Modification of Maleic Anhydride–Styrene Copolymer with Noradrenaline by Chemical and Enzymatic Methods

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ABSTRACT: Maleic anhydride copolymer was modified with another biologically active agent, noradrenaline (NA), using both chemical and enzymatic methods. The modification and synthesized products were named as follows: chemical modification, MASTNAc; enzymatic modification, MASTNAe; enzymatically synthesized MASTNA from individual monomers, MASTNAem. Chemical and enzymatic reactions were performed at 70°C and 38°C, respectively. In the chemical reactions azobisisobutyronitrile was used as the initiator. In the enzymatic reactions, an extracellular extract, including an enzyme with peroxidase-like activity, was used. All the reactions were performed in an organic medium, methyl ethyl ketone. Structural characterization of the copolymer and modified copolymer were carried out by Fourier transform infrared (FTIR) and nuclear magnetic resonance (^{1}H NMR). FTIR and ^{1}H NMR spectra confirmed that NA was successfully covalently bound onto the MAST copolymer backbone by both chemical and enzymatic methods. Surface morphology of the samples was studied by scanning electron microscopy. Results obtained indicated that chemical and enzymatic addition of NA to MAST backbone yielded products having quite similar physical and chemical properties. On the other hand, MASTNA-modified copolymer synthesized by individual monomers appeared to be different in its chemical structure. Furthermore, enzymatic modification and synthesis appeared to provide a good alternative method because it required much milder conditions such as low temperature, and better product qualities: higher solubility in water, higher yield and purity. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 122: 2821–2828, 2011

Key words: maleic anhydride–styrene copolymer; enzymatic polymerization; modification; water-soluble polymers; peroxidase

INTRODUCTION

Maleic anhydride (MA) containing copolymers have often been used as reactive polymers in the preparation of functional polymers to which the active agents consisting of amino or hydroxyl groups (as nucleophiles) can be bound by ring opening reactions.[1] Chemically synthesized maleic anhydride–styrene (MAST) copolymer has also been known to confer antitumor activity. Modification products of MA-containing copolymers have been considered to be versatile materials, promising new applications in many diverse areas of industry.[2] For example, MA copolymers, such as methyl methacrylate or vinyl acetate, show many and various biological activities.[3] In biological systems, these polymer complexes can modify proteinaceous drugs.[4] A covalent binding between amino group of antiseptic agent acriflavine and MA group of MAST has been reported to alter pharmacokinetics of acriflavine.[5] Modification of MAST with hydroxyl-containing compounds,[6] antimicrobial 4-aminobenzoic acid, 4-hydroxybenzoic acid,[7] or pirarubicin[8] can yield products that are readily soluble in water. Solubility in water is of great importance in biological systems. A number of water-soluble copolymers, containing carboxyl functional groups, for example, have found many pharmaceutical applications.[9] Complete hydrolysis of the anhydride groups of copolymers is often accompanied by the formation of strong hydrogen-bonded fragments. Besides, this process also increases the number of carboxylic acid groups available for covalent modification. Antitumor activity of such modified copolymers has been demonstrated to be dependent upon the amount of hydrogen-bonding between carboxyl groups and the nature of their distribution along the side chains. It becomes obvious therefore that the presence of carboxylic acid residues along the macromolecular chain is important both in polymer science and macromolecular engineering.[9]

Noradrenaline (NA) is a catecholamine acting as a hormone and a neurotransmitter in both central and sympathetic nervous systems. As it is made up of a distinct benzene ring with two hydroxyl groups, an intermediate ethyl chain, and a terminal amine group,

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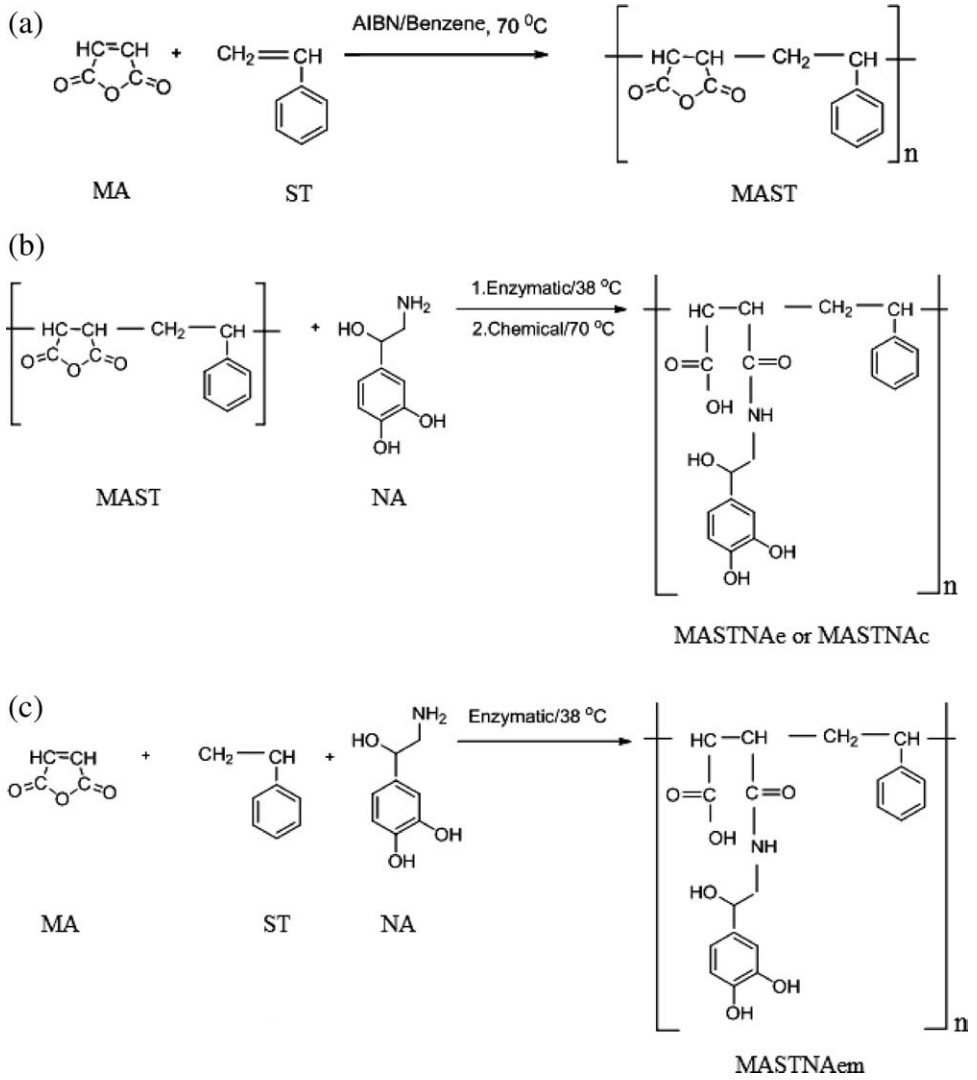

Scheme 1 (a) The polymerization reaction of MAST copolymer, (b) the reaction mechanism recommended for MAST copolymer with NA, and (c) the reaction mechanism recommended for MA and ST monomers with NA.

it has a proteinaceous structure.[10] The presence of amino and phenol groups gives the molecule both amphoteric and polar character. In this study, it was observed that nucleophilic amino end of NA [Scheme 1(b)] was more reactive, and it was evidenced that this group was involved in the modification of MAST copolymer by a ring-opening reaction.

Enzymatic synthesis has made an important progress in recent years.[11,12] Crude or isolated forms of enzymes prepared from biological systems can be used in very specific biotransformation reactions in nonaqueous media.[13] Biocatalytic plastics, for example, can be prepared directly by the copolymerization of vinyl monomers using organic solvent-stable enzymes.[11]

In this study, for the first time MAST copolymer was enzymatically modified by adding NA, and some properties of the resulting modification product, such as yield, color, and solubility in aqueous media, were compared with those of chemically modified MAST copolymer (MASTNAc). Cytotoxicity of these two modification products was also compared by using a cell proliferation assay.

A fermentation product, having peroxidase-like activity, was used for the modification of MAST with NA. Peroxidase enzymes are often used as the source of free radicals in polymerization reactions (peroxidation), and the reactions occur as chain-radical polymerization.[14,15] These chain-radical polymerizations include initiation, propagation, and termination steps.[15,16] Horseradish peroxidase (HRP) and soybean peroxidase (SBP), for example, are the members of typical peroxidases family for the catalysis of free-radical polymerization of vinyl monomers.[12,17–19] Various vinyl monomers have been used in enzymatic polymerization reactions and the main advantages of vinyl polymerization, over chemical polymerization, include mild reaction environments such as much lower temperatures in nonaqueous media, and high reaction specificity to generate functionalized-modified polymers by free-radical polymerization.[13,19,20] The

polymerization behavior often depends on the monomer structure. Peroxidase enzymes prefer to break carbon–carbon double bonds on some of the vinyl monomers such as ST[13] at temperatures very close to the body temperature. In the initiation step, the active center of the radical removes an unpaired electron from the double bond and then behaves as a new active center at the end of the chain. Addition can occur at either end of the monomer. The process of electron transfer and the motion of the active center one monomer along the chain constitute the propagation step which could continue until the supply of monomers is exhausted. Finally, the growth of a polymer chain is halted by the termination reaction.[21] Because NA has both amino and phenolic hydroxyl groups, chemical and enzymatic modification of MAST copolymer with this agent was possible. Enzymatic modification and synthesis appeared to provide better product features because the products could completely dissolve in water and did not exert any toxicity on a mouse fibroblast cell line (L929), which is also the predominant cell type in the human body.

EXPERIMENTAL

Materials

Benzene, MA, methyl ethyl ketone, and styrene (138°C, bp; purified before use), were obtained from Merck (Germany). Ethyl alcohol and petroleum ether were obtained from Smyras (Teknik, Turkey). Azobisisobutyronitrile was obtained from Teaxm (Russia) and was then purified by re-crystallizing twice in chloroform (Merck, Germany). NA was purchased from Sigma (St. Louis, MO).

Source and preparation of the biocatalyst (peroxidase enzyme)

Ten grams of 20-day-old infant feces taken aseptically into sterile plastic containers were homogenized by pipetting in peptone water containing 20% glycerol and were then stored at −80°C in 2 mL cryotubes. One milliliter of the fecal samples was inoculated into 2-L growth medium (pH 6.8) (per liter: 2 g peptone; 2 g yeast extract; 0.1 g NaCl; 0.04 g K_2HPO_4; 0.04 g KH_2PO_4; 0.01 g $MgSO_4.7H_2O$; 0.01 g $CaCl_2{\cdot}2H_2O$; 2 mL Tween 80). Fermentation process was carried out at 37°C in a 5-L fermenter (BioFlo110, New Brunswick, NJ). Incubation was carried on until the culture pH was reached 4.6. Cells were removed by centrifugation (Sigma 6K15, USA). Extracellular proteins in the supernatant were precipitated by the addition of two volumes of 99% ethanol and by incubation at −20°C overnight. The precipitate was then centrifuged at 5000 rpm, and protein pellet was dried at room temperature. Four hundred and fifty micrograms of the pellet were dissolved in 250-μL distilled water and used immediately for each of the polymerization reactions.

Synthesis of mast copolymer

Mast copolymer was synthesized by using the methods as described in Ref. 22.

Chemical modification of mast copolymer with NA

Modification of MAST copolymer with NA was carried out at 70°C, 24 h in methyl ethyl ketone. The calculated molar ratio of the MAST unit and NA was $\sim 1 : 2$.[5,22] After the completion of reaction, the product was precipitated with petroleum ether and decanted. The precipitate was dried under pressure and at room temperature.[23]

Enzymatic modification of mast copolymer with NA agent

Enzymatic modification of MAST copolymer with NA was carried out for 24 h at 38°C, in methyl ethyl ketone. This method contained $\sim$ 0.2 mg/mL final extract concentration, and the calculated molar ratio of MAST:NA was $\sim 1 : 2$.[5,17,22]

Enzymatic MA-ST-NA synthesis from individual MA and ST monomers, and NA agent

Enzymatic synthesis of MAST-NA modified copolymer from the monomers of MA, ST, and NA agent was also carried out for 24 h at 38°C, in methyl ethyl ketone. This method contained $\sim$ 0.2 mg/mL final extracellular extract concentration and the calculated molar ratio of the MA, ST monomers, and NA was $\sim 1 : 1 : 2$, respectively.[5,17,22]

Characterization

MAST and modified MAST-NA copolymer samples were prepared as KBr pellets (2-mg sample in 100-mg KBr) and were then analyzed in a Fourier transform infrared (FTIR) spectrophotometer (Mattson 1000, Unicam, USA) at wavelengths between 400 and 4000 cm^{-1} and at 4 cm^{-1} resolution. For the nuclear magnetic resonance (^1H NMR) spectra analysis, 6 mg of the sample were dissolved in 0.8 mL dimethyl sulfoxide and were analyzed in an NMR spectrometer at 400 MHz (Bruker Avance III, Karlsruhe, Germany). Sample surface morphology was visualized by using scanning electron microscopy (SEM; LEO 440 Mensegi England). The characterization studies were carried out at Technology Research and Developing Centre, Erciyes University, Kayseri, Turkey.

Cytotoxicity

Cultivation of L929 mouse fibroblast cells

A mouse connective tissue fibroblast cell line, L929 (ATCC cell line, NCTC clone 929) was cultured in Dulbecco's minimum Eagle medium (Sigma) supplemented with 10% fetal calf serum (Sigma) and 2 m*M*/mL L-glutamine. No antibiotics were added to the cell culture medium. The cultures were cultivated in an incubator at 37°C and 5% CO_2, until the cell monolayer attained confluence, after ~ 7 days. Assays were always performed in the exponential growth phase of the cells.

Fibroblast cells were selected because they are the predominant tissue type in the body and are easy to cultivate and because of their favorable doubling time of 24 h. Moreover, these cells are recommended by many standard institutions.[24]

Cell proliferation assay

The proliferation assay analyzes the number of viable cells by the cleavage of tetrazolium salts added to the culture medium, using the Cell Proliferation Reagent WST-1 (Cayman, MI).[25] During the assay, tetrazolium salts are cleaved to formazan by cellular enzymes. An expansion in the number of viable cells results in an increase in the overall activity of mitochondrial dehydrogenases in the sample. This augmentation in enzyme activity leads to an increase in the amount of formazan dye formed, which correlates directly with the number of metabolically active cells in the culture.[25] The formazan dye produced by the metabolically active cells was quantified by a scanning multiwell spectrophotometer by measuring the absorbance of the dye solution at 450 nm. Cells were seeded in 96-well microtiter plates at a concentration of 1×10^5 cells/mL in a final volume of 100 μL per well. For the cytotoxicity of the modified products 500, 250, 125, 62.5, 31.2, and 15.6 μg/mL concentrations were used. Cells were then incubated for 24 h with both enzymatic and chemically modified copolymers in a humidified atmosphere (37°C, 5% CO_2). After the incubation period, 10 μL of the Cell Proliferation Reagent WST-1 were added to 10 μL of culture medium in each well, and the absorbance of the samples was measured at 450 nm against the control (the same cells without any treatment) using a microtiter plate reader (Thermo Scientific Microplate Photometer, Multiskan, FC). The same volume of culture medium and Cell Proliferation Reagent WST-1 (10 μL of Cell Proliferation Reagent WST-1/100 μL of culture medium) was added to one well to use as a background control (absorbance of culture medium plus WST-1 in the absence of cells) as a blank position for the ELISA reader. The absorbance was measured after 2 h from the start of the tetrazolium reaction. The experiments were conducted in quadruple. The optical density (OD) of the samples was compared with that of the negative control to obtain the percentage viability, as follows: cell viability (%) = [(OD450 (sample)/OD450 (negative control)) × 100].

The results, expressed as mean ± SD from four replicates, were analyzed statistically by using one-way analysis of variance at 95% confidence levels for multiple comparisons and student's *t*-test for two-group comparisons.

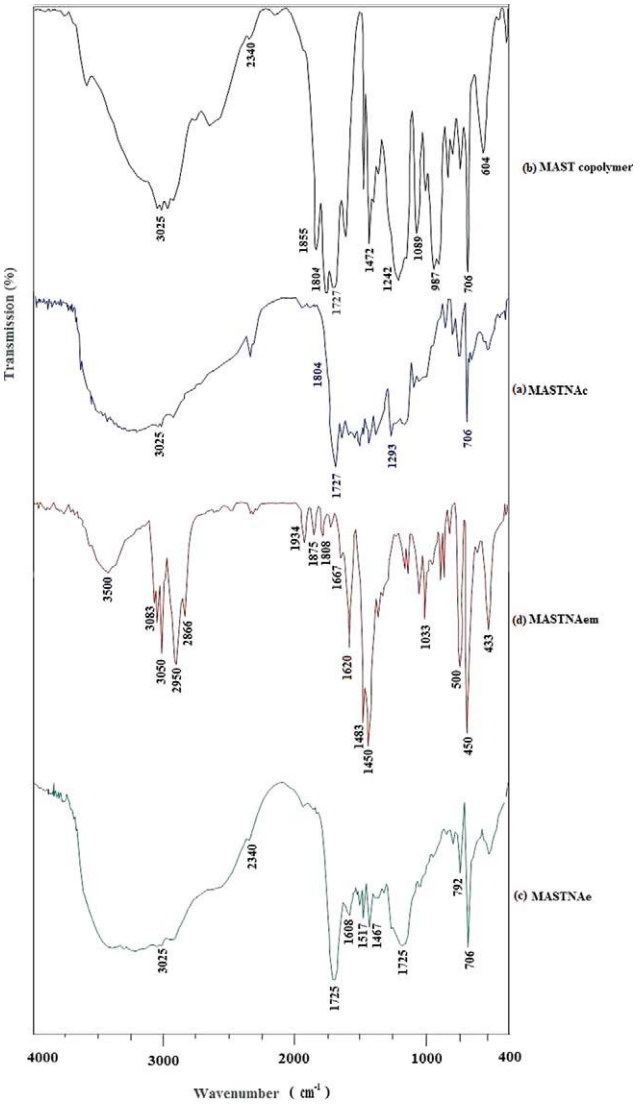

Figure 1 FTIR spectra of (a) MASTNAc, (b) MAST copolymer, (c) MASTNAe, and (d) MASTNAem. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

RESULTS AND DISCUSSION

FTIR analysis

MAST copolymer had anhydride units at 1780 cm^{-1} and 1855 cm^{-1}, as expected.[9,23,26] In MASTNA samples, this peak was present with much reduced intensity or disappeared completely [Fig. 1(a,d), respectively]. Complete disappearance of this characteristic anhydride

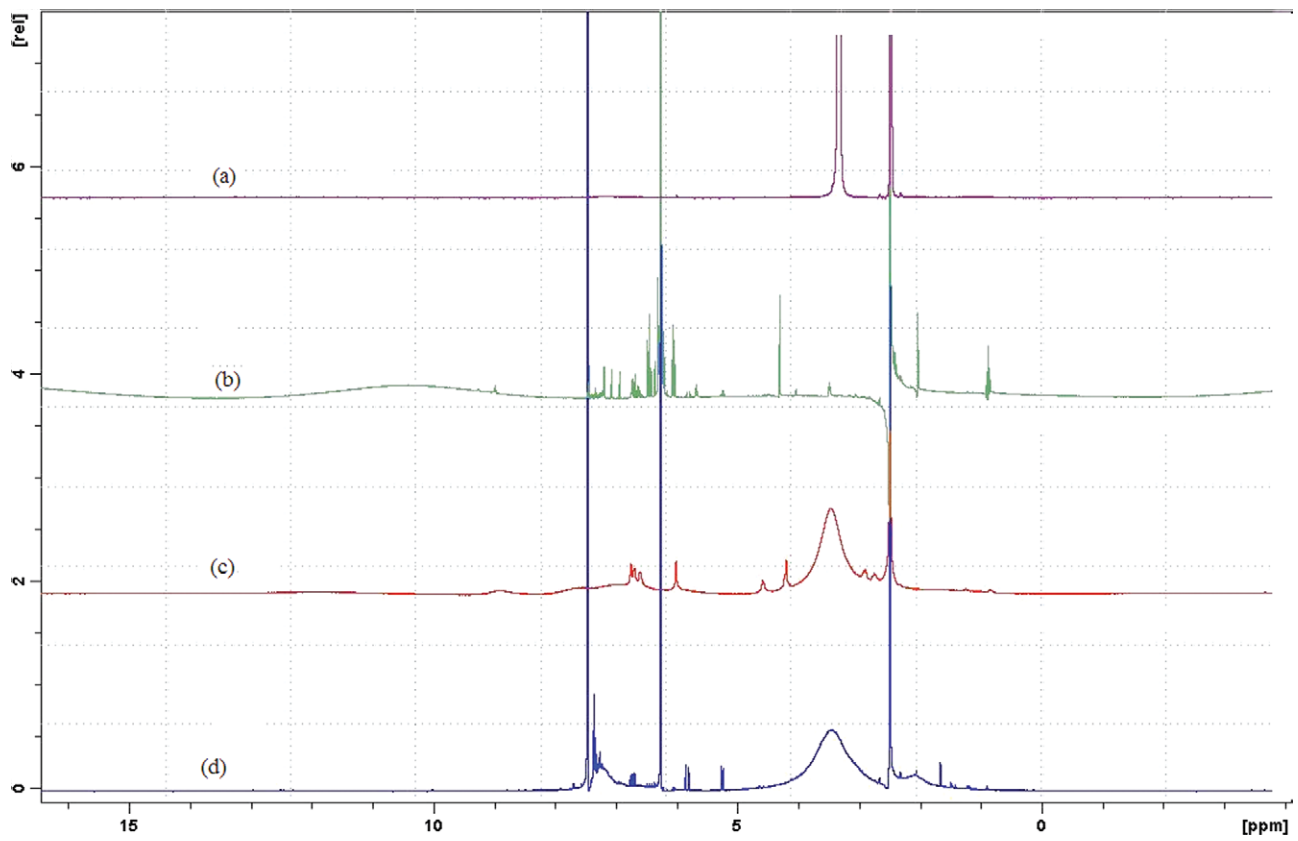

Figure 2 NMR spectra of (a) MASTNAc, (b) MASTNAem, (c) MASTNAe, and (d) MAST copolymer. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

peak in the enzymatic modified copolymer might indicate that almost all of the anhydride rings were opened by NA, because of its nucleophilic amino group.[7] The ring-opening reaction generally resulted in the formation of a carboxylic group and amide or ester structure.[7] In the IR spectra, however, the carbonyl stretching within —COOR at 1500–1600 cm^{-1}, and —NH stretching of NH_2 group at 3000–3700 cm^{-1} were not observed in either of the modified copolymers [Fig. 1(a,c,d)]. The —NH stretching of —CONHR mono-substituted amide group peak was observed in both of the enzymatically modified and synthesized products [MASTNAe and MSTNAem; Fig. 1(c,d)] at 1715 cm^{-1}. The intensity of the absorption bands due to the C=O of the anhydride groups, at C—O—C:1855 cm^{-1} and C=O:1780 cm^{-1}, decreased after modification, and the band position was shifted to lower wave-numbers, 1718 cm^{-1} [Fig. 1(c,d)] and 1651 cm^{-1} [Fig. 1(d)] due to the amide and free acid formation.[26] In addition, the peaks at 1855 cm^{-1} and 1780 cm^{-1} were also disappeared while the peak intensity of acid units at 3500 cm^{-1} increased[9] [Fig. 1(d)]. These findings indicated that an amidization reaction took place in the MASTNAem reaction. Another evidence for the resulting amide structure was the presence of a group of peaks indicating an amide C=O stretching at 1651 cm^{-1}, an —NH bending 1554 cm^{-1},[6] and a —CN stretching at 1551 cm^{-1} (C—N—H),[23] again related to —CONHR mono-substituted amide group [Fig. 1(d)]. In the MASTNAc sample, carbonyl (C=O) group peaks were observed at 1780 cm^{-1} [Fig. 1(a)], and this can be accounted for by the partial modification of MAST.[27] The MASTNAem sample yielded the most complicated FITR result by having various wave-numbers: at 410 cm^{-1}, 615–640 cm^{-1} (double band), 790 cm^{-1}, 872 cm^{-1}, 928 cm^{-1}, 1271 cm^{-1}, 1890 cm^{-1}, 2039 cm^{-1}, 2169 cm^{-1}, and 2684 cm^{-1} [Fig. 1(d)]. Thus, the interpretation of chemical structure of this product deserves another study.

NMR analysis

^1H NMR spectroscopy confirmed the FTIR results. In MAST copolymer, two methine protons on furan unit at 6.3 ppm[28] and aromatic hydrogen of benzene ring of styrene at 6.7–7.4 ppm[29,30] were obtained [Fig. 2(d)]. The peaks between 7.2 and 7.4 ppm belonged to aromatic ring hydrogen of styrene,[4,6] indicating a proton signal of the styrene residue [Fig. 2(a,d)]. These aromatic hydrogen peaks indicated a partially modified MAST copolymer. ^1H NMR spectra of enzymatic modified copolymers included a carboxylic acid and an —NH peak approximately at 12 ppm and 9 ppm that were taken as the indications of an amide structure [Fig. 2(b,c)],

because there was no indication for an amine proton in the spectra.[4] Ester groups expected to appear at 4.1 ppm[26] were not observed in any of the modified copolymers [Fig. 2(a–c)].

SEM images of the MASTNA-modified copolymers

SEM analyses indicated that MAST copolymer [Fig. 3(a)], and MASTNAe and MASTNAem [Fig. 3(b,c)] had both characteristic and uniform surface structures. Since the chemical synthesis of MASTNA produced the lowest amount of modified copolymer, there was not enough material to do the SEM analysis. The surface of MAST copolymer seemed to have more cavities [Fig. 3(a)], indicating a relatively lower particle density, than MASTNAe [Fig. 3(b)]. The spacing between the chains of MASTNAem was much shorter than that of MASTNAe. Furthermore, individual polymer particles with rectangular appearance, $\sim$ 20 μm in length, could be discerned in the MASTNAem image [Fig. 3(c)].

Physical and chemical properties of the products

The physical and chemical properties of the produced MAST copolymer and its amide derivatives were significantly different (Table I). As can bee seen, these differences included powder color, solubility in water, and yield and purity of the products. For example, MAST copolymer was white, MASTNAc was dark brown, MASTNAe was light brown, and MASTNAem was yellow. Yellow and light brown colors indicated that the reactions reached completion, using up almost all the anhydride groups, and the brown color implied that there were significant amounts of unreacted anhydride groups in MASTNAc. This color difference could also be correlated with the solubility patterns of the materials: MAST (white) being insoluble, MASTNAc (dark brown) partially soluble (+, Table I), MASTNAe (light brown) being more soluble (++) than the dark brown product, and MASTNAem (yellow) completely soluble (+++) in water. Furthermore, yields of the reactions appeared to increase in the same order (13, 44, and 91%, respectively) (Table I). Water solubility is a very important feature of biologically active copolymers and high solubility often promises higher activities in biological systems by means of, for example, high numbers of carboxylic groups available for hydrogen bonding.[3,16] FTIR and ^{1}H NMR results demonstrated that MAST copolymer contained a benzene ring in each repeating unit, coming from ST monomers. The access of NA to the polymer backbone could be restricted by this benzene ring, which might impose steric hindrance, and lead to partial modification of MAST.

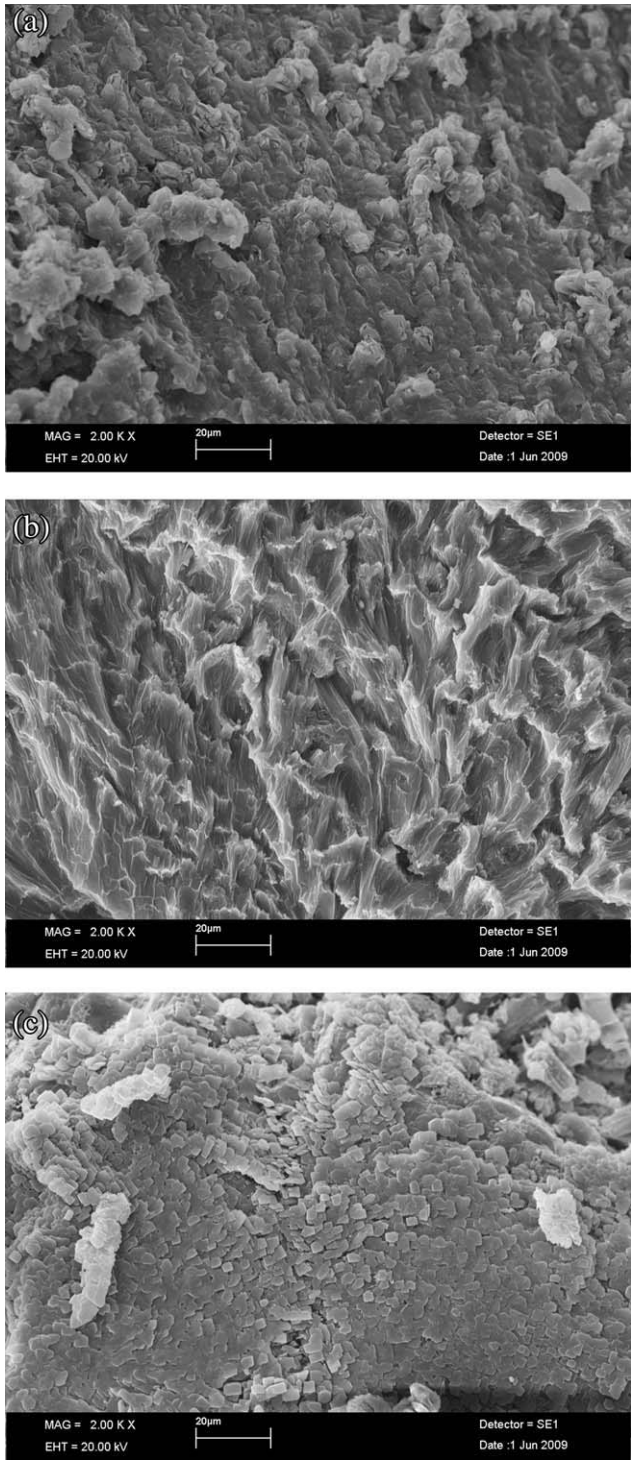

Figure 3 SEM images of (a) MAST copolymer, (b) MASTNAe, and (c) MASTNAem.

Higher cytotoxicity values recorded for the MASTNAc sample could result from the unreacted anhydride rings, and these appeared to be in correlation with the less solubility of MASTNAc in water. On the other hand, during the enzymatic modification reactions nearly all of the anhydride rings seemed to have reacted with NA, rendering MASTNAe to be almost completely soluble in water and nontoxic.

TABLE I
Reaction Conditions and Yield, and Some of the Physical and Chemical Properties of Products

Polymer code	Total weight of reactants (mg)	Extracellular extract (μg)	Temp. (°C)	Time (h)	Yield (mg)	Yield (%)	Color	Solubility in water
MASTNAc	90	–	70	24	12	13	Dark brown	+
MASTNAe	90	450	38	24	40	44	Light brown	++
MASTNAem	4040	400	38	24	3700	91	Yellow	+++

A mechanism suggested for the modification reaction

Nucleophilic amino end of catecholamines is the main functional group posing a target for another chemical agent.[10] NA contains this nucleophilic amino group as well as a phenol group. Over these two functional groups, either an ester[6] or an amide reaction[23] would be possible. A close inspection of the FTIR results demonstrated that the reaction product had an amide structure [Scheme 1(b,c)]. This finding was also supported by a decrease in the peak intensity at 1855 cm^{-1} in the FTIR spectra [Fig. 1(c,d)]. This finding was also in agreement with a modified copolymer structure suggested in a similar study.[23]

Three reaction mechanisms for the formation of the products (MAST copolymer, MASTNAc and MASTNAe, and MASTNAem) were proposed below, respectively.

From the above formulae, it can be calculated that molecular weight of the repeated units of MAST copolymer and MASTNA is 202 g mol^{-1} and 371 g mol^{-1} respectively.

Cell proliferation assay

Cytotoxicity of MASTNAc, MASTNAe, and MASTNAem were tested for 24 h by quantitative analysis using WST-1 test (Fig. 4). As can be seen MASTNAe had no negative effect on cell viability even at the highest concentration (500 μg/mL) used. The cytotoxicity values of MASTNAe were compared with those of other modification products, and control culture, and found to be statistically significant ($P < 0.05$).

CONCLUSIONS

It was shown for the first time that MAST copolymer could be modified with NA by both chemical and enzymatic methods. The most important finding was that NA could open almost all of the MA rings during the formation of MASTNAe. It should also be emphasized that the enzymatic modification and synthesis products (MASTNAe and MASTNAem) appeared to have attained much better physical and physiological properties such as solubility in water, higher yield and purity, and almost no toxicity.

Cytotoxicity measurements are often taken as the first step before identifying a compound as the drug candidate. Therefore, all of the products obtained were tested for their cytotoxicity by using a fibroblast cell line. Because MASTNAe showed almost no toxicity, even at 500 μg/mL concentration, it was believed that this modification product deserves to be investigated further to assess its antitumor, antimicrobial, antifungal, antiamibisidal, or angeonese inhibition effect, and by *in vivo* systems such as animal models.

The enzyme preparation used in this work was dissolved in a small volume of water (50 μL) and was then added into organic reaction medium (1 mL) in which the substrate materials were soluble. In the reaction mixture, it could be seen by the naked eye that water sample containing the enzyme was dispersed in the organic medium as spherical water particles. The enzyme molecules most probably exerted their catalytic activity on the surfaces of these aqueous vesicles. Thus, it might be possible to replace these water vesicles with an immobilizing agent, having a nonporous structure, for example,

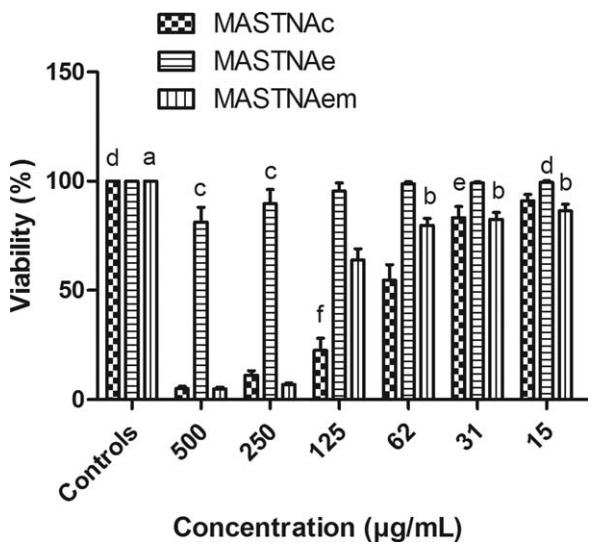

Figure 4 Effect of MASTNAc, MASTNAE, and MASTNAem on viability of L929 cell. Data were presented as mean ± SD. [a]$P < 0.05$ versus all concentrations; [b]$P < 0.05$ versus 500, 250, and 125 μg/mL; [c]$P < 0.05$ versus125, 62, 31, and 15 μg/mL; [d]$P < 0.05$ versus 500, 250, 125, 62, and 31 μg/mL; [e]$P < 0.05$ versus 500, 250, 125, and 62 μg/mL; [f]$P < 0.05$ versus 500 and 250 μg/mL.

and with much smaller radius, providing a base for the catalytic activity through its surface.[31] Such immobilization efforts could enable the recovery and reuse of the catalyst. Immobilization techniques have also been used to maximize product yield or to optimize reaction conditions.[31] Within the limits of this work, however, immobilization studies could not be performed. In the future studies, this technology will also be exploited.

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