Chemical Modification of Poly(styrene-alt-maleic anhydride) with Antimicrobial 4-Aminobenzoic Acid and 4-Hydroxybenzoic Acid

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Abstract: Poly(styrene-alt-maleic anhydride) (SMA) is one of the most appropriate intermediate polymers that can be converted into bioactive polymers as its succinic anhydride units can react with any bioactive agents with low molecular weights containing amino and hydroxy groups. In this study, SMA was reacted with antimicrobial 4-aminobenzoic acid and 4-hydroxybenzoic acid to obtain P-1 and P-2, respectively, with reasonably high yields. The glass transition temperatures of P-1 and P-2 were higher than that of SMA due to hydrogen-bonding interactions. The polymers became crosslinked on heating at about 300 °C. The new polymers were found to exhibit excellent bactericidal activities even though their antifungal activities were not satisfactory.

Keywords: poly(styrene-co-maleic anhydride), bioactive agent, 4-aminobenzoic acid, 4-hydroxybenzoic acid, antimicrobial polymer

Introduction

Antimicrobial agent-bound polymers exhibit antimicrobial activities by slowly releasing active agents through hydrolysis, while some polymers are also antimicrobial by themselves [1]. Such polymers have certain advantages over low molecular weight active agents because they are more stable against volatilization, dissolution, and diffusion to the surface of the material to be protected.

Antimicrobial agents can be incorporated in linear or crosslinked carrier polymers via covalent bonds [2,3]. Linear polymers release the active agents faster than the crosslinked polymers because the duration period required for water penetration into the labile bonds and hydrolysis is normally shorter for linear polymers compared to crosslinked polymers [4,5]. As such linear polymers are more useful additives especially when blended with soluble polymers in organic solutions.

There are several strategies for preparing antimicrobial polymers from bioactive agents with low molecular weights [3]. For example, an active agent containing a reactive group can be linked to a suitable site on a carrier polymer backbone. If the active agent and/or the preformed carrier polymer do not have the proper reactive groups, they used to be derivatized to effect attachment. Alternatively, the active agent can be converted into a polymerizable derivative, which can be subsequently polymerized. Finally, an active agent containing more than one reactive group can form a polymer via step-growth polymerization.

Tani and coworkers proposed a hydrophilic-hydrophobic model for the hydrolysis of bound agents in a polymer matrix [6]. The release mechanism consists of the following three steps: (A) permeation of the surrounding medium into the polymer matrix, (B) hydrolysis of the bound agent to produce free active agents, and (C) diffusion of the free agents out of the polymer matrix. Accordingly, the extent of the hydrophilicity of the antimicrobial polymers is very important in terms of the controlled release of the bound agents from the polymer matrix.

Any bioactive agent containing a reactive group can be
covalently attached to poly(styrene-alt-maleic anhydride) (SMA) through a ring-opening reaction between the active agent and the succinic anhydride unit. Consequently, SMA is an appropriate intermediate polymer for the preparation of antimicrobial polymers.

In the current study, an intermediate polymer was prepared, and its conversion into different polymers tested, including its antimicrobial activities. SMA was reacted with model compounds such as 4-amino benzoic acid (ABA) and 4-hydroxy benzoic acid (HBA) to obtain P-1 and P-2, respectively, as shown in Scheme 1.

The model compounds were chosen to react with SMA as they are known to exhibit antimicrobial activities. For example, the antibacterial activity of ABA against certain microorganisms is greater than that of commonly used acidulants such as formic, propionic, acetic, lactic, and citric acids [7]. HBA [8] and its esters [9] have also been reported to inhibit certain microbial and fungal growth. This paper mainly describes the synthesis and some general characteristics of the new polymers. A preliminary result on their antimicrobial activity test is also briefly described.

Experimental

Materials

The styrene (99+%), maleic anhydride (99%), ABA (99%), HBA (99%), N,N-dimethylformamide (DMF, anhydrous, 98%), and triethylamine (99%) were all purchased from Aldrich. The 2,2'-azobisisobutyronitrile (AIBN) (98%) was purchased from Junsei Chemicals and purified by recrystallization using methanol before use. The rest of the materials were used as received.

Instruments

The FT-IR spectra were obtained with a JASCO FT-IR spectrophotometer. The 1H-NMR spectra were obtained with a JEOL-JMN 400 (400 MHz) spectrometer. The differential scanning calorimetry (DSC) and thermal gravimetric analysis (TGA) experiments were performed using a DuPont 2000 differential scanning calorimeter. The gel permeation chromatography (GPC) data were obtained with a Waters 440 HPLC calibrated with polystyrene standard samples.

Chemical Modification of SMA

The SMA was prepared following previously reported procedures [10,11]. Briefly, a solution of styrene (15.6 g; 0.149 mol) and maleic anhydride (14.7 g; 0.149 mol) in a 1:1 molar ratio in anhydrous DMF (70 mL) was polymerized in the presence of AIBN (1.6 mol%) at 60–70 °C for 12 h under a nitrogen atmosphere. A part of the reaction mixture was withdrawn, cooled to room temperature, and precipitated from diethyl ether. The precipitate was reprecipitated into acetone/diethyl ether and dried under a vacuum to obtain SMA.

Another part of the above reaction mixture was withdrawn and added to a solution of ABA in anhydrous DMF, followed by the addition of triethylamine [12,13]. The calculated molar ratio of the succinic anhydride unit, ABA, and triethylamine was approximately 1:1:5.2. The mixture was heated at about 90 °C for 48 h under a nitrogen atmosphere, cooled to room temperature, and finally precipitated into tetrahydrofuran. The precipitate was redissolved in DMF, and precipitated into tetrahydrofuran to obtain P-1. The remaining reaction mixture was also reacted with HBA at 90 °C for 24 h under a nitrogen atmosphere to obtain P-2.

Accelerated Fungi Growth Test

Antifungal test was carried out according to ASTM G-21. P-1 and P-2 were dissolved in DMF (4.0 wt%). Each filter paper soaked with the polymer solution (0.2 mL) was irradiated with UV light for 1 h. A potato-dextrose agar (20 mL) was dried and inoculated with A. niger (1.0 × 10⁵ ~ 1.5 × 10⁵ CFU/mL) in each Petri dish. Each circular filter paper (diameter, about 0.6 cm) was then placed in the center of the Petri dish and pressed gently. The samples were incubated at 30 °C for 72 h. The growth of fungi on the surface of the test specimens was observed by the naked eye.

Shake Flask Test

The biocidal activity test of the new polymer by the contact method was performed for two different types of bacteria species such as E. coli (KCTC 1682) and S. aureus (KCTC 1916) [14,15]. A certain amount of pure DMF for a blank (control) sample or a solution (1.0 mL) of either AP or SMA-AP in DMF (10 wt%) was transferred into each wide-mouth glass bottle. A bacterial
culture suspension (5.0 × 10³/mL) was added dropwise onto the above DMF solutions and DMF control, followed by the addition of 70 mL phosphate buffer solution (PBS, pH 7.2). After the bottles were incubated at 37 °C for 24 h under shaking (150 rpm), 0.1 mL of each suspension was plated out on a Luria Broth (LB) agar. After incubation for 1 day, the number of bacteria colony was counted. The percent reduction of the bacteria cells was calculated from the following formula:

\[
\% \text{ reduction} = \left( \frac{N_b - N_i}{N_b} \right) \times 100
\]

where \(N_i\) is the number of the bacterial cells recovered from the inoculated suspension which contains either AP or SMA-AP in the bottle, and \(N_b\) is the number of bacteria recovered from the inoculated suspension which contains only DMF without the biocides in the bottle.

**Results and Discussion**

**Synthesis**

Styrene and maleic anhydride underwent radical polymerization to yield SMA [10,11]. The FT-IR spectrum of SMA showed the two peaks at about 1850 and 1780 cm⁻¹, as shown in Figure 1, corresponding to the stretching vibrations of the anhydride moiety. The peaks within a range of 3100 ~ 3000 cm⁻¹ were due to aromatic C-H stretching vibrations.

The SMA was reacted with ABA and HBA in the presence of triethylamine to yield P-1 and P-2, respectively [12,13]. The ring-opening reaction resulted in the formation of a carboxylic group and amide (P-1) or ester (P-2) bond. The FT-IR spectra clearly indicated that the absorption peaks of the anhydride carbonyl groups at about 1850 and 1780 cm⁻¹ disappeared and instead new carboxyl group peaks appeared within a range from 1694 cm⁻¹ to 1727 cm⁻¹. It has been previously reported that carboxylic acid groups exhibit peaks at 1728 when they are hydrogen-bonded to ether oxygens [10] and at 1710 ~ 1700 cm⁻¹ when they exist as hydrogen-bonded parfs [16-18]. Thus, the peak at 1709 cm⁻¹ indicates that hydrogen-bonded carboxylic acid pairs were present in P-1. The peak at 1665 cm⁻¹ is probably due to amide carbonyl groups.

In contrast, interestingly, there were two absorption peaks at 1727 and 1694 cm⁻¹ in P-2. The peak at 1727 cm⁻¹ may have been due to carboxyl groups, hydrogen-bonded to the ester groups, and the other peak at 1694 cm⁻¹ due to carboxyl groups which are existing as hydrogen-bonded pairs. The ester groups may have also been hydrogen-bonded with some of the carboxyl groups as mentioned above, thereby shifting their absorption peaks to a lower frequency region. Consequently, some of the absorption peaks of the ester groups are probably overlapped with those of the carboxyl groups. Similar observations have been reported for monomethoxy poly(ethylene glycol)-grafted SMA [10].

It is well known that styrene and maleic anhydride can form alternating copolymers within a wide range of feed ratios due to the donor/acceptor interaction or rapid cross-propagation reactions [10]. To avoid an overlapping of the polymer and solvent proton peaks, SMA was reacted with butanol at 60 °C. The resulting polymers did not include any unreacted succinic anhydride units, thereby indicating that the ring-opening reaction of the succinic anhydride units with butanol was almost complete. The integral ratio of phenyl (5H) to the methylene group (2H) bonded directly to the oxygen atom of the butyl ester bond was 2.5 : 1, as shown in Figure 2. This result partially supports the fact that SMA is an

![Figure 1. FT-IR spectra of SMA, P-1, and P-2 (KBr).](image)

![Figure 2. ¹H-NMR spectra of SMA modified with butanol in DMSO-d6.](image)
alternating copolymer since even random copolymers can have the same overall composition.

The number- \((M_n)\) and weight-average molecular weights \((M_w)\) estimated using GPC were 13000 and 23000, respectively. The isolation yields of P-1 and P-2 were about 90 and 85\%, respectively. The yields are reasonably high considering that the reaction mixtures were purified with the repeated precipitation method.

The extent of amidation and esterification between SMA and ABA or HBA was attempted to be estimated by elemental analysis. The elemental percentages of carbon, hydrogen and nitrogen in P-1 were determined to be 65.9, 5.1, and 4.5\% while the calculated percentages of those were 67.3, 5.1, and 4.1\%, respectively. The experimental values are in good agreement with the calculated values, indicating that the amidation of SMA with ABA was almost quantitative. However, the elemental percentages of carbon and hydrogen in P-2 were determined to be 60.7 and 6.2\% while the calculated percentages were 67.1 and 4.7\%. The less amount of carbon indicates that the esterification of with HBA was not completed. The FT-IR spectrum of P-2 indicated the remaining anhydride peak more clearly. This result is probably due to the shorter reaction time because HBA reacted for only 24 h while ABA did for 48 h.

**Thermal Properties**

The thermal transitions of SMA, P-1, and P-2 were studied using DSC, and the results are shown in Figure 3. The glass transition temperature \((T_g)\) of SMA was measured to be about 202 °C, which is very close to a previously reported value \((M_8, 83000; T_g, 201 °C)\) [10]. In contrast, the \(T_g\) of P-1 and P-2 was 251 [13] and 253 °C, respectively. The increased \(T_g\) of the new polymers probably resulted from enhanced molecular interactions due to hydrogen bonding, as mentioned above.

The thermal stability of SMA, P-1, and P-2 was studied using TGA, and the results are shown in Figure 4. SMA began to decompose at about 300 °C. In contrast, the two new polymers were decomposed with two steps. The initial weight loss in P-1 and P-2 at temperatures near 250 °C was 8.7 and 10.8\%, respectively. The calculated weight of water to be formed via formation of an anhydride from two carboxylic groups in P-1 and P-2 were 9.1 and 10.4\%, which are somewhat close to the calculated values. In the case of P-1, the dehydration can also occur via imidation between an amide group and neighboring carboxyl group as the imidation is well-known to occur on heating SMA with primary amines [12,19]. In order to prove this hypothesis, the polymers were heated at about 300 °C for 1 h under the identical conditions as above. The FT-IR spectrum of the heated P-1 sample clearly showed one of the characteristic two peaks of anhydride carbonyl groups at 1780 cm\(^{-1}\), as shown in Figure 5. However, the absorption peak (usually ~1710 cm\(^{-1}\)) of the imide carbonyl groups could not be observed because the region was complicated with other carbonyl peaks. Even though the imidy carbonyl group was not detected from the IR spectrum, the imidation cannot be rule out completely in P-1. In order to see the resonance signals of imidy carbons in \(^{13}\text{C}\)-NMR spectrum, the annealed P-1 samples were attempted to be dissolved in solvents such deuterated dimethyl sulfoxide, chloroform, acetone, THF, and DMF. However, they were not dissolved in those solvents, suggesting that P-1 became crosslinked due to the intermolecular reactions. Similarly, the annealed P-2 samples were not dissolved in the organic solvents, probably due to the intermolecular anhydride formation.

**Antifungal Test**

The antifungal properties of P-1 and P-2 against *A. niger* were studied in an agar dish test according to ASTM G-21 as mentioned in the experimental section. The control filter papers soaked in pure DMF or a SMA solution in DMF were completely covered with the fungi while the ABA or HBA samples only exhibited fungal growth on the agar up to the edge of the papers. The P-1 and P-2 samples showed a sparse growth (~10% covered) and moderate growth (~30% covered) on the filter papers, respectively.

The activity of the polymers was somewhat lower than that of the corresponding free active agents. This result indicates that the release rate of the active agents from the polymer backbones via hydrolysis was not fast enough to show a comparable activity under the experimental conditions employed. However, it should be noted that the release rate is largely dependent on the experimental conditions such as pH, ionic strength, temperature, etc.
Figure 4. TGA curves of SMA, P-1, and P-2. The heating rate was 20 °C/min.

Figure 5. FT-IR spectra of P-1 after heating at 300 °C for 1 h.

Antibacterial Test

The antibacterial activity of P-1 and P-2 was investigated with the shake flask test method toward *E. coli* and *S. aureus*. The number of bacterial cells in the bacterial culture suspension was $5.0 \times 10^3$/mL. After their contact with either bioactive agents (ABA, HBA) or polymers (P-1, P-2) in diluted PBS, the suspensions were incubated at 37 °C for 24 h, and the number of the bacteria colony was counted. The bacteria cells/mL was calculated by multiplying the number of colonies by the dilution factor.

The number of *E. coli* cells which were in contact with either ABA or P-1 was compared with the cells initially added, and the reduction was calculated to be about 93 and 45%, respectively, as shown in Table 1. In contrast, the reduction in the specimens containing HBA or P-2 was 100%. On the other hand, the number of *S. aureus* cells in the specimens containing either the bioactive agents or polymers was reduced to be in a range from 95 to 99.99%. This result indicates that the antibacterial activities of the polymers were similar to those of the corresponding bioactive agents and excellent at least against *E. coli* and *S. aureus*.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Sample</th>
<th>Bacteria/mL (24 h contact)</th>
<th>Reduction (%)</th>
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<tr>
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Conclusions

P-1 and P-2 were successfully synthesized by reacting SMA with ABA and HBA, respectively. The T<sub>g</sub> of the polymers were higher than that of SMA because of hydrogen bonding interactions between the polymer chains. The polymers became crosslinked and insoluble in organic solvents on heating at about 300 °C. This preliminary result indicates that the new polymers exhibited excellent bactericidal activities against *E. coli* and *S. aureus* even though their antifungal activities against *A. niger* were not satisfactory. Accordingly, this synthetic strategy can be conveniently used in synthesizing more bioactive polymers, as SMA can react with any active agent containing amino or hydroxy group.

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