Ferulic Acid as a Comonomer in the Synthesis of a Novel Polymeric Chain with Biological Properties

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ABSTRACT: In this study, ferulic acid was copolymerized with methacrylic acid to obtain a useful material with both antioxidant and antifungal properties. An ascorbic acid/hydrogen peroxide redox pair was used as a water-soluble and biocompatible initiator system to prime the one-pot polymerization reaction. The obtained material was characterized with gel permeation chromatography, Fourier transform infrared spectroscopy, ultraviolet spectroscopy, and fluorescence analyses, and the disposable phenolic group content was determined to verify the insertion of ferulic acid into the polymeric backbone. Finally, the antioxidant and antifungal activities were demonstrated toward the hydroxyl radical and *Aspergillus niger*, respectively. © 2009 Wiley Periodicals, Inc. J Appl Polym Sci 115: 784–789, 2010

Key words: antioxidants; biological applications of polymers; radical polymerization

INTRODUCTION

Ferulic acid (FA; i.e., 4-hydroxy-3-methoxycinnamic acid) is one of the most abundant phenolic acids in plants.[1,2] FA, which is rarely found in the free form, is usually found as ester crosslinks with polysaccharides in the cell wall, such as in arabinoxylans in grasses, pectin in spinach, and sugar beet and xyloglucans in bamboo.[3] It also can crosslink with proteins.[4] The crosslinking properties of FA with both polysaccharides and proteins suggests that it can be used in the preparation of complex materials to be used in biomedical,[5] pharmaceutical,[6] food,[7] and cosmetic applications.[8]

In recent years, there have been an increasing number of reports on the physiological functions of FA and its derivatives in humans, including antioxidant, antifungal, anti-inflammatory, antithrombosis, and anticancer activities.[9–12]

Free FA is a good antioxidant because of its ability to donate the hydrogen atoms of phenolic hydroxyl groups in reactions with peroxyl radicals. In this way, stabilized phenoxyl radicals are produced and thus terminate lipid peroxidation chain reactions.[13] FA has shown high scavenging activity for hydrogen peroxide (H_2O_2), superoxide anions ($^{-}O_2^{\bullet}$), hydroxyl radicals (·OH), and nitrogen dioxide free radicals.[14] Free radicals, such as $^{-}O_2^{\bullet}$, H_2O_2, and ·OH, are constantly formed in the human body by normal activities such as in immunological defenses and metabolic reactions.[15] Their excess is opposed by a balanced system of antioxidant defenses, including antioxidant compounds and enzymes. Upsetting this balance causes oxidative stress, which can lead to cell injury and death.[16] Therefore, much attention has been focused on the use of antioxidants to inhibit lipid peroxidation or to protect against the damage of free radicals.[17]

FA, as well as phenolic acid derivatives, is also known to play an important role in antifungal activity.[18] It could potentially serve as an effective alternative to conventional antifungal agents, which are frequently perceived to present hazards to human health and the environment.[19]

Because of its important properties, the goal of this study was to evaluate the biological activity of an FA polymeric derivative. This kind of macromolecular system has shown a higher stability and slower degradation rate than compounds with a lower molecular weight.[20,21]

Antioxidant polymers could be applied in those fields in which the use of a single molecule is prohibitive[22]; for example, they can be used in hemodialysis applications, in particular, by their introduction in dialysis membranes. Hemodialysis patients, indeed, are exposed to oxidative stress, which contributes to cardiovascular disease and accelerated atherosclerosis, the major causes of mortality in these patients.[23]

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Another field of application of this kind of materials could be cosmetic formulations, to avoid the oxidation of their components, but they can also be used as preservative agents in food packaging.

Antifungal polymers, because of their high stability, could be very useful in all environments that susceptible to contamination by pathogenic fungi, which have a strong ability to survive on different surfaces.[24] Contaminated materials are often associated with increased risk of infections. Medically important fungi, such as *Candida, Aspergillus, Fusarium, Mucor*, and *Paecilomyces* spores, survive on hospital fabrics from 1 day to several weeks. In response to these challenges, much effort has been devoted to the development of infection-resistant materials for hospital, medical, pharmaceutical, bioprotective, and related hygienic applications.[25]

In our study, FA was copolymerized with methacrylic acid (MAA) to obtain a useful material with both antioxidant and antifungal properties. The synthetic strategy we used was an one-step radical polymerization based on the use of water-soluble redox initiators, which allows us to obtain the copolymer through the direct polymerization of FA and MAA. The choice of MAA as a comonomer was related to the broad application field of methacrylate polymers in biomedicine and biotechnology. Acrylate and methacrylate polymers, indeed, have been applied in drug-delivery systems, contact lenses, food technology, quality control systems, and synthetic membranes for biosensors.[26]

The obtained material was characterized by gel permeation chromatography (GPC), Fourier transform infrared (FTIR) spectroscopy, and ultraviolet–visible and fluorescence analyses, and then, its antioxidant and antifungal activities were evaluated.

EXPERIMENTAL

Materials

FA, MAA, poly(methacrylic acid) [poly(MAA)], sodium salt standards, *N,N*-dimethylformamide, H_2O_2, ascorbic acid (AA), deoxyribose, $FeCl_3$, ethylenediaminetetraacetic acid disodium (EDTA) salt, dipotassium hydrogen phosphate, potassium dihydrogen phosphate, thiobarbituric acid, trichloroacetic acid, Folin–Ciocalteu reagent, and sodium carbonate were obtained from Sigma–Aldrich (St. Louis, MO). All solvents were reagent-grade or high preformance liquid chromatography (HPLC)-grade. MAA was purified before use by distillation under reduced pressure.

Synthesis of the methacrylic acid–ferulic acid copolymer [poly(MAA–FA)]

The polymerization of MAA with FA by AA/H_2O_2 redox initiators was carried out as follows: in a 10-mL glass tube, 0.50 g of FA was dissolved in 5.50 mL of dimethylformamide, and then, MAA (3.50 g) and 2 mL of distilled water containing 10 m*M* AA and 7 m*M* H_2O_2 were added. The mixture was maintained at 25°C for 3 h under atmospheric air.

The obtained polymer was precipitated by the addition of the polymeric solutions to an excess volume of diethyl ether (5:1) under agitation at room temperature. The suspensions were filtered by a sintered glass filter funnel (Pyrex, Ø30 mm; porosity = 3) and washed with diethyl ether, and the recovered polymer was dried in a vacuum oven at 40°C. The samples were then further purified by dissolution in water and precipitation in diethyl ether (5:1) three times.

The copolymer was checked to be free of unreacted FA and any other compounds by HPLC analysis after each purification step.

The blank polymer [poly(MAA)] was prepared under the same conditions without FA.

Instrumentation

The liquid chromatography consisted of a Jasco BIP-Ipump and a Jasco (Tokyo, Japan) UVDEC-100-V detector set at 240 nm. A 250 × 4 mm^2 C-18 Hibar column (particle size = 5 μm, Merck, Darmstadt, Germany) was used. As reported in the literature,[27] the mobile phase was methanol at a flow rate of 0.5 mL/min and at room temperature. IR spectra were recorded as films or KBr pellets on a Jasco FTIR 4200. A PerkinElmer (Waltham, MA) Lambda 900 spectrophotometer was used to obtain the absorption spectra, whereas the corrected emission spectra, all confirmed by excitation ones, were recorded with a PerkinElmer LS 50B spectrofluorimeter, equipped with a Hamamatsu R928 photomultiplier tube.

The molecular weight distributions of the synthesized polymers were analyzed by a GPC system composed of μBondagel E-125 and E-500 GPC columns (Millipore, Water Associates (Eschborn, Germany)) connected in series, a Jasco PU-2080 Plus liquid chromatograph equipped with a Rheodyne 7725i injector (fitted with a 20-μL loop), an Agilent (Santa Clara, CA) ELSD 1200 light-scattering detector, and a Jasco–Borwin integrator. The mobile phase that we used was phosphate-buffered saline (pH 7.4) at a rate of 0.8 mL/min calibrated with six individual poly (MAA)s, sodium salt standards with peak molecular weights ranging from 1670 to 236,000 Da and polydispersity index values ranging from 1.02 to 1.11. The mobile phase was phosphate-buffered saline buffer (pH 7.5) at a flow rate of 0.8 mL/min.

Determination of the disposable phenolic groups

The amount of total phenolic groups was determined with the Folin–Ciocalteu reagent procedure

according to the literature with some modifications.[28]

Poly(MAA–FA) (100 mg) was dissolved in distilled water (6 mL) in a volumetric flask. Folin–Ciocalteu reagent (1 mL) was added, and the contents of flask were mixed thoroughly. After 3 min, 3 mL of Na_2CO_3 (7.5%) was added, and then, the mixture was allowed to stand for 2 h with intermittent shaking.

The absorbance was measured at 760 nm against a prepared control with the blank polymer under the same reaction conditions. The amount of total phenolic groups in the polymeric materials was expressed as an FA equivalent concentration with an equation that was obtained from an FA calibration curve. This one was recorded with five different FA standard solutions. Each solution (0.5 mL) was added to the Folin–Ciocalteu system to raise the final concentrations to 0.03, 0.06, 0.09, 0.12, and 0.15 m*M*. After 2 h, the absorbance values of the solutions were measured to record the calibration curve, and the correlation coefficient, slope, and intercept values of the regression equation obtained were calculated by the method of least squares.

Evaluation of the antioxidant activity on ·OH

Poly(MAA–FA) (75 mg) was incubated with 0.5 mL of deoxyribose (3.75 m*M*), 0.5 mL of H_2O_2 (1 m*M*), 0.5 mL of $FeCl_3$ (100 m*M*), 0.5 mL of EDTA (100 m*M*), and 0.5 mL of AA (100 m*M*) in 2.5 mL of potassium phosphate buffer (20 m*M*, pH7.4) for 60 min at 37°C.[28] Then, to 1 mL of sample, 1 mL of thiobarbituric acid (1% w/v) and 1 mL of trichloroacetic acid (2% w/v) were added, and the tubes were heated in a boiling water bath for 15 min. The contents were cooled, and the absorbance of the mixture was read at 535 nm against the control reagent without polymer.

The antioxidant activity was expressed as a percentage of scavenging activity on ·OH according to eq. (1):

$$\text{Inhibition}(\%) = \frac{A_0 - A_1}{A_0} \times 100 \qquad (1)$$

where A_0 is the absorbance of a standard that was prepared in the same conditions, but without any polymer and A_1 is the absorbance of polymeric sample.

The same reaction conditions were applied for the blank polymer poly(MAA) to evaluate the interference of the polymeric material on a deoxyribose assay. All samples were assayed in triplicate, and the data were expressed as means (±standard errors of the mean).

Evaluation of the antifungal activity

The synthesized polymeric material was screened *in vitro* for their antifungal activities against *Aspergillus niger.* Antifungal assays were performed as follows.[29] Poly(MAA–FA) was dissolved in distilled water at a concentration of 100 mg/mL. Then, the solution was added to sterilized potato dextrose agar to give a final concentration of 10 mg/mL. After the mixture was cooled, the mycelium of the fungi was transferred to this test plate and incubated at 29°C for 3 days. The antifungal index was calculated according to eq. (2):

$$\text{Antifungal index}(\%) = \left(1 - \frac{D_t}{D_c}\right) \times 100 \qquad (2)$$

where D_t is the diameter of the growth zone in the test plate and D_c is the diameter of growth zone in the control plate (without antifungal agent).

The same reaction conditions were applied for the blank poly(MAA) to evaluate the interference of the polymeric material.

Each experiment was performed three times, and the data were averaged.

RESULTS AND DISCUSSION

Synthesis of poly(MAA–FA)

Our approach for the synthesis of poly(MAA–FA) involved the direct copolymerization of MAA and FA (Fig. 1), which acted as comonomers without any derivatization procedure.

This procedure allowed us to overcome the limitations of common synthetic strategies for the preparation of functionalized polymers, which involve several different steps. A first strategy is based on the functionalization of a molecule with antioxidant properties by the insertion of a polymerizable group and its subsequent polymerization or copolymerization.[30] Another procedure involves the derivatization of a preformed polymeric structure with an antioxidant.[31] Finally, a third approach is the grafting of a synthesized monomeric antioxidant onto a polymeric chain via melt processing with free-radical initiators.[32] All of these strategies are time-consuming because they require the purification of intermediate products and a difficult optimization of the reaction conditions. On the contrary, our single-step procedure allowed us to obtain highly performing materials in a shorter time.

As explained before, an AA/H_2O_2 redox pair, a water-soluble and biocompatible system, was used as the initiator. Compared to conventional initiator systems (i.e., azo compounds and peroxides), which require relatively high polymerization temperatures

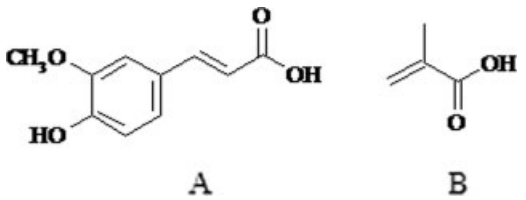

Figure 1 Structures of (A) FA and (B) MAA.

to ensure their rapid decomposition, redox initiators show several advantages. It is possible, indeed, to perform polymerization processes at lower temperatures, with all of the polymer chains initiated almost instantaneously because of the reduction of the induction time.[33] Furthermore, the lower polymerization temperature reduced the risks of FA degradation, and the generation of any kind of toxic reaction products was avoided.

A possible mechanism of redox reagents interaction is shown in Figure 2: AA was oxidized by H_2O_2 to form ·OH and ascorbate radical intermediates that initiated the polymerization.[34]

The reactivity of FA toward free-radical polymerization could be explained on the basis of its chemical structure, which is characterized by a carbon–carbon double bond in the styrenic position and a phenolic group. It is known, indeed, that the styrenic group of cinnamic acid can undergo free-radical polymerization under a wide range of conditions.[35] Phenolic group compatibility with this kind of polymerization was also proven in a different study[36]: monomers with active functional groups (phenolic groups) as side substituents, indeed, were used for the preparation of chelating[37] or grafted polymeric systems with free-radical initiators. On the other side, a phenolic group could be directly involved in polymerization process; it was indeed reported that the phenolic radical undergoes a dimerization processes by a reaction between ·OH and an aromatic ring.[38]

On the basis of these considerations, our hypothesized polymerization mechanism involved the reaction of both styrenic carbon–carbon double bonds and phenolic oxygen for FA insertion into the polymeric chain.

In the prepolymerization feed, a ratio of 1 : 7 w/w between FA and MAA was chosen because it represented the optimal value for obtaining the polymer with the highest efficiency. A higher amount of FA, indeed, made it difficult to carry out the formation of oligomers, and they were difficult to purify by the conventional purification technique.

Characterization of poly(MAA–FA)

Poly(MAA–FA) and the respective control polymer were characterized by FTIR, ultraviolet–visible, and fluorescence analyses, and the molecular weight distributions were analyzed by GPC.

The incorporation of FA in the polymeric backbone was proven by the appearance in the FTIR spectrum of poly(MAA–FA) of a peak at 1544 cm^{-1} attributable to carbon-to-carbon stretching within the aromatic ring of FA. Further confirmation of FA insertion into the polymeric chain was obtained by comparison of the UV spectra of FA (25 μ*M*) and poly(MAA–FA) in ethanol (4 mg/mL), as shown in Figure 3. In the poly(MAA–FA) spectrum, the presence of absorption peaks in the aromatic region was related to the presence of FA in the sample. In addition, the formation of a covalent bond between MAA and FA was proven by the fact that the wavelengths of the aromatic peaks were shorter in poly (MAA–FA) (280 and 305 nm) than in FA (290 and 315 nm).

The emission spectra of free antioxidant and synthesized polymer also confirmed the presence of FA in the polymer. In the spectra of poly(MAA–FA), a hypsochromic shift of the emission peak of FA (from 358 to 334 nm) was detected (Fig. 4). This spectral blueshift was due to the presence of FA covalently bonded to the polymeric chain because no emission peak was detected in the same wavelength range for blank polymer.

The molecular weight distributions of the synthesized polymers were analyzed by GPC, and the results show average molecular weights of 70,000 and 40,000 Da for poly(MAA) and poly(MAA–FA), respectively.

Because the properties of poly(MAA–FA) were derived from FA, it was important to characterize

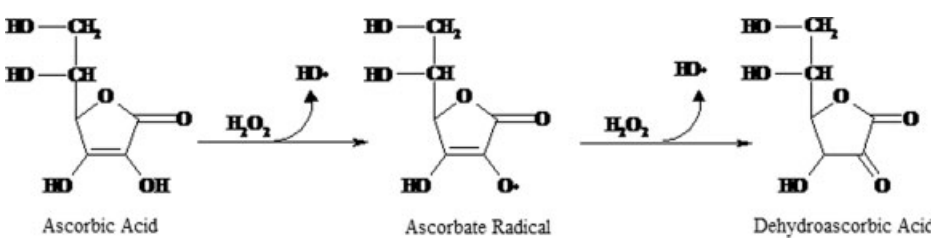

Figure 2 AA/hydrogen reaction mechanism.

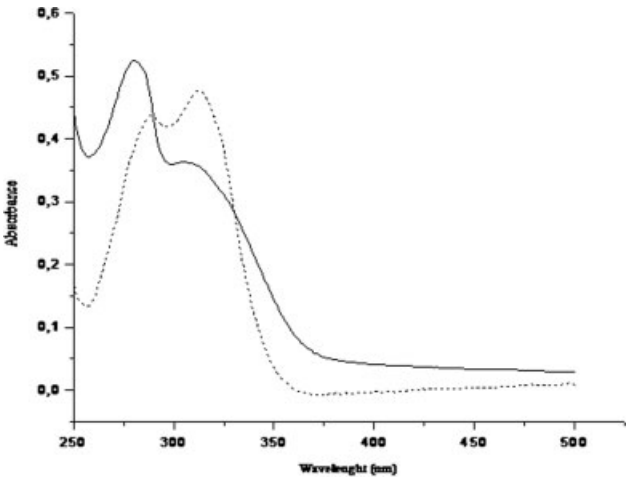

Figure 3 UV absorption spectra of (- - -) FA and (—) poly (MAA–FA).

the synthesized copolymers in terms of phenolic content. The Folin–Ciocalteu phenol reagent was used to obtain a crude estimate of the amount of disposable phenolic groups present in the polymeric chain. Phenolic compounds undergo a complex redox reaction with phosphotungstic and phosphomolybdic acids present in the Folin–Ciocalteu reactant. The color development is due to the transfer of electrons at basic pH to reduce the phosphomolybdic/phosphotungstic acid complexes to form chromogens, in which the metals have a lower valence.

Disposable phenolic groups in the samples were expressed as milligram equivalents of FA, and this value was 3.21 mg/g of dry polymer. Control experiments were also performed with the blank polymer, and no interference was recovered.

Evaluation of the antioxidant properties

·OH groups exhibit very high reactivity and tends to react with a wide range of molecules found in living cells. They can interact with the purine and pyrimidine bases of DNA. They can also abstract hydrogen atoms from biological molecules (e.g., thiol compounds), which leads to the formation of sulfur radicals able to combine with oxygen to generate oxysulfur radicals, a number of which damage biological molecules.[16] Because of the high reactivity, the radicals have a very short biological half-life. Thus, an effective scavenger must be present at a very high concentration or must possess a very high reactivity toward these radicals. Although ·OH formation can occur in several ways, by far, the most important mechanism *in vivo* is the Fenton reaction, where a transition metal is involved as a pro-oxidant in the catalyzed decomposition of $^{-}O_2^{\bullet}$ and H_2O_2. These radicals are intermediary products of cellular respiration, phagocytic outburst, and purine metabolism. ·OH can be generated *in situ* by the decomposition of H_2O_2 by a high-redox-potential EDTA–Fe^{2+} complex, and in the presence of a deoxyribose substrate, it forms thiobarbituric acid reactive substances (TBARS) that can be measured. Antioxidant activity is detected by decreased TBARS formation, which can come about by the donation of hydrogens or electrons from the antioxidant to the radical or by direct reaction with it. Consequently, the ability of poly(MAA–FA) to scavenge ·OH was evaluated by a Fenton-mediated deoxyribose assay.[7]

Good antioxidant properties were found, with inhibition values of 95 ± 2.1% for poly(MAA–FA) and 19 ± 1.7% for poly(MAA).

Evaluation of the antifungal activity

A. niger is less likely to cause human disease than some other *Aspergillus* species, but if large amounts of spores are inhaled, a serious lung disease, aspergillosis, can occur. Aspergillosis is particularly frequent among horticultural workers who inhale peat dust, which can be rich in *Aspergillus* spores. *A. niger* is one of the most common causes of otomycosis (fungal ear infections), which can cause pain, temporary hearing loss, and, in severe cases, damage to the ear canal and tympanic membrane.[19]

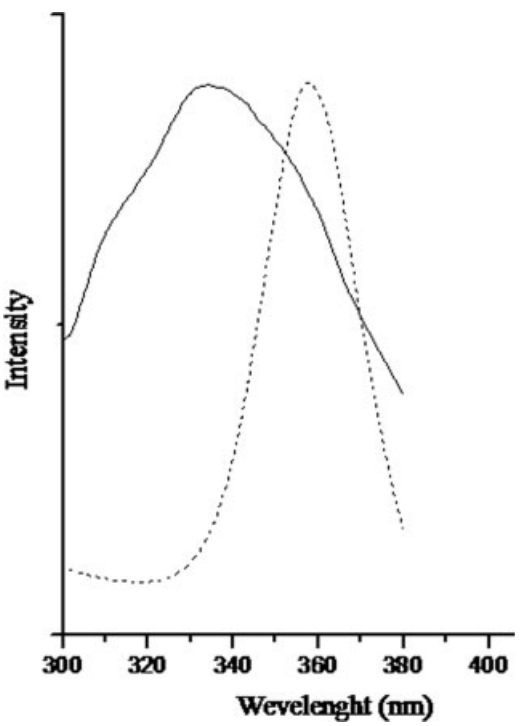

Figure 4 Emission spectra of (- - -) FA and (—) poly (MAA–FA).

Poly(MAA–FA) showed antifungal activity, as it inhibited the mycelial growth of *A. niger* on potato dextrose agar. The growth declined with the increase in polymer concentration, and growth was completely inhibited at a concentration of 10 mg/mL (antifungal index = 97 ± 1.4%). No antifungal activity was found in the poly(MAA)-treated samples.

CONCLUSIONS

FA and MAA were copolymerized by a one-pot free-radical reaction to obtain a material with high antifungal and antioxidant properties. The incorporation of FA into the polymeric chain was confirmed by FTIR and UV analyses. After the determination of disposable phenolic groups, the macromolecular system was tested in terms of scavenging activity toward ·OH and antifungal properties against *A. niger.* These good results, together with the biocompatibility and improved stability of the polymer, clearly shows the utility of the proposed polymeric device in hospital, medical, pharmaceutical, bioprotective, and related hygienic applications. The use of poly (MAA–FA) would make it possible to create good protection against free-radical damage and fungal contamination.

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