to leak, resulting in cell death.[23−28]

PGAIC is formed via multiple ionic bonds between the pyridinium cations of the HDP^+ molecules and the carboxyl anions of PGA (Figure 1), suggesting that it lacks the function as microbicides. The rates of dissociation and diffusion of HDP^+ from stable PGAIC are probably limited or very slow, allowing for its resistance to degradation by chemicals (Figure 3; see also Figure S3 in the Supporting Information). Nevertheless, the MICs of PGAIC for *C. albicans* and *A. niger* were the same as those of HDP^+ alone.[28] These indicate that PGAIC retains the antifungal performance of HDP^+ and acts as a microbiostat (nonmicrobicide). In formulating antimicrobial agents, polymeric materials are generally more efficient and selective (thus safer) than the smaller molecules,[29] and facilitate prolonged activity owing to the controlled release of toxic moieties from the polymer networks. Accordingly, long-acting microbiostatic polymers may reduce the spread of drug-resistant microbes.

PGAIC has the potential to serve as a bioplastic material possessing a broad spectrum of antimicrobial activity that will be specifically useful to the food-related and hygiene industries. It is usually safer to use L-PGA as the core polymer, because it is isolated from nonsporulating microorganisms[12] and not, for example, from *Bacillus* species.[11] Moreover, the use of cationic surfactants, such as HDP^+, is likely to provide a promising strategy for fabricating water-friendly anionic polymers, including PGA.

We are hopeful that archaeal L-PGA and its versatile PGAIC-type derivatives will generate considerable interest among researchers seeking to develop advanced biomaterials for industrial applications.

ASSOCIATED CONTENT

Supporting Information

Data for the solid-state NMR (Table S1)/2D HETCOR (Figure S1) analyses and the calorimetric measurements (Figure S2) of PGAIC, and its resistance to alkaline conditions (Figure S3). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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