

## Enzymatic Polymerization of Natural Anacardic Acid and Antibiofouling Effects of Polyanacardic Acid Coatings

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Received: 17 February 2008 / Accepted: 13 May 2008 /  
Published online: 1 July 2008  
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**Abstract** Anacardic acid, separated from cashew nut shell liquid, is well known for its strong antibiotic and antioxidant activities. Recent findings indicate that phenolic compounds from plant sources have an effect on Gram-negative bacteria biofilm formation. In this work, a polyphenolic coating was prepared from anacardic acid using enzymatic synthesis and tested for its effects on biofilm formation of both Gram-negative and Gram-positive bacteria. Natural anacardic acid was enzymatically polymerized using soybean peroxidase. Hydrogen peroxide and phenothiazine-10-propionic acid were used as an oxidizing agent and redox mediator, respectively. Nuclear magnetic resonance and Fourier transform infrared (FTIR) analyses showed the formation of oxyphenylene and phenylene units through the phenol rings. No linkage through the alkyl chain was observed, which proved a high chemo-selectivity of the enzyme. Aqueous solvents turned out to play an important role in the polymer production yield and molecular weight. With 2-propanol, the highest production yield (61%) of polymer (molecular weight=3,900) was observed, and with methanol, higher-molecular-weight polymers (5,000) were produced with lower production yields (43%). The resulting polyanacardic acid was cross-linked on a solid surface to form a permanent natural polymer coating. The FTIR analysis indicates that the cross-linking between the polymers took place through the unsaturated alkyl side chains.

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The polyanacardic acid coating was then tested for its antibiofouling effect against Gram-negative and Gram-positive bacteria and compared with the antibiofouling effects of polycardanol coatings reported in the literature. The polyanacardic acid coating showed more reduction in biofilm formation on its surface than polycardanol coatings in the case of Gram-positive bacteria, while in the case of Gram-negative bacteria, it showed a similar reduction in biofilm formation as polycardanol.

**Keywords** Enzymatic polymerization · Anacardic acid · CNSL · Antibiofouling · Soybean peroxidase · Redox mediator

## Introduction

Anacardic acid is a natural phenolic lipid, which is well known for its strong antibiotic and antioxidant activities [1–3]. Its antibiotic effects have long been reputed, but little systematic studies of it have been reported until the late 1960s except the outstanding work by Eichbaum in 1946 [4]. Its antioxidant activity was recognized also and has been studied for its mechanisms and applications since the early 1990s [5–7].

Anacardic acid research has focused on separation [8–14], antimicrobial antioxidant mechanisms [15–17], structure–activity relationship [18], and synthesis [19] and has successfully made great advances over the last two decades. However, the applications have rather been limited to its use as supplements in clinical [20–22], food [23, 24], and health care [25, 26] fields. Cardanol, another component in cashew nut shell liquid (CNSL), was successfully polymerized through enzymatic polymerization, and the antibiofouling effect of a surface coating formed by the cross-linked polycardanol was presented recently [27].

In an attempt to expand its applications to industrial and medical fields, a polymer of anacardic acid (i.e., polyanacardic acid) and its coating were synthesized. Cross-linked polyanacardic acid may form a permanent coating on a solid surface and is expected to have higher activity than polycardanol. Enzymatic polymerization was used to control the resulting polymer structures, which are directly related to antimicrobial activities.

In this study, we extracted anacardic acid from untreated raw CNSL and polymerized it using soybean peroxidase. Generally, in enzymatic polymerization of most of the phenols and aromatics, oxidative enzymes are used for high selectivity and production yields [28, 29]. Peroxidases catalyze oxidative radical polymerization of phenolic compounds, and due to the strong regio-/chemo-selectivity of peroxidases, polymerization takes place at specific positions of the phenolic compounds. Uniform structures of resulting polymers are helpful in determining the structure–activity relationships of the polymers and cross-linked polymers.

Although polymerization of anacardic acid to produce phenolic resins via chemical synthesis schemes was reported [19], there have been no detailed studies on the enzymatic polymerization of natural phenolic compounds including anacardic acid. The enzymatic polymerization of phenolic compounds have been considered challenging mainly because of phenolic compounds' enzyme inhibition effects, such as anacardic acid being well known for inhibiting many enzymes such as tyrosinase, lipoxygenase, and other oxidases [30–33].

Peroxidases were not observed to be inhibited by anacardic acid in our study. However, their activities were not sufficient enough to induce the oxidation of anacardic acid for oxidative polymerization, and as a result, the production yields and molecular weights were not high. To enhance the redox reaction between the substrate and the enzyme, a redox mediator was successfully used for the enzymatic polymerization of anacardic acid. Redox

mediators are low-molecular-weight compounds that mediate oxidation reactions between a substrate and an enzyme. Mediators basically act as a good electron carrier between the enzyme and substrate. Recently, some redox mediators have been used to oxidize and polymerize substrates that are difficult to be oxidized by enzymes [34–36]. The mediator used in this study was phenothiazine-10-propionic acid, which has been successfully used for enzymatic polymerization of cardanol recently [35].

The resulting polymers have been cross-linked on a glass slide to make a permanent antimicrobial coating. The molecular weights and structures of the polymer and coating were analyzed using nuclear magnetic resonance (NMR), Fourier transform infrared (FTIR), and gel permeation chromatography (GPC). We compared the antibiofouling effect of polyanacardic acid coating with that of polycardanol coatings on both Gram-negative and Gram-positive bacteria and observed that polyanacardic acid had more significant effects on biofilm formation of Gram-positive bacteria. Within the best of our knowledge, it is the first time that natural anacardic acid is polymerized and cross-linked using enzymatic method and tested for antibiofouling effects.

## Experimental

### Enzymatic Polymerization

Untreated raw CNSL was obtained from Sun Food, Andhra Pradesh, India. Anacardic acid was extracted from CNSL according to the method described in Paramashivappa et al. [10]. The soybean peroxidase enzyme has been kindly donated by Bio Research Products, USA. All other materials were commercially available and used as received.

The polymerization of anacardic acid (300 mg) 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) at room temperature under open air. Phenothiazine-10-propionic acid was used as a redox mediator in the reaction. It was added (1,000  $\mu$ M) in the beginning of polymerization. Hydrogen peroxide was continuously added as an oxidizing agent during the polymerization. An infusion pump (Model 780100, KD Scientific) was used to inject  $\text{H}_2\text{O}_2$  (30% aqueous solution) at 50  $\mu$ L/h for 3 h.

After 24 h, the reaction was terminated by centrifugation, and the residue was washed with methanol and water to remove any unreacted anacardic acid and enzyme, respectively. The residue was then dried in a desiccator and weighed to calculate the yield.

### Cross-linking of Polyanacardic Acid

The obtained polymer was 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 to form a smooth coating. The hardness of the samples was determined by a pencil scratch hardness test.

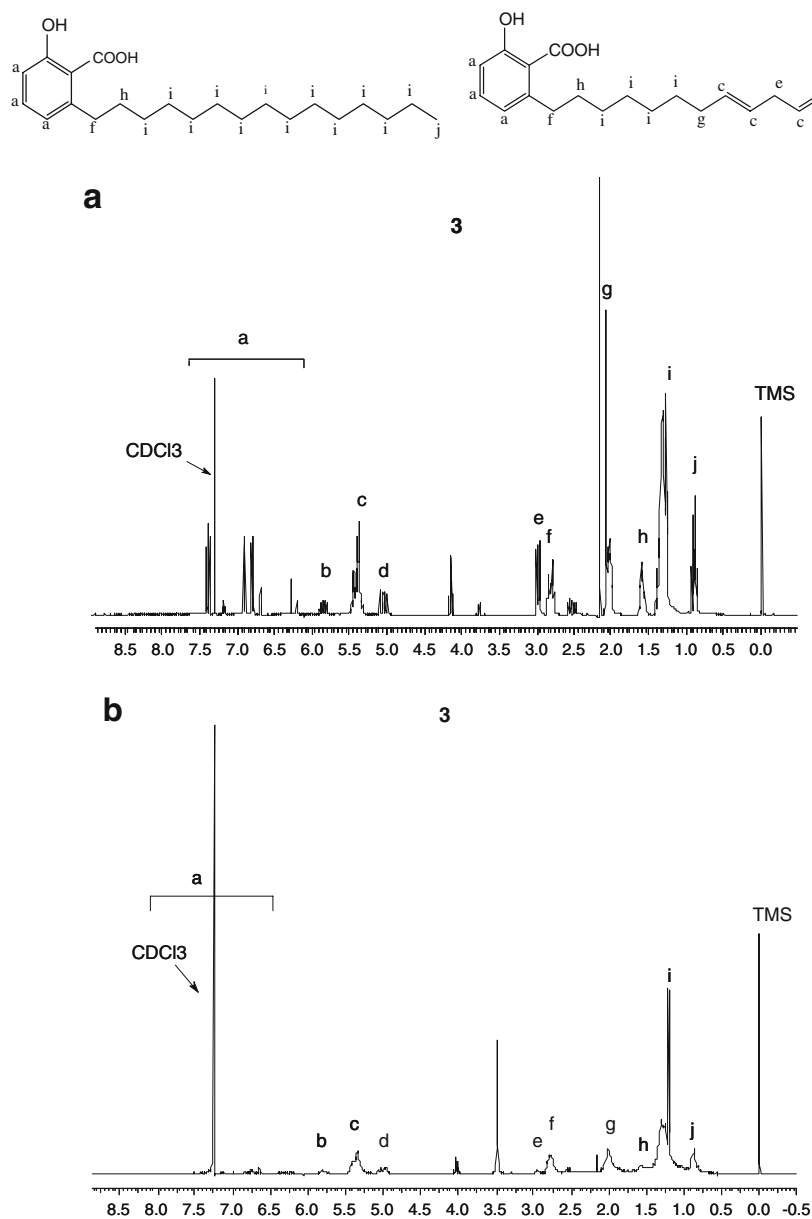
### Characterization of Polyanacardic Acid

The resulting polymers obtained from enzymatic polymerization were characterized for molecular weight and structure using  $^1\text{H}$  NMR, FTIR, and GPC. The curing of the polymer was also monitored by FTIR spectroscopy. The polymer samples were prepared in a  $\text{CDCl}_3$  solvent for NMR (NMR Varian 400 MHz [VXRS-400]) analysis. FTIR (FTS 4000, DIGILAB) analysis was performed using Ge micro attenuated total reflectance. GPC

analysis was done using a Phenogel column (particle size: 5  $\mu\text{m}$ , Phenomex, Torrance, CA, USA).

### Antibiofouling Effects of Polyanacardic Acid Coating

To investigate the antibiotic effects of the polyanacardic acid coating, *Leuconostoc mesenteroides* (Gram-positive) and *Pseudomonas aeruginosa* (Gram-negative) were used



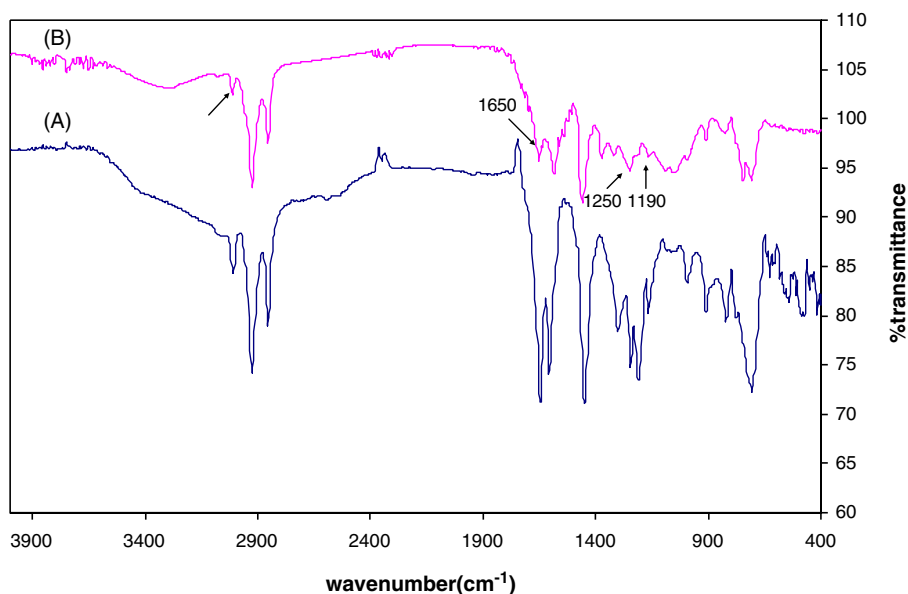
**Fig. 1**  $^1\text{H}$  NMR spectra of anacardic acid (a) and polyanacardic acid (b)

for forming biofilms on the coated and uncoated slides. *P. aeruginosa* was grown in a tryptone soya broth medium (50 g/l) and *L. mesenteroides* in a deMan–Rogosa–Sharpe medium (55 g/l) supplemented with sucrose (10 g/l). The stains were grown on the polycardanol- and polyanacardic acid-coated surfaces and on plastic slides (polypropylene) in separate Petri plates for 1 week. Polypropylene slide was chosen because it is a commonly used material in artificial medical devices and implants. The culture broths in all the cases were replaced with fresh nutrient solutions every 2 days. After a week, the biofilm formed on the uncoated and coated plates were pretreated for scanning electron microscope observation as described in the references [37, 38].

## Results

In the NMR results of the polymer shown in Fig. 1, all the peaks in the polymer spectrum were observed to be broader than those in the monomer peaks, which indicate that the polymer formation took place. However, the ratio of the integral areas of the peaks of the unsaturated and saturated groups in the monomer remained the same in the polymer spectrum. This clearly indicates that the polymerization occurred only in the aromatic ring and not in the unsaturated groups in the alkyl chain. The proton peak of the benzene ring in the region “a” almost disappeared in the polymer spectrum, which indicates that the coupling took place through the aromatic ring.

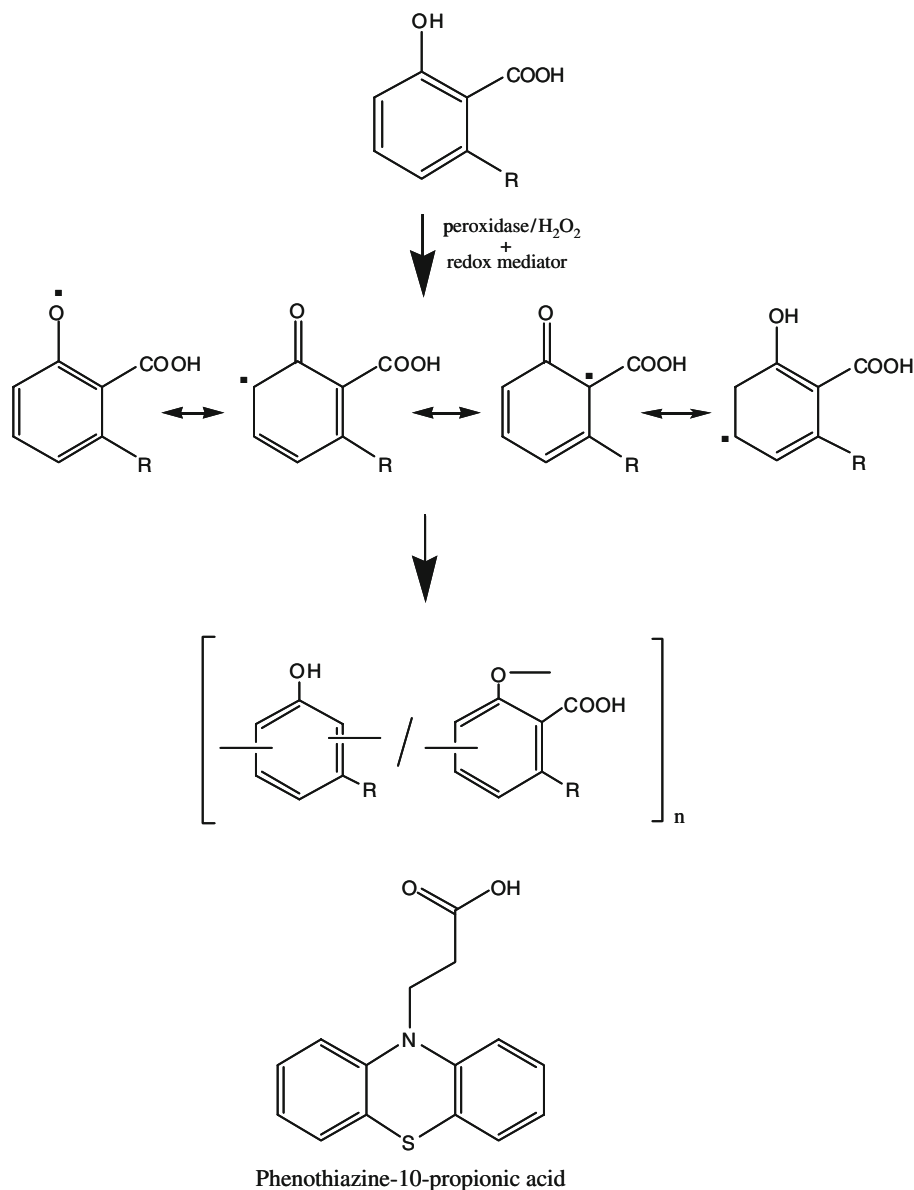
In the FTIR spectra of the monomer and polymer shown in Fig. 2, the peaks at  $3,010\text{ cm}^{-1}$  remained the same indicating no reaction of the unsaturated group during the polymerization. The broad peak at  $1,250\text{ cm}^{-1}$  and the peak at  $1,190\text{ cm}^{-1}$  in the polymer spectrum are ascribed to the vibration of C–O–C and C–OH linkages. The peak at  $1,650\text{ cm}^{-1}$  indicates the vibration of C=O in the COOH group of anacardic acid. This peak



**Fig. 2** FTIR spectra of anacardic acid (A) and polyanacardic acid (B)

decreased in its intensity in the polymer spectrum, which indicates some decarboxylation taking place during the polymer formation. A similar type of decarboxylation was reported to happen during enzymatic polymerization of 4-hydroxybenzoic acid derivatives to poly(phenylene oxide) using horse radish peroxidase (HRP) [39].

According to the FTIR and NMR results, it is suggested that the polymerization produces a mixture of oxyphenylene and phenylene units with some decarboxylation that



**Fig. 3** Reaction scheme of polymerization catalyzed by peroxidase

took place during the polymerization as shown in Fig. 3. The polymers are produced by a free radical oxidative coupling process. A phenoxy free radical is first generated by the enzyme, which can transfer and exist in one of the four forms as shown in Fig. 3. The radicals then combine to form a dimer. A radical transfer then takes place between a monomer radical and polyphenol or oligomer (dimer) to form a polymeric radical. Finally, the polymeric radicals and the monomer radicals undergo oxidative coupling to form polyphenols of higher molecular weight.

There was no polymerization observed to happen in the unsaturated side chain of the monomer, which demonstrated high chemo-selectivity of the enzymes used in enzymatic polymerization.

The effects of the solvent and mediator on the polymer molecular weight are summarized in Table 1. It turned out that without the mediator, no polymerization occurred. The highest production yield (61%) of the polymer was resulted when a 2-propanol aqueous solvent was used. Methanol produced higher-molecular-weight polymers than 2-propanol.

The curing of polyanacardic acid resulted in a smooth brown coating. The structure of the coating was analyzed using FTIR (Fig. 4). The peak at  $3,010\text{ cm}^{-1}$  due to the unsaturated group disappeared in the cured product. The peak at  $914\text{ cm}^{-1}$  due to the C–H vibration of the terminal vinyl group also indicates that the cross-linking between the polymers took place through the unsaturated alkyl side chains.

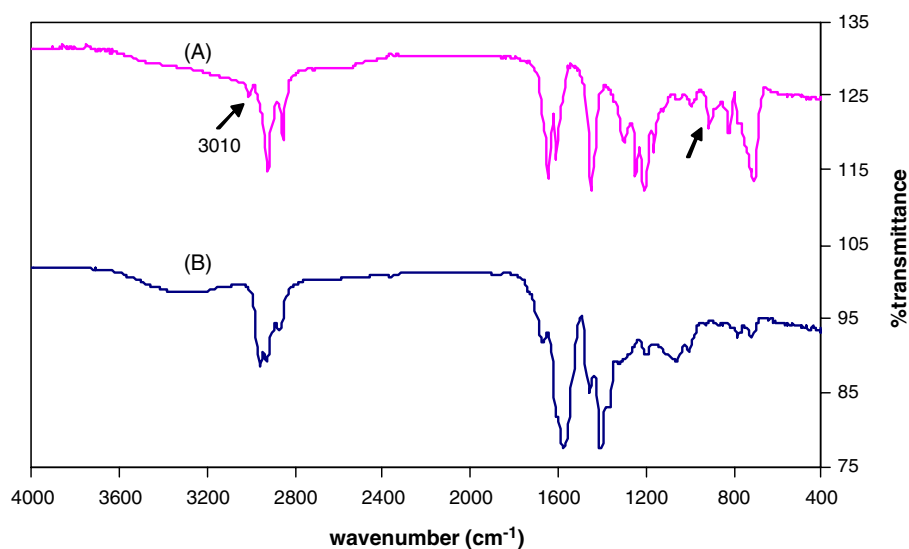
The hardness of the coating was tested using the pencil scratch hardness test as described in Guevin [40]. The result is summarized in Table 2.

The scanning electron microscopy (SEM) images of the biofilm formation on the polyanacardic acid- and polycardanol-coated surfaces and plastic slides after 7 days of bacterial growth are shown in Figs. 5 and 6. The images clearly show a decrease in biofilm formation on all the coated slides with polycardanol and polyanacardic acid. In the case of the Gram-negative bacteria, *P. aeruginosa*, there was not much difference between the polycardanol-coated and polyanacardic acid-coated slides. In the case of the Gram-positive bacteria (*L. mesenteroides*), however, the slide coated with polyanacardic acid showed more reduction in biofilm formation compared to the polycardanol-coated slide. The SEM images (Figs. 7 and 8) of the biofilm of *P. aeruginosa* and *L. mesenteroides* on the plastic surface consists of a lot of gelatinous exopolymers, whereas the polycardanol- and polyanacardic acid-coated slides showed only individually attached cells without exopolymers.

To compare the effect of hydrophilicity/hydrophobicity of the surfaces on the biofilm formation, an air–water contact angle analysis was conducted on all the surfaces. The polycardanol-coated surface turned out to be the most hydrophobic surface with a contact angle of  $124.6 \pm 4$ . The polyanacardic acid was slightly less hydrophobic with a contact angle of  $110 \pm 6$ , and the plastic surface had a contact angle of  $117.3 \pm 2$ .

**Table 1** Effects of solvents and mediator on the production yield and molecular weight of polyanacardic acid.

Solvent	Mediator	Catalyst (mg)	Yield (%)	MW
Methanol	Without	10	0	–
Propanol	Without	10	0	–
Methanol	With	10	43	5,000
Propanol	With	10	61	3,900



**Fig. 4** FTIR spectra of *A* polyanacardic acid and *B* cured product after 9 days

## Discussion

The enzymatic polymerization of anacardic acid proceeded in the presence of a redox mediator to produce a polymer consisting of a mixture of phenylene and oxyphenylene units as shown in Fig. 3. When the redox mediator was absent in the reaction, no polymerization was observed. NMR and FTIR results confirm that the polymerization took place only through the phenol ring and not through unsaturated side chain of the monomer, which proves the high chemo-selectivity of the enzyme. Soybean peroxidase with the help of the redox mediator generates free radicals from anacardic acid, which combine and grow into a polymer through a chain growth process. The redox mediator acts as an electron carrier between the enzyme and the substrate. The polymerization mechanism and the role of the redox mediator are summarized in Fig. 3.

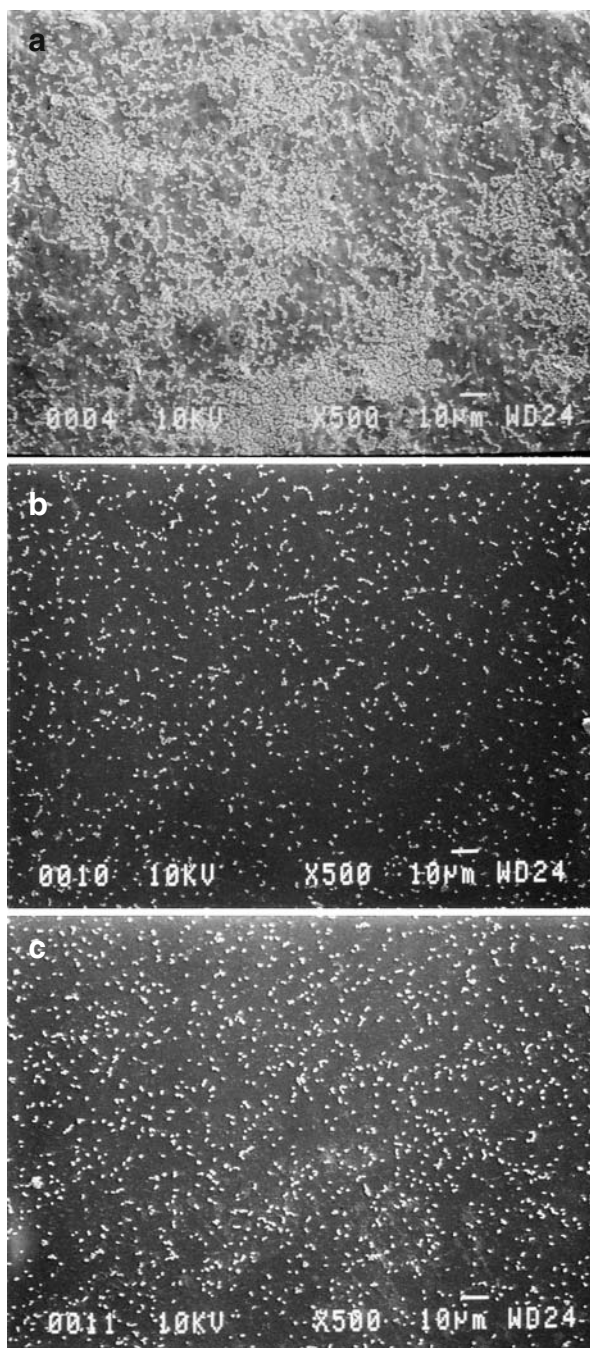
The polymer molecular weight could be easily controlled by varying the nature of the solvent as shown in Table 1. The difference in molecular weight with different solvents is attributed to the change in enzyme activity in different solvents as described in Kim et al. [24].

**Table 2** Pencil scratch hardness of cured polyanacardic acid and commercial coating paint.

Catalyst composition (% w/w)	Curing time			
	1 day	3 days	5 days	9 days
None	—	—	—	—
Cobalt Naphthenate (0.5)/possible to touch methyl ethyl ketone peroxide(MEKP)	2H	4H	5H	5H
Commercial coating paint possible to touch	2H	2H	3H	4H

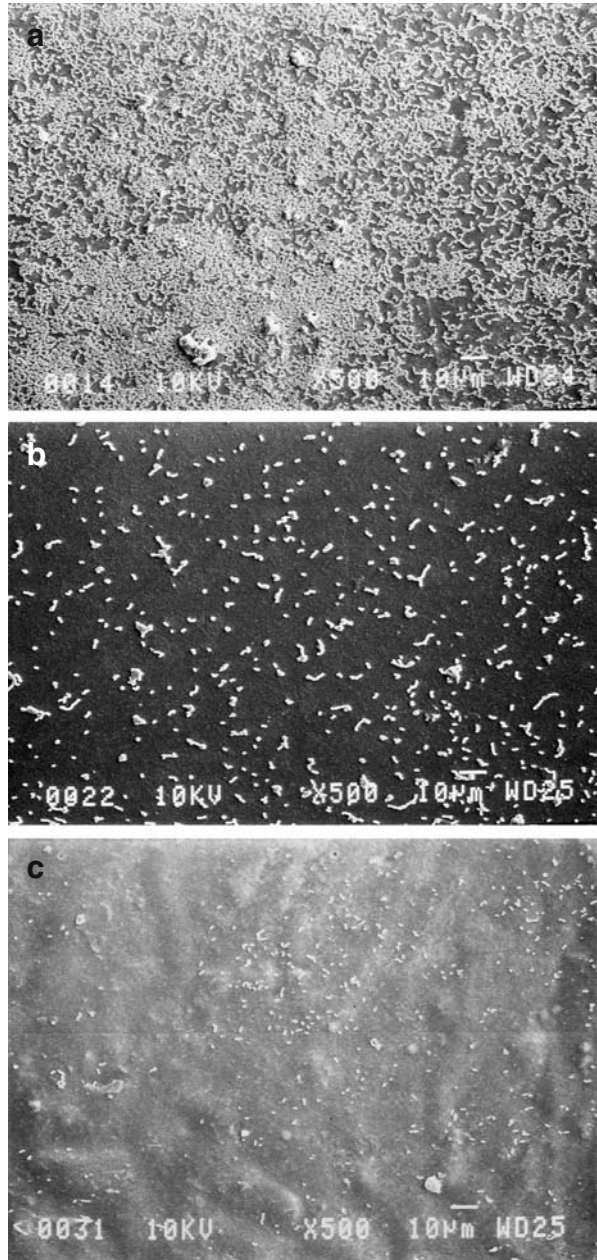


**Fig. 5** *Pseudomonas aeruginosa* biofilm formation on the coated materials observed after 7 days on SEM aft. Plastic surface (scale bar=10  $\mu$ m,  $\times$ 500) (a), plastic surface coated with polycardanol (scale bar=10  $\mu$ m,  $\times$ 500) (b), plastic surface coated with poly-anacardic acid (scale bar=10  $\mu$ m,  $\times$ 500) (c)



Curing (i.e., cross-linking) of the polymer was done to form a nice and smooth coating with the help of cobalt naphthenate. The FTIR analysis of the cured polymer indicates the involvement of unsaturated groups in the cross-linking of the polymer. The curing of the polymer might be taking place by a free radical coupling through the unsaturated side

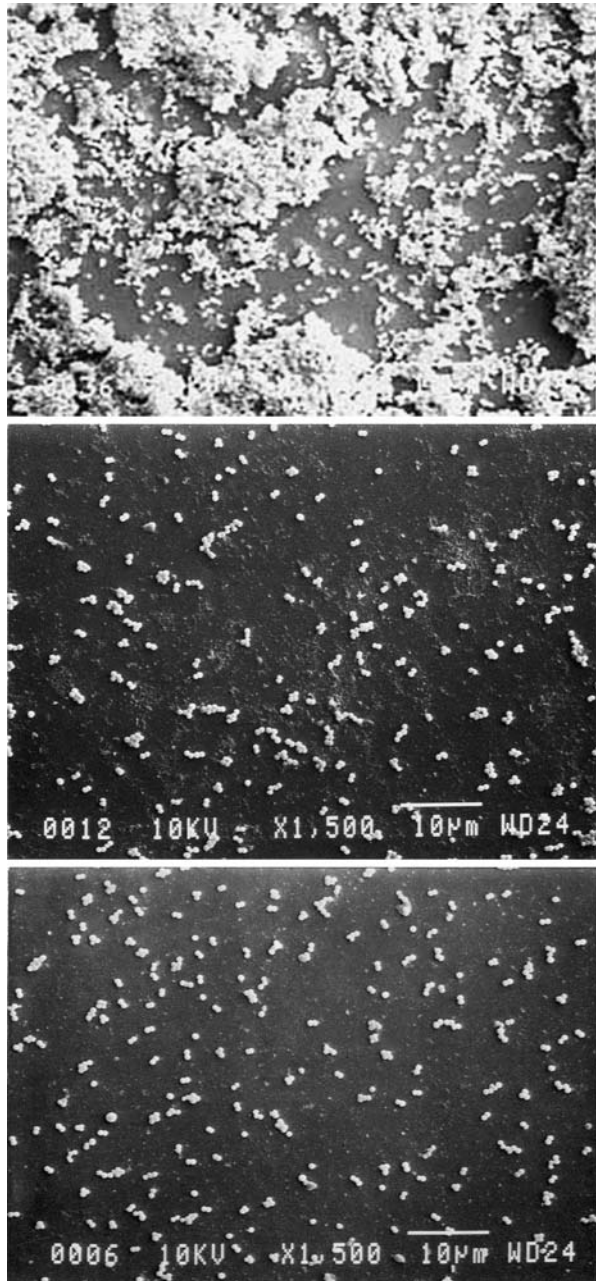
**Fig. 6** *Leuconostoc mesenteroides* biofilm formation on the coated materials observed after 7 days on SEM aft. Plastic surface (scale bar=10  $\mu$ m,  $\times$ 500) (a), plastic surface coated with polycardanol (scale bar=10  $\mu$ m,  $\times$ 500) (b), plastic surface coated with polyanacardic acid (scale bar=10  $\mu$ m,  $\times$ 500) (c)



chain. The curing mechanism of a monoene of the polymer is indicated in Fig. 9. The coating hardness reached the hardness value of a commercially available paint in 9 days, which shows that coating can be used for commercial purposes.

The SEM images of biofilm formation of *P. aeruginosa* and *L. mesenteroides* on the polycardanol- and polyanacardic acid-coated slides show that the polyanacardic acid coating was more effective against both Gram-negative and Gram-positive bacteria,

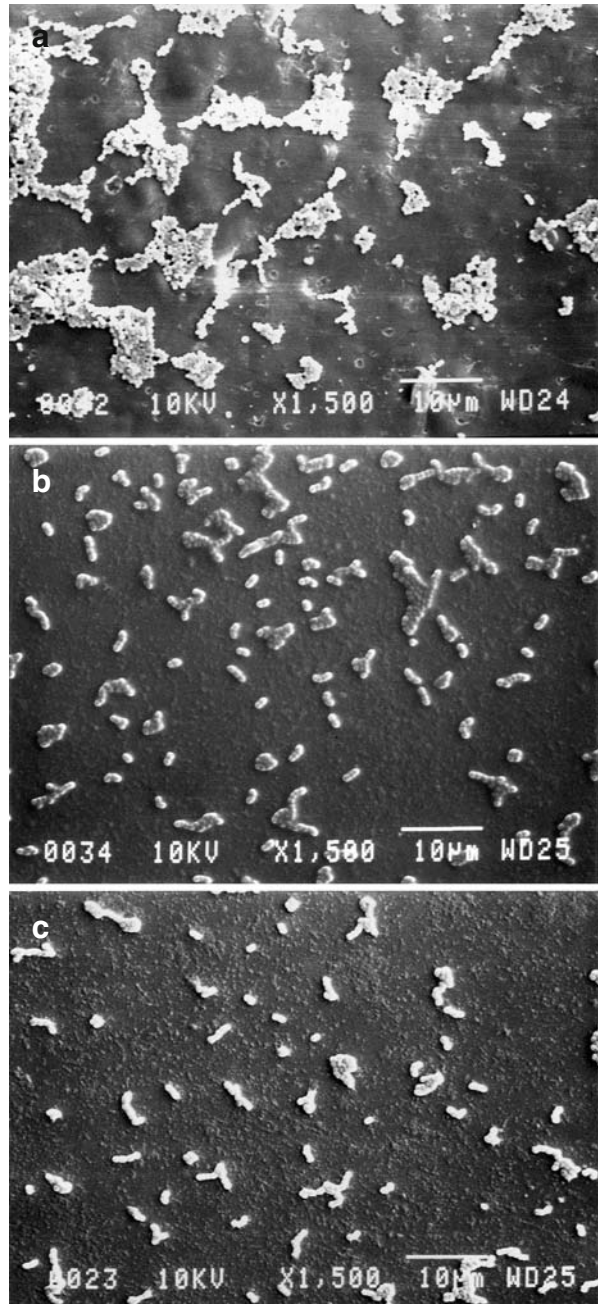
**Fig. 7** *Pseudomonas aeruginosa* biofilm formation on the coated materials observed after 7 days on SEM aft. Plastic surface (scale bar=10  $\mu\text{m}$ ,  $\times 1,500$ ) (a), plastic surface coated with polycardanol (scale bar=10  $\mu\text{m}$ ,  $\times 1,500$ ) (b), plastic surface coated with polyanacardic acid (scale bar=10  $\mu\text{m}$ ,  $\times 1,500$ ) (c)



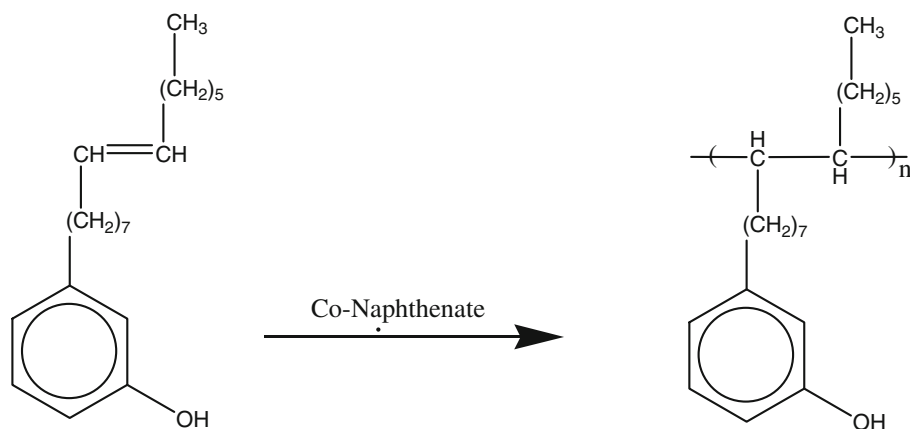
whereas the polycardanol coating was only effective against Gram-negative bacteria. In biological systems, hydrophobic interactions are usually the strongest of all long-range noncovalent forces, and the adhesion to surfaces is often mediated by these types of interactions. It has been demonstrated that hydrophobicity plays an important role in a wide range of microbial infections [41, 42]. As stated in the result section, the polycardanol coating was observed to be more hydrophobic (contact angle=124.6 $\pm$ 4) than the plastic



**Fig. 8** *Leuconostoc mesenteroides* biofilm formation on the coated materials observed after 7 days on SEM aft. Plastic surface (scale bar=10  $\mu$ m,  $\times 1,500$ ) (a), plastic surface coated with polycardanol (scale bar=10  $\mu$ m,  $\times 1,500$ ) (b), plastic surface coated with polyanacardic acid (scale bar=10  $\mu$ m,  $\times 1,500$ ) (c)



surface ( $117.3 \pm 2$ ). However, the polycardanol coating resulted in less numbers of adhered cells on the surface than the plastic surface. This result indicates that hydrophobicity is not a main antibiofouling mechanism of the polycardanol coating. On the other hand, the polyanacardic acid coating was slightly less hydrophobic ( $110 \pm 6$ ) than the plastic surface. Therefore, it seems possible that the antibiofouling effect of the polyanacardic acid coating



**Fig. 9** Curing mechanism of the polymer

is due to low hydrophobicity. However, considering the small difference of contact angles between the polyanacardic acid and plastic surfaces and yet the significant difference in biofilm formation, the effect of hydrophobicity of the phenolic coatings on biofilm formation is considered either negligible or marginal. Therefore, the observed antibiofouling activities of polyphenolic coatings are concluded to be not caused by the hydrophilic/hydrophobic interaction between the cells and the coating surface.

Anacardic acid is known to have good antimicrobial activity against Gram-positive bacteria rather than against Gram-negative bacteria [12–14], and therefore, the polyanacardic acid coating might possibly act as an antimicrobial coating against *L. mesenteroides*, the Gram-positive bacteria. In the case of the Gram-negative bacteria, according to Figs. 7 and 8, the coatings still successfully reduced the cells on the surface, which cannot be explained with antimicrobial effects of the coatings. It is thought that the phenolic coatings interfere with the biofilm formation by inhibiting the exopolymer production mechanisms of both Gram-positive and Gram-negative cells.

## Conclusions

Natural anacardic acid was successfully polymerized using enzymatic polymerization. Soybean peroxidase induced the polymerization of anacardic acid in the presence of a redox mediator and produced soluble cross-linkable polyphenolic compounds. Poly-anacardic acid had a uniform structure and was readily cross-linked (i.e., cured) by adding a mixture of cobalt naphthenate and methyl ethyl ketone peroxide to form a smooth hard coating on a glass slide.

The structure analysis indicated that the polymerization took place mostly through the phenol ring, resulting in mostly oxyphenylene and phenylene polymers. No coupling between the alkyl side chains was observed, which demonstrated strong chemo-selectivity of the enzyme.

When an aqueous methanol solvent was used, a higher molecular weight (~5,000) of polyanacardic acid was resulted than when 2-propanol was used (~3,900). However, 2-propanol produced higher production yields (61%) of polyanacardic acid than methanol (43%).

The polyanacardic acid coating was effective as an antibiofouling coating material against both Gram-positive and Gram-negative bacteria, whereas the polycardanol coating was more effective against Gram-negative bacteria than against Gram-positive bacteria. The present research has been conducted by the funding support from National Science Foundation (CTS-0626022).

**Acknowledgment** The present research has been conducted by the funding support from National Science Foundation (CTS-0626022).

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