Investigation of Cytotoxic Effects of New Maleic Anhydride Binary and Ternary Copolymers on L929 Mouse Fibroblasts

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ABSTRACT: The purpose of this study was to investigate the cytotoxic effects of poly(MA-*alt*-AA) and poly[MA-*co*-AA-*co*-(VA)] in cell culture model. Complex-radical copolymerization (CTC) of maleic anhydride (MA), and acrylic acid (AA) and ternary polymerization of (MA), vinyl acetate (VA) and AA, considered as acceptor−donor−acceptor systems, were carried out. For the cytotoxicity study, cells were incubated at initial density of 50,000 cells/mL in 24-well plates. After 24-h incubation, the culture medium was removed and fresh medium containing five different dilutions of polymers were added separately. Cell viability and proliferation were assessed on 1st and 5th days. Apoptosis was assessed using propidium iodide/acridine orange (PI/AO) staining at the 1st and 5th days of incubation. Kruskal-Wallis test was applied to compare the effects of different dilutions on cell viability at each evaluation period. The results showed that there were differences between *co*- and terpolymer in view of cell proliferation and apoptotic response. The results suggest that functional groups in *co*- and terpolymer group may cause apoptotic cell death without effecting cell proliferation. © 2009 Wiley Periodicals, Inc. J Appl Polym Sci 115: 1366–1370, 2010

Key words: poly(MA-*alt*-AA); poly(MA-*co*-AA-*co*-(VA) (L929; cell proliferation; hydrophilic polymer; biological applications of polymers; apoptosis; propidium iodide/acridine orange

INTRODUCTION

Functional polymers have gained much attention over the past four decades as more and more polymers find applications beyond their traditional use in commodity. Although there are only few significant samples of functional polymers, the range of functional polymers available today is enormous: from very simple structures obtained in single step to polymers with complex architectures prepared through multi-step syntheses.[1] This diversity gives a highly versatile and diverse group of macromolecules, many of them which have been applied specifically in the wide range of arena for example, solubilizing agents, nanoparticulate formation, surface modification, macromolecular drug carriers, diagnostic imaging agents, and implants. In addition, the majority of these polymers show a multitude of biological activities, as well as inhibition of efflux pumps such as P-glycoprotein.[2]

Functional groups, properly located on a polymer as well as its structure, are usually responsible for its biological activity, biocompatibility and/or biodegradability and may impart on it either therapeutic or/and toxic character. According to the goal, appropriate functional group and ligand can be designed. For example, carboxylic groups induce therapeutic activity of many drugs, and so novel biologically active anhydride containing polymers is expected to show considerably high biological activities because the anionic character of the polymers formed after their hydrolysis.[3]

Several synthetic polyanions have become of interest to investigators for comparison of their physiological properties with those of naturally occurring polyanions. Synthetic polycarboxylic acid polymers were found to produce a broad spectrum of immunological effects. The synthetic carboxylic acid polymers first investigated were poly(acrylic acid), poly(methacrylic acid), poly(ethylene-*co*-maleic anhydride), and oxidized polysaccharides. Subsequently, the copolymer of divinyl ether and maleic anhydride (MA) has shown significant biological activity.[4,5] Among these polymers hydrolyzed form of the divinyl ether MA copolymer (DIVEMA), which contains carboxylic acid groups, exhibits high antitumor activity together with toxic side effects.[6–8] Recently, antitumor activity of prepared anion-active copolymers hydrolyzed poly(MA-*alt*-AA), poly[MA-*co*-AA-*co*-(VA)][9]

and poly(DHP-*alt*-MA) and poly(DHP-*co*-MA-*co*-VA)[10] were studied using methyl-thiazol-tetrazolium (MTT) colorimetric assay and LD_{50} dose of each copolymer and terpolymer were calculated. They have sufficiently high antitumor activity, which depends on amount of hydrogen bonding carboxylic groups and their regular distribution in side chain of functional macromolecules.

In the present study, poly(MA-*alt*-acrylic acid) and poly(MA-*co*-vinyl acetate-*co*-acrylic acid) were prepared by the ternary and binary copolymerization of the corresponding monomers. The structural properties of these polymers were identified by FTIR, DSC, and TGA analyses. An *in vitro* model of L929 mouse fibroblasts was used to investigate the effects of new co- and terpolymer on cell viability and apoptosis.

MATERIALS AND METHODS

Preparation of polymers

Copolymerization of MA (Fluka, Germany) with acrylic acid (AA), (Fluka, Germany) as well as terpolymerization of vinyl acetate (VA) (Fluka, Germany), MA, and AA was carried out in similar conditions, in *p*-dioxane in the presence of benzoyl peroxide (BPO) (Fluka, Germany) (0.1 %) as an initiator at 70°C under a nitrogen atmosphere. The poly[MA-*alt*-acrylic acid] and poly[MA-*co*-acrylic acid-*co*-(vinyl acetate)] were isolated from the reaction mixture by repreccipitation with anhydrous methanol and *n*-hexane, respectively. Copolymers were purified by twice repreccipitating from *p*-dioxane solution with *n*-hexane and by washing with several portions of hexane, benzene, and diethyl ether, and were dried in vacuum at 50°C to a constant weight with almost quantitative yields . (~75%). Terpolymers were purified by several repreccipitating from anhydrous acetone solution with *n*-hexane and by washing with *n*-hexane and benzene, and were dried in vacuum at 50°C to a constant weight with quantitative yields . (~95%).[9] The used reagents including organic solvents were purified by ordinary methods.

Characterization anhydride containing *co*- and terpolymer

Fourier transform infrared (FTIR) spectra of copolymer films or thin coatings on KBr pellet were recorded with FTIR Nicolet 510 spectrometer in the 4000–400 cm^{-1} range, where 30 scans were taken at 4 cm^{-1} resolution. The acid number (AN) of the anhydride-containing polymer samples were determined by known non-aqueous titration method. Intrinsic viscosities of synthesized polymers were determined in *p*-dioxane at 25 ± 0.1°C in the concentration range of 0.1–1.0 dLg^{-1} using an Ubbelohde viscometer. Differential scanning calorimetric (DSC)

and thermogravimetric (TGA) analyses of polymers were carried out with a DuPont V4.1C 2000 and DuPont TA 2000 in nitrogen atmosphere at a heating rate of 5°C/min. The copolymer and terpolymer synthesized by the use of 1 : 1 and 1 : 2 : 1 molar ratio of initial monomers, respectively, had following characteristics.[11]

Poly[maleic anhydride-alt-acrylic acid]
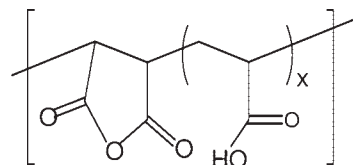
where $x = 1.12$ (AA unit = 52.83), yield 75%, glass transition temperature T_g 111°C and T_m 153°C (by DSC analysis), intrinsic viscosity $[\eta]_{in}$ in *p*-dioxane at 25°C 1.25 dL g^{-1}, acid number (AN)= 878 mg KOH/g, monomer unit ratio in copolymer $(m_1 : m_2) = 1 : 1.12$.

FTIR spectra (film), cm^{-1} : νOH 3060 (broad, in ─COOH), νCH$_3$ 2950 (as) and 2880 (s), νCH$_2$ 2930 (as) and 2870 (s), νCOOH 2545 (broad), νC=O 1836 (as) and 1766 (s) (C=O in anhydride unit), νC=O 1730 (C=O in ester group), νC=O 1585 (as) (in COO$^-$), δCH$_2$ 1478 and 1443 (doublet), δCH$_3$ 1385 and 1357 (doublet), δC─O 1240–1170 (ester and carboxyl), νC─O─C 1035 (in anhydride unit), δOH 943 (out-of-plane OH bending), δCH 886 and 871 (doublet), δCH$_3$ 842 (rock), δCH$_2$ 720 (rock), δCH 645 (in main chain from anhydride unit), and δO─C=O 560 (s) (bend of COOH); 645 (in main chain from anhydride unit), and δO─C=O, 578 (s) (bend of ester group). In the FTIR spectra of hydrolyzed copolymers, there are disappeared characteristic bands for anhydride units, and appeared new bands in field of 1970, 1585, and 1630 cm^{-1} relating to ─COOH groups, as well as increased in intensity of 3060 and 2545 cm^{-1} broad bands.

Poly[maleic anhydride-co-acrylic acid-co-(vinyl acetate)]
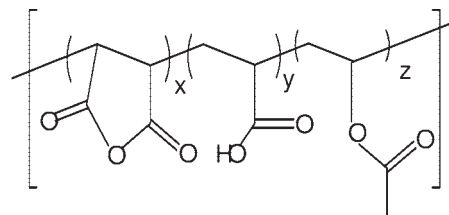
where x, y,z (MA unit = 50.02, AA unit = 20.97, VA unit =30.34) yield 95%, glass transition temperature T_g 186.5°C and T_m 201°C (by DSC analysis), intrinsic viscosity $[\eta]_{in}$ in *p*-dioxane at 25°C 1.36 dL g^{-1}, acid number (AN) = 710 mg KOH/g, monomer unit ratio in copolymer $(m_1 : m_2 : m_3) = 1 : 2 : 1$.

FTIR spectra (film), cm^{-1}: νOH 3060 (broad, in —COOH), νCH$_3$ 2950 (as) and 2880 (s), νCH$_2$ 2930 (as) and 2870 (s), νCOOH 2545 (broad), νC=O 1836 (as) and 1766 (s) (C=O in anhydride unit), νC=O 1730 (C=O in ester group), νC=O 1585 (as) (in COO$^-$), δCH$_2$ 1478 and 1443 (doublet), δCH$_3$ 1385 and 1357 (doublet), δC—O 1240–1170 (ester and carboxyl), νC—O—C 1035 (in anhydride unit), δOH 943 (out-of-plane OH bending), δCH 886 and 871 (doublet), δCH$_3$ 842 (rock), δCH$_2$ 720 (rock), δCH 645 (in main chain from anhydride unit), and δO—C=O 560 (s) (bend of COOH).[11]

Assessment of cell proliferation

Poly(MA-*alt*-AA) and poly[MA-*co*-AA-*co*-(VA)] were prepared in five different dilutions (Dilution 1: 0.00114 g/mL; Dilution 2: 0.00057 g/mL; Dilution 3: 0.00028 g/mL; Dilution 4: 0.00014 g/mL; Dilution 5: 0.00007 g/mL). L929 fibroblastic cell line was plated in 24-well culture plates at an initial density of 50,000 cells/mL and incubated in Dulbecco's Modified Eagle Medium/Ham's F12 (DMEM/F12) (Biochrom AG, Germany) supplemented with 10% fetal bovine serum (FBS) (Biochrom AG, Germany). After a 24 h of incubation, the culture medium was removed and fresh medium containing poly(MA-*alt*-AA) and poly[MA-*co*-AA-*co*-(VA)] was added. Untreated cells served as controls. The cultures were maintained at 37°C in a humidified atmosphere of 5% CO$_2$ and 95% O$_2$. To measure cell proliferation, dye exclusion method was used. At the end of each period (1st and 5th days), the culture medium was removed, and the cells were collected from the surface of the culture plates with trypsin-EDTA solution (Biochrom AG, Germany). L929 cells were counted with trypan blue and counted under an inverted microscope (Olympus IX70, Japan). Each experiment was repeated six times for each test material and the control group.

Assesment of apoptosis

Propidium iodide/acridine orange (PI/AO) (Sigma Chemical Co., St. Louis, MO) staining was used to assess apoptosis of treated cells and of the control. At each evaluation period, the cell culture medium was removed and the cells were washed briefly in sterile phosphate buffered saline (PBS) (Sigma Chemical Co., St. Louis, MO). Approximately 25 μg/mL AO and 25 μg/mL PI were mixed at a v : v ratio of 1 : 1 and added to the cells for 20 s. Thereafter, cells were washed in PBS for 10 s and mounted in PBS : glycerol (v/v, 1 : 1). The cells were visualized by fluorescence microscopy (Olympus IX70, Japan). Apoptotic cells were evaluated by counting red cells with fragmented nuclei. AO-stained cells were

observed under a FITC filter (520–560 nm) in green color, and PI-stained cells were observed under rhodamine filter (510–560 nm) as stained red. Cells were visualized and photographed under the fluorescence microscope at 40x (BH2-RFL-T3 Model fluorescence attachment, Olympus, Japan).

Statistical analysis

For each dilution, statistical analysis of cell proliferation within time was performed using Kruskal Wallis Test. Kruskal Wallis test was applied to compare the effect of different dilutions of poly(MA-*alt*-AA) and poly[MA-*co*-AA-*co*-(VA)] on cell survival at each evaluation day. The comparison was made between control and dilution 1 (0.00114 g/mL), dilution 2 (0.00057 g/mL), dilution 3 (0.00028 g/mL), dilution 4 (0.00014 g/mL), dilution 5 (0.00007 g/mL) of the poly(MA-*alt*-AA) and poly[MA-*co*-AA-*co*-(VA)] samples.

RESULTS

Assessment of cell proliferation

For each dilution tested, cell proliferation with respect to the evaluation periods is presented in Figures 1 and 2. Cell numbers at day 1 (Fig. 1) and 5 (Fig. 2) were lower in poly(MA-*alt*-AA) group than poly[MA-*co*-AA-*co*-(VA)] for all dilutions. At day 1 and 5, pairwise comparisons of cell proliferation between all groups showed significant difference ($P < 0.05$).

Assessment of apoptosis

Apoptotic response was observed with morphological changes of fibroblasts. Cells displayed different patterns of apoptotic response in *co*- and terpolymer group. Starting from the first day of incubation, cells
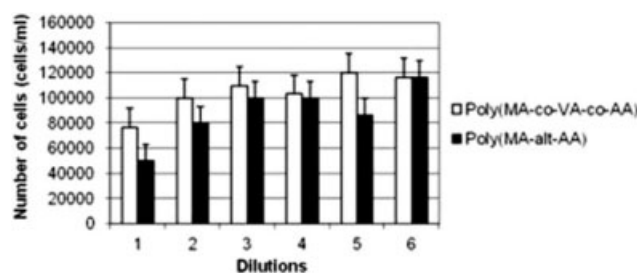

Figure 1 Cell proliferation of L929 mouse fibroblasts for five different dilutions of test materials poly[MA-co-AA-co-(VA)] and poly(MA-*alt*-AA) and the control group at the first day of incubation 1: Dilution 1 (0.00114 g/mL); 2: Dilution 2 (0.00057 g/mL); 3: Dilution 3 (0.00028 g/mL); 4: Dilution 4 (0.00014 g/mL); 5: Dilution 5 (0.00007 g/mL); and 6: Control.
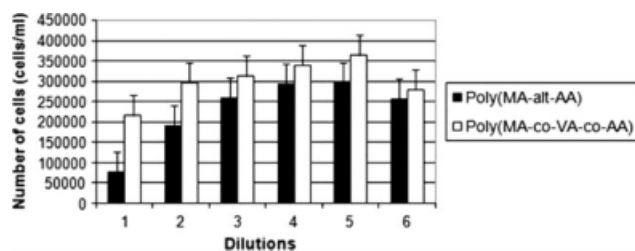
Figure 2 Cell proliferation of L929 mouse fibroblasts for five different dilutions of test materials poly[MA-*co*-AA-*co*-(VA)] and poly(MA-*alt*-AA) and the control group at the fifth day of incubation.

in all dilution groups demonstrated a rapid apoptotic response, as evidenced by yellow, orange, and red-staining, round cell morphology and fragmented nucleus in poly(MA-*alt*-AA) group and poly[MA-*co*-AA-*co*-(VA)]. In all dilution groups, healthy and normal cells stained green and had normal fibroblastic morphology. However, apoptotic cells displayed in the poly[MA-*co*-AA-*co*-(VA)] group were less than the copolymer group in the same dilution and day. Apoptotic and healthy cells were shown in the first dilution group at day 1 in Figure 3(a–c).

DISCUSSION

The evaluation of cytotoxic and cytostatic effects of a chemical compound is very important in order to determine the first risk assessment when working new biomaterials.[2] Most current tests include the level of cell viability assays after exposure to a chemical compound or a biomaterial. Many cell culture techniques are used to assess cell damage caused by biomaterials. These methods are based on cell cultures with established or diploid cell lines and primary tissue explants techniques. In the present study, an *in vitro* model of L929 mouse fibroblasts placed in direct contact with the anhydride containing water-soluble copolymers was used. This cell line is well established and has been commonly used for cytotoxicity evaluation of biomaterials.

Application of conventional chemotherapeutic drugs is often hampered by their lack of cell selectivity, resulting in undesired side effects.[3] One approach to overcome this limitation is to use alternative anti tumor drugs.[4,12,13] Drug delivery systems such as liposomes and micelles, water soluble polymer-drug conjugates, biodegradable gels and
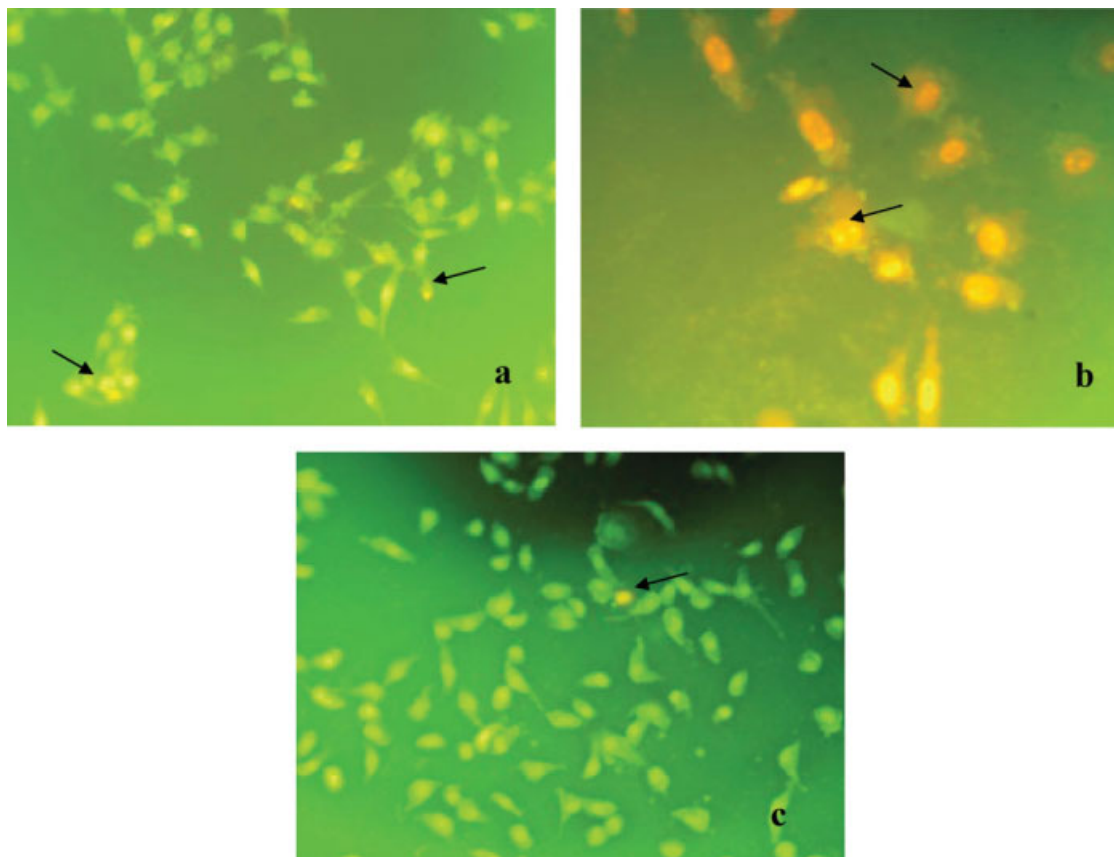

Figure 3 Cell morpology at day 1 in dilution 1 of the test materials. a. Poly[MA-co-AA-co-(VA)], b. Poly(MA-*alt*-AA), c. Control (X40) (black arrows indicates apoptotic cells). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

microparticles, and drug formulations can be thought as alternative systems.[14]

Recently, water-soluble polymeric materials have had an increasing importance. Water-soluble polycations such as poly(ethylenimine) (PEI), poly-(dially1-dimethyl-amonium chloride) (DADMAC), and polyanions may be used in various biomedical applications.[15–17] In biomedical applications, they are under investigation as micro- and nanoparticulate drug carrier systems for proteins and peptides as well as transfer of DNA or RNA into the cells.[18–20]

Polymer-drug conjugates are also an alternative to therapy of tumorogenic tissues. These conjugates are mono-sized hybrid constructs that covalently combine a bioactive agent with a polymer to ensure not only its efficient delivery to the required intracellular compartment but also its availability within a specific period of time.[21] It has already been demonstrated that the polymer-drug conjugation promotes tumor targeting.[22] Therefore, these conjugates have the potential to improve the therapy of drug-resistance tumors by reducing undesired side effects.[23] Duncan et al.,[24] Satchi-Fainaro[25] have been used polymer-drug conjugates as intravenously as anticancer agents.

In the present study, water-soluble anhydride containing poly(MA-*alt*-AA) and poly[MA-*co*-AA-*co*-(VA)] were prepared as a potential anticancer drug and functional groups of these polymers have biological activity on tumor cells in copolymer samples.[9,10] The hydrolysis mechanism of MA copolymers have great importance due to the close connection with to form carboxylic acid or anion, depending on reaction medium.[17]

The observed apoptotic response from this study can be expressed polyanionic character of the *co*- and ternary polymers. The carboxylic and anionic character of poly[MA-*co*-AA-*co*-(VA)] depicts the low cytotoxicity behavior. Also, VA fragments in the terpolymer gives the immobility to the polymer chains, and the solubility and forming of carboxylic character are lowered. On the other hand, cells showed apoptotic response in dilution 1 starting from the first day of incubation. This finding is also important to determine the critical dose of the polymers. However, further experiments need to be performed in order to establish and enlighten the route of apoptotic mechanism. Consequently, this study can be the initial and basic step for testing apoptotic effects of poly(MA-*alt*-AA) and poly[MA-*co*-AA-*co*-(VA)] polymers.

CONCLUSION

In the present study, poly(MA-*alt*-acrylic acid) and poly(MA-*co*-vinyl acetate-*co*-acrylic acid) were prepared by the ternary and binary copolymerization of the corresponding monomers by charge-transfer complex (CTC) polymerization. Used poly(MA-*co*-AA) and poly[MA-*co*-AA-*co*-(VA)] were prepared in five different dilutions. Proliferation and apoptotic response of the cells were evaluated at the 1st and 5th days of incubation at five different dilutions of polymers. The results suggest that functional groups in water-soluble polymer groups may cause apoptotic cell death without effecting cell proliferation. For clarifying the apoptotic cell death and the route of apoptotic mechanism; further experiments will be our motivation to realize upcoming studies.

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