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Molecular design of anti-biofouling materials from natural phenolic compounds

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Abstract−Many natural phenolic compounds found in plants are well known for their antibiotic and antioxidant activities. It has been hypothesized that these activities of natural phenols could be used for developing permanent anti-biofouling coatings. In this study, two phenolic components, anacardic acid and cardanol, were extracted from cashew nut shell liquid, and tested for their antibiotic and anti-biofouling activities against *Pseudomonas fluorescens*. Both compounds killed all the cells within 18 hours (anacardic acid) and 30 hours (cardanol) after the addition to the culture media at a concentration of 800 μg/ml. To form a stable permanent coating of these compounds, first they were polymerized by enzymatic polymerization, and the polymers were cross-linked on a glass slide. *P. fluorescens* were cultured on the coated and uncoated glasses for two weeks, and the images of the cells grown on the surfaces were taken by SEM. The coated surfaces clearly demonstrated anti-biofouling activities, showing not only fewer numbers of cells but also less exopolymer than the uncoated surfaces. Based on these results, a phenolic compound with a similar structure of anacardic acid was synthesized by using propylene diamine and fluorocarboxylic acid with cardanol. The synthesized phenolic compound was polymerized and cross-linked on a glass slide to test the anti-biofouling activity. The SEM images of the cells on the coated surface showed considerable decreases in the number of adhered cells and the amount of exopolymers even more than the anacardic acid and cardanol coatings. It is thought that the natural phenolic compounds with active functional groups can be used for anti-biofouling agents.

Key words: Biofilm, Natural Phenolic Compounds, Anti-biofouling Coatings, Anacardic Acid, Cardanol

INTRODUCTION

Many natural phenolic compounds found in plants and fruits are known to have antibiotic and antioxidant activities [1-3]. Flavonoids (Fig. 1a) probably are the most extensively investigated natural phenolic compounds that show strong antioxidant and antibiotic activities [4-7]. Some plant phenolic lipids are used as pharmaceutical-grade food supplements for reducing high blood pressure (anacardic acid from seeds of ginkgo tree (Fig. 1b [8]) and some are used for controlling diabetes (ferulic acid from turmeric and curcumin [9]). To better utilize the functionality of these materials, it is necessary to understand how the structure of the compounds enables these activities to occur. In this study, two different natural phenolic compounds were used for anti-biofouling effects against bacteria, and the effects were analyzed. Based on the experimental results, a new polyphenol has been designed and synthesized for more effective anti-biofouling effect.

There are two types of natural phenolic compounds in general, resorcinol and catechol lipids, depending on the phenol ring structures. We used anacardic acid and cardanol obtained from cashew nut shell liquid (CNSL) as model compounds (Figs. 1c and d). Anacardic acid has been chosen to represent the resorcinol lipids that have -COOH substituent, and cardanol for catechol with -OH substituent. Both of them have 15-carbon alkyl chain with 1-3 double bonds at the meta position. These compounds have simple and unique structures of phenolic compound and are easy to manipulate. They are abundant in the waste CNSL, and also known to have strong antioxidant, antibiotic activities, and inhibitory effects on carcinogenesis, tumorigenesis, and enzymes such as tyrosinase that catalyzes melanoma and other ailments [10-14]. Furthermore, unlike many other natural phenols, these compounds have a long alkyl side chain that makes the compounds amphiphilic, which results in many advantages in antibiotic activity and in the formation of anti-

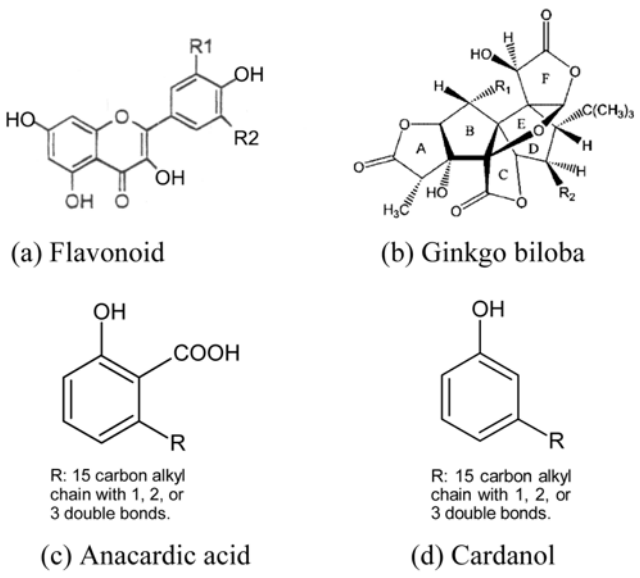

Fig. 1. Examples of natural phenolic compounds.

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biofouling coatings. We compared the structural differences between the -COOH and -OH groups for antibiotic and anti-biofouling activities.

First, we tested the antibiotic effects of pure anacardic acid and cardanol. *Pseudomonas fluorescens* was used as a model strain. Then, these compounds were polymerized and cross-linked on glass slides to form permanent coatings. FTIR analyses showed that the polymers have uniform structures that have links between the functional groups, not through the alky chains. The antimicrobial and anti-biofouling activities of the coatings were tested with *P. fluorescens*. Enzymatic polymerization was used to maintain the uniform structures of the compounds. The effects of the functional groups, i.e., -OH and -COOH, on antimicrobial and anti-biofouling activities were discussed using the experimental results. The newly designed coating showed enhanced anti-biofouling activities, and could possibly be used to explain the possible antimicrobial and anti-biofouling mechanisms of the phenolic compounds and their coatings.

EXPERIMENTAL METHODS

1. Natural Phenols

Untreated CNSL, which contained 70-80% of anacardic acid, was obtained from Sun Food Corporation, Andhra Pradesh, India. Anacardic acid was extracted from CNSL according to the method described in Paramashivappa et al. [15]. Cardanol was obtained from Palmer International, Inc. (USA).

2. Antimicrobial Activity Measurements for the Natural Phenols

Pseudomonas fluorescens was used to determine the antimicrobial activities of cardanol and anacardic acid. The minimal inhibitory concentration (MIC) against microorganisms was used by two-fold serial broth dilution [11]. MIC was the lowest concentration of the antimicrobial agent at which no cell growth was observed. The cells were grown at 25 °C in the culture medium prepared for *P. fluorescens* according to [16], and in the middle of the exponential growth phase (at 8 hours after the inoculation of cells), cardanol and anacardic acid were added at various concentrations, from 10 μg/ml to 800 μg/ml (the solubility limit in the water-based medium). The cells were taken every 4 hours for colony count. The minimum concentrations that killed the cells completely were taken as an MIC.

3. Enzymatic Polymerization of the Phenolic Compounds

Each component was polymerized by using peroxidase enzymes. Polymerization and cross-linking of the polymers are necessary to form a stable coating on solid surfaces. The components were first polymerized in a beaker and then cross-linked on glass slides. Both the enzymatic polymerization and cross-linking were performed at room temperature and atmospheric pressure.

Enzymatic polymerization of both anacardic acid and cardanol (300 mg each) was performed using 10 mg of soybean peroxidase in an equivolume mixture of methanol (12.5 ml) and a pH 7 phosphate buffer solution (12.5 ml). Phenothiazine-10-propionic acid (PPA) was used as a redox mediator in the reaction. The detailed procedures for enzymatic polymerization of cardanol and anacardic acid are described in Kim et al. [17] and Chelikani et al. [18], respectively.

4. Cross-linking of the Polymers

The obtained polymers were applied on a glass slide with a mixture of cobalt naphthenate and methyl ethyl ketone peroxide (3 wt%), and then kept under air at ambient conditions for 24 hours to form a smooth coating. A pencil scratch test was used to determine the hardness of the coatings.

5. Antimicrobial and Anti-biofouling of the Coatings

The coated slides prepared in 2.4 and uncoated slide were cut into square tabs (1 cm×1 cm). Each slide tab was placed in two separate flasks containing 200 ml of the nutrient broth, and the flasks were inoculated with the *P. fluorescens*. The cells were cultured for 2 weeks. Part of the nutrient was replaced with a fresh one every two days. This part of the experiment was done in duplicate, one for the antimicrobial test and the other one for SEM.

The first set of slides was taken from the culture media and the cells were scraped with a cotton swab. The cells were taken from the cotton swab by using a high speed tissue homogenizer (Brinkman PT1200E) at 20,500 rpm for 30 seconds and were spread on an agar plate after serial dilution to count the number of live cells. The duplicate slides were pretreated for SEM imaging [19]. Polycardanol coated slides were tested in the same manner as poly-anacardic acid coatings as stated above.

6. Designing of Anti-biofouling Coating

To verify the effect of the functional groups on anti-biofouling activity, cardanol was modified with propylene diamine and fluorocarboxylic acid to form highly reactive acetyl amide group, and then polymerized with peroxidase. The polymers were cross-linked (i.e., cured) on the solid surface with MEKP. It took about 24 hours for curing.

Fig. 2 describes the polymerization and cross-linking schemes of the new polymer (poly N-(N-(2'-hydroxy-6'-alkyl-benzamide)-

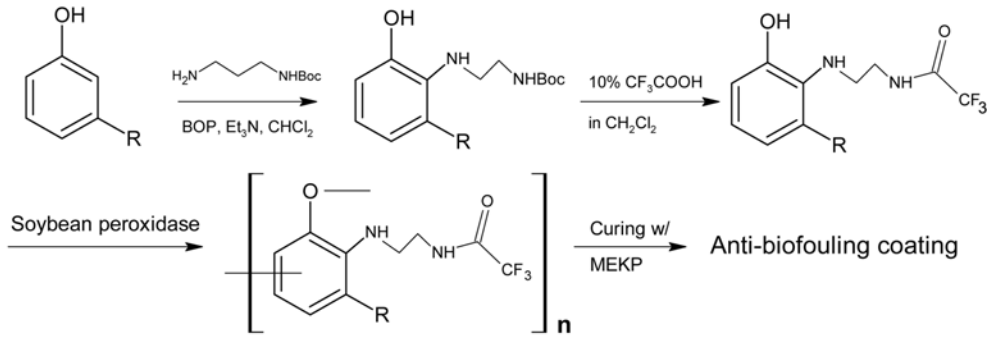

Fig. 2. Structure modification, and the polymerization and curing schemes for cardanol to synthesize a novel anti-biofouling agent.

3-amino-propyl)-acrylamide). Ter-butoxycarbonyl (Boc) was added to protect the amino group in the presence of BOP reagent and Et_3N [20,21], and the hydroxyl group in the carboxylic acid was first replaced with a polymerizable amine group to form peptide bond. The reaction proceeded further to replace the trifluoroacetate with polymerizable acrylamide [22], and to polymerize the monomers by using the same enzymatic polymerization with soybean peroxidase as in 2.3 above. The new polymer was coated on a glass slide by the same method described in 2.4.

7. Test of Anti-biofouling Coating

Poly-cardanol, poly-anacardic acid, and the newly designed phenolic polymer were cured on the glass slides, and tested for anti-biofouling effects against *P. fluorescens* (Gram-negative). This species was selected because of their tendency to produce a large amount of exopolymers within a short period of time.

RESULTS AND DISCUSSION

1. Antibiotic Effect of Anacardic Acid

Anacardic acid and cardanol were added into the *P. fluorescens* culture media at various concentrations, and the minimum concentrations that killed the cells completely were determined as the MIC. The MIC's of anacardic acid and cardanol were both 800 μg/ml. Neither of them showed considerable antimicrobial activities up to 800 μg/ml. It took 18 hours after the addition of anacardic acid to completely kill the cells. For cardanol, no live cells were observed after 30 hours of addition. Fig. 3 compares the cell counts between the three cases. The MICs of anacardic acid and cardanol were added at 8 hours after the inoculation. The MICs of cardanol and anacardic acid against *P. fluorescens* were close to the values reported in Himejima and Kubo [11].

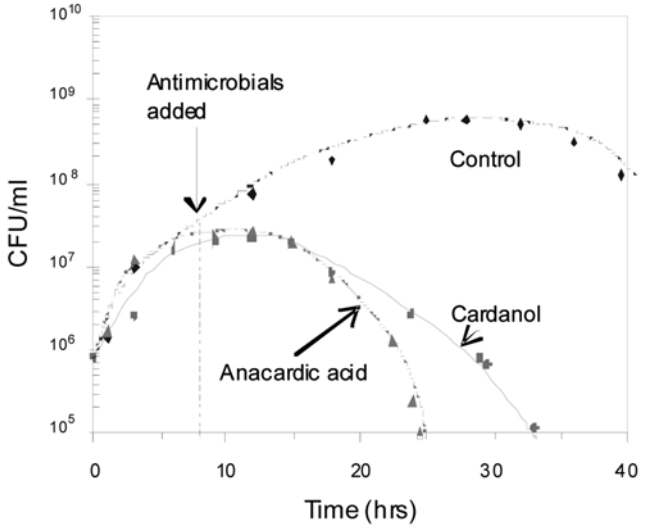

Fig. 3. Antibiotic activities of anacardic acid and cardanol.

2. Enzymatic Polymerization and Cross-linking

The polymerized anacardic acid and cardanol (i.e., poly-anacardic acid and poly-cardanol) were obtained from enzymatic polymerization. The obtained polymers were analyzed for molecular weights and structure by using GPC and FTIR spectroscopy. GPC analysis was done with a Phenogel column (particle size: 5 μm, Phenomonex, Inc., Torrance, CA), and FTIR (FTS 4000, DIGILAB) analysis was performed on a Ge micro ATR. The molecular weights of poly-cardanol and poly-anacardic acid were found to be 2,700 and 3,900 with the production yields of 75% and 61%, respectively. FTIR analyses for cardanol and poly-cardanol (Fig. 4) show that the peak at 1,189 cm^{-1}, representing an ether-link (-O-) vibration in cardanol, became larger and broader in poly-cardanol. Therefore, it appears that poly-(phenylene oxide) was formed through the -OH group predominantly and no linking through the alkyl chain as a result of the enzymatic polymerization. The FTIR analysis of poly-cardanol reaffirms the suggested structure by Ikeda et al. [23] as shown in Fig. 5(a).

FTIR results for anacardic acid and its polymer showed a similar pattern to those of cardanol. The FTIR analyses were published elsewhere [18]. Polymerization of anacardic acid appeared to undergo decarboxylation. Therefore, it is suggested that the polymerization produced a mixture of oxyphenylene and phenylene units with some decarboxylation that took place during the polymerization. The suggested structures of poly-anacardic acid are shown in Fig. 5(b).

3. Polyphenol Coatings and their Antibiofouling Effects

The resulting phenolic polymers were used to form a coating on a glass slide by cross-linking them with cobalt naphethanate and MEKP.

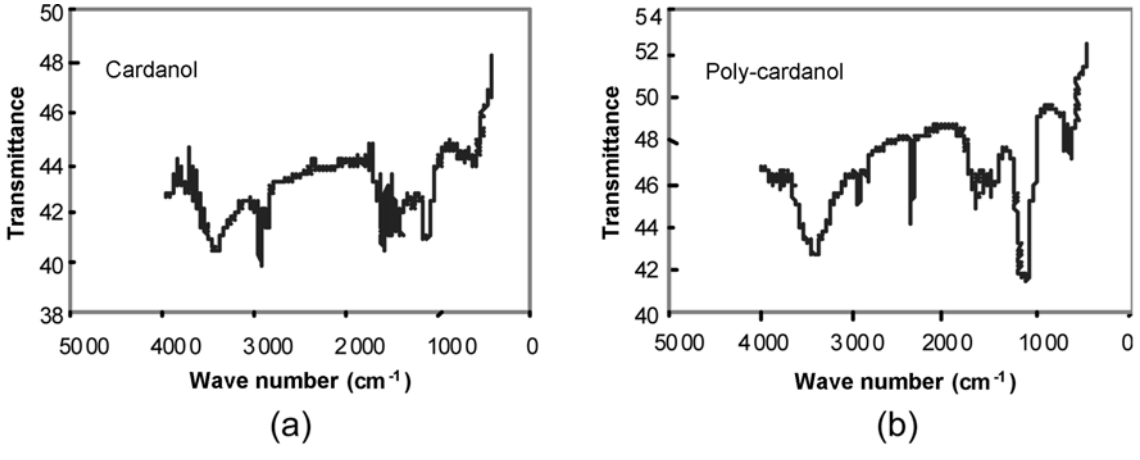

Fig. 4. FTIR analyses for cardanol and poly-cardanol: (a) Cardanol, (b) Poly-cardanol.

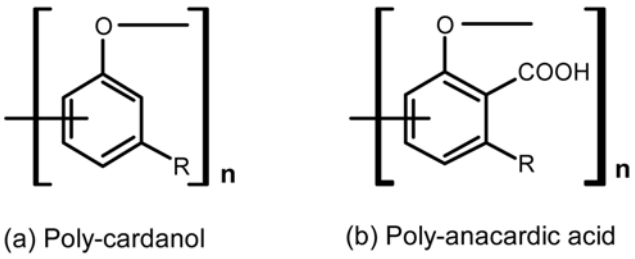

Fig. 5. Suggested structures of poly-cardanol and poly-anacardic acid.

Brownish transparent glossy coatings resulted. Pencil scratch hardness tests showed that the cured surfaces were of the range of 4H-5H hardness. As stated earlier, these coated slides were cut 1 cm× 1 cm tabs and immersed in a culture medium inoculated with *P. fluorescens*. Uncoated slides were cut in the same size and inoculated as a control.

Fig. 6 shows the cells and morphology of the biofilms formed on the uncoated and poly-cardanol coated glass slides. We observed a dramatic difference in the biofilm formation in these two groups. The uncoated slides were completely covered with a thick biofilm consisting of extracellular biopolymers (exopolymers, in short) as shown in Fig. 6(a), whereas the coated slides had very few cells and exopolymers on the surface (Fig. 6(b)). Very little exopolymer was observed on the coated plates, even though there was some lumping of the cells with exopolymers. Without enough exopolymers, the cells on the coated surfaces are only weakly attached, and therefore can be easily removed from the surface by fluid flow. The poly-anacardic acid coating also showed similar results (SEM images are not shown here). No antimicrobial activity was observed on the surface of the polyphenol coatings.

4. Structure Effects on Antimicrobial and Anti-biofouling Activities

Although both the polycardanol and polyanacardic acid showed similar anti-biofouling effect, it was observed that anacardic acid demonstrated stronger antimicrobial activity than cardanol, as shown in Fig. 3. The structural difference between the two is the carboxyl group at the ortho position in anacardic acid. In our design of anti-biofouling agents, we added carboxyl and amide group to enhance the antimicrobial activity. Diaminopropane was added to cardanol and the N-terminal, NH_2, was acetylated with fluorocarboxylic acid. The polymerization was performed in the same manner as in the enzymatic polymerization for poly-cardanol and poly-anacardic acid. The average polymer production yield was 63% and average molecular weight was 5,400.

The newly designed polymer was cross-linked in the same manner as in the curing of poly-anacardic acid using MEKP on a glass slide. *P. fluorescens* was cultured on the coated surface by using the same inoculation method described in 2.5. Fig. 7 shows the SEM images of the cells on the glass slide and the new polymer coating. It is clear that the new phenolic polymer coating reduces the number of cells on the surface compared to the glass slides in Fig. 7(a), which is completely covered with a thick biofilm. The new phenol polymer coating also showed lesser number of cells than the uncoated glass slide, and moreover, fewer cells than the poly-cardanol and poly-anacardic acid coatings shown in Fig. 7(b). It is noted that there are differences between the morphologies of the attached cells on the new polyphenol coating in Fig. 7(b) and poly-cardanol coating in Fig. 6(b). First, the cells in Fig. 7(b) are more uniformly distributed than those in Fig. 6(b). Fig. 6(b) shows some lumping of the cells. Lumping of the cells is due to exopolymer production of the cells. Therefore, the new polyphenol coating appears to suppress (or delay) exopolymer production more than the polycardanol and polyanacardic acid coatings.

However, no significant changes were observed in the numbers of live and dead cells on the coated surfaces. It was concluded that the coatings had no antimicrobial activities. However, the amide groups and carboxylic group appear to have enhanced anti-biofouling effects as they suppress or delay exopolymer production of the cells. Further studies are needed to understand how these groups contribute to the reduction of biofouling [24].

CONCLUSIONS

Both of the natural phenolic compounds, anacardic acid and car-

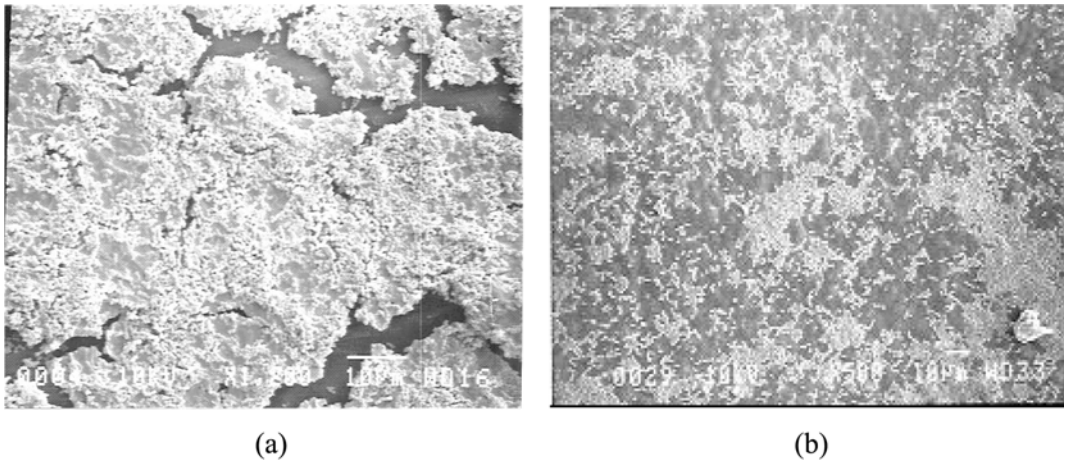

Fig. 6. *P. fluorescens* on (a) uncoated glass and (b) poly-cardanol coated glass (×500). (a) The white patches are the biofilm and dark cracks are the glass surface. (b) The white dots are bacterial cells.

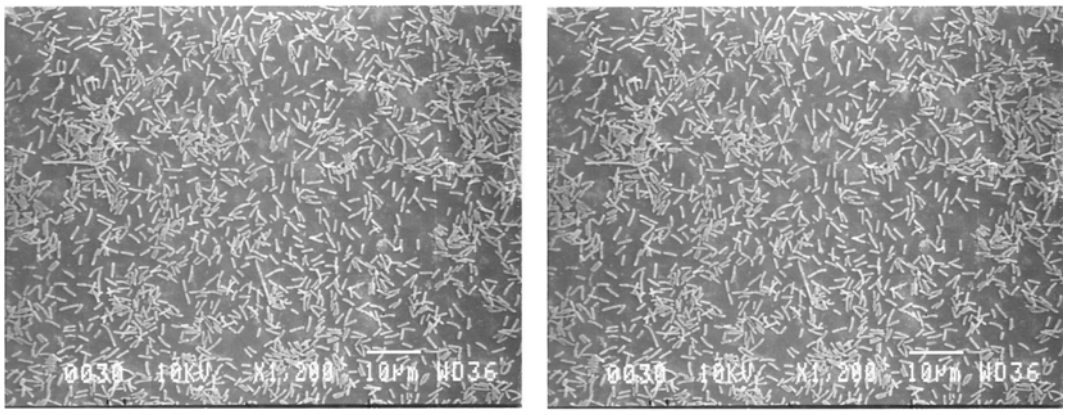

Fig. 7. Newly designed phenolic coating: (a) *P. fluorescens* biofilm was formed on the entire uncoated glass. (b) *P. fluorescens* cells were scattered on the new phenolic coating (×500). The white dots are bacterial cells.

danol, showed good antibiotic activities against *P. fluorescens.* Permanent coatings of the polymers of these two phenolic compounds showed considerable reductions of the adhered cells and exopolymers on the coated surfaces. However, the phenolic coatings showed no observable antimicrobial activities against the adhered cells. The phenolic component with a highly reactive functional group of acetyl amide group was successfully synthesized by modifying cardanol using propylene diamine and fluorocarboxylic acid, and its polymer coating showed dramatic reductions of the adhered cells and exopolymers.

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