Amoebicidal Effect of Poly (Maleic Anhydride-Co-Vinyl Acetate) Copolymer on Entamoeba Histolytica Trophozoites

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Abstract

Poly (maleic anhydride-co-vinyl acetate) (MAVA) copolymer was synthesized by free-radical copolymerization in methyl ethyl ketone (MEK) at 80°C, using benzoyl peroxide (BPO) as the initiator. The radical chain copolymerization was confirmed by Fourier Transform Infrared (FTIR) and Nuclear Magnetic Resonance (1H-NMR) spectra. Copolymer surface morphology was visualized by scanning electron microscopy (SEM). After these characterization studies, the amoebicidal effect of MAVA was investigated on E. histolytica trophozoites using differing copolymer concentrations and periods of time. Results indicated that MAVA killed all the trophozoites at 32 mg/mL concentration in 3 h.

Keywords: Amoebicidal effect; Entamoeba histolytica; Poly (maleic anhydride-co-vinyl acetate) copolymer; Radical chain reaction; Trophozoite.

Introduction

First biological studies have been carried out with a relatively simple polymer, maleic anhydride-divinyl ether (DIVEMA), using tumor cell lines [1]. Later on it has been shown that DIVEMA also possessed antiviral, antibacterial, and antifungal activities [2,3].

Vinyl based monomers have the necessary requirements for the design of polymer-drug conjugates: (1) availability of suitable functional groups for covalent coupling with drugs, (2) biocompatibility, non-toxicity, and non-immunogenicity, (3) biodegradability or having molecular weights below the renal excretion limit [4]. Maleic anhydride (MA) containing copolymers have often been used as reactive macromolecules in the preparation of functional polymers by radical chain polymerisation reactions. For example, MA-styrene, -vinyl acetate or MA-methyl methacrylate have been shown to display various biological activities [1,5]. The copolymers could act as mitotic inhibitors, thus having important consequences in neo-plastic process and in immunology, and they could also be used in the treatment of viral infections [6,7].

Entamoeba histolytica is an anaerobic protozoan that can cause serious digestive tract infections, predominantly amoebic dysentery or liver amoebic abscess. Amoebiasis is the second leading cause of death among parasitic diseases and is responsible for 100,000 deaths per year worldwide [8]. The infection is more common in developing countries, although cases have also been reported in developed countries among homosexual men, immigrants, HIV-infected patients and travelers visiting endemic areas [9,10]. *E. histolytica* is the only pathogenic intestinal amoeba among the *Entamoeba* species. Its life cycle consists of two stages, a vegetative trophozoite stage and a dormant cyst stage. The trophozoite is highly motile and multiplies asexually by binary fission within the wall of the large bowel [11]. Trophozoites often become encysted in the lumen of colon and create protective walls around their small, round center. The trophozoites when passed in the stool are rapidly destroyed outside the body.

*E. histolytica* infection is primarily treated by tissue active agents, like metronidazole, tinidazole and chloroquine or more toxic emetine derivatives, including dehydroemetine (Figure 1).

So far the amoebicidal effect of MAVA on *Entamoeba histolytica* trophozoites has not been investigated [5,12-14].

In this study the amoebicidal effect of MAVA was investigated on *Entamoeba histolytica* trophozoites. Results indicated that MAVA inhibited the proliferation of trophozoites at 32 mg/mL concentration in 3 h.

Experimental

Materials

Maleic anhydride (MA), methyl ethyl ketone (MEK), and benzoyl peroxide (BPO) were obtained from Merck (Germany). Vinyl acetate (VA) and ethyl acetate were obtained from Sigma-Aldrich (USA). Ethyl alcohol and petroleum ether were obtained from Smyras (Teknik, Turkey).

Synthesis of MAVA

MAVA was synthesized by free radical polymerization of maleic anhydride (MA) and vinyl acetate (VA) at 1:1 molar ratio in a methyl ethyl ketone (MEK) using benzoyl peroxide (BPO) as an initiator, at 80°C for 24 h (Table 1) [5]. The reaction sample was purged with nitrogen gas continuously and allowed to continue until the first white precipitate products observed after the addition of 5 volumes of ethyl alcohol. The mixture was then cooled to room temperature. Unreacted vinyl acetate or its homo-polymers were removed by dissolving the precipitate in ethyl acetate for 24 h and reprecipitating with petroleum ether. The final sample was filtered under vacuum and then dried at 55°C for 24 h [15].

Structural characterization

MAVA copolymer was prepared as KBr pellets (2 mg sample in 100 mg KBr) and analyzed by FTIR spectrometry (Mattson 1000, Unicam, USA).


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USA) at 400-4000 cm⁻¹ with 4 cm⁻¹ increment. ¹H-NMR analysis was performed at 400 MHz (Bruker Avance III, Karlsruhe, Germany) using 6 mg of the copolymer sample dissolved in 0.8 mL dimethyl sulfoxide (DMSO). Sample surface morphology was visualized by SEM using 6 mg of the copolymer sample dissolved in 0.8 mL dimethyl sulfoxide (DMSO) at 400 MHz (Bruker Avance III, Karlsruhe, Germany). The characterizations were carried out at Technology Research and Developing Centre, Erciyes University, Kayseri, Turkey.

**Preparation of *Entamoeba histolytica* trophozoites**

*E. histolytica* was a laboratory strain was kindly provided by Dr. John Clark (London School of Hygiene & Tropical Medicine). Trophozoites were prepared in Robinson medium at 35.5 °C and harvested on the sixth day by centrifugation at 500g for 5 min. The final concentration was adjusted to 10x10⁴ trophozoites/mL in PBS and used immediately in the assay.

**Assessment of the amoebicidal effect**

Serial dilutions of MAVA (2, 4, 8, 16, 32, and 64 mg/mL) were prepared in sterile distilled water. Two hundred microliters of the trophozoite suspension and 200 µL test solutions were mixed thoroughly in micro-centrifuge tubes via pipetting up and down. The tubes were then incubated for 1, 2, 3, 6, 8, 24, 48 or 72 h at 35.5 °C. Cell growth was monitored periodically by light microscopy.

Trypan blue dye exclusion test was used to determine the inhibitory effect of MAVA on the proliferation of *Entamoeba histolytica*. The principle of the test is that live cells possess intact cell membranes which exclude the dye, whereas dead cells do not. 25 µL cell suspensions were simply mixed with the same volume of 0.5% trypan blue solution in a hemocytometer. The mixture was incubated for 3 min at room temperature. Unstained (viable) and stained (nonviable) cells were then counted. Cell viability was calculated by dividing the number of viable cells by the number of total cell number determined experimentally. The same procedure was applied to control sample containing only trophozoite suspension and distilled water.

**Results and Discussion**

**FTIR analysis**

MAVA had anhydride units at 1880 cm⁻¹ and 1804 cm⁻¹ (Figure 2), indicating symmetric and asymmetric C=O stretching vibrations, respectively [16-18]. In addition, characteristic C=O stretching vibrations at 1242 cm⁻¹ and 1012 cm⁻¹ and a CH₃ group of VA stretching vibration at 1395 cm⁻¹ were also observed [19].

**NMR analysis**

¹H-NMR result confirmed that of FTIR. Characteristic features of the MAVA spectrum were a chemical shift of two protons, on MA group, at 5.4 ppm; three methyl protons at 2 ppm [12,13]; -CH₃ protons, on VA unit, approximately at 2 ppm; and a multiplet peak for -CH₂ bound to oxygen, at 1.1 ppm (Figure 3) [5,18].

**Surface morphology**

Surface features of MAVA were visualized by SEM at differing magnifications (Figure 4; (a) 20.00 KX-1 µm, (b) 20.00 KX-2 µm, (c) 10.00 KX-2 µm, and (d) 5.00 KX-10 µm; KX, magnification; µm, resolution). In the images it was obvious that MAVA had surfaces characterized by evenly distributed cavities.

**Amoebicidal effects of MAVA**

Amoebiasis is a common infection that affects the human gastrointestinal tract. The disease still remains a major public health concern especially in Africa and South Asia [20,21]. Etiological agent of the disease is *E. histolytica*, a protozoon parasite. Drugs such as metronidazole [1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole] are currently used for the treatment of acute invasive amoebic dysentery. In some cases, however, such treatments often remain ineffective. The above-mentioned drugs also have some adverse effects. Metronidazole, for example, is a proven mutagen in bacteria and its high doses could lead to carcinoma in rodents [22]. It has been estimated that metronidazole had a minimum inhibition concentration (MIC) of 2 µg/mL after 24 h [23]. Metronidazole, with 50% inhibitory concentration (IC50, 0.17–0.37 µg/mL), has often been used for the treatment of *E. histolytica* infections [24]. Its cytotoxic dose has been reported to be 855.77 mg/mL [25]. In this study, it was for the first time shown that MAVA displayed the best antiamebic activity at 32 mg/mL within 3 h and that no viable trophozoites were present after 8 h incubation at 8 mg/mL polymer concentration. Cytotoxicity of MAVA has also been estimated and it has been found that it exerted almost no toxicity on mouse L929 fibroblast cells at 500 µg/mL concentration [unpublished.

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**Table 1:** Reaction conditions for the synthesis of MAVA copolymer.

<table>
<thead>
<tr>
<th>COPOLYMER</th>
<th>MOLECULAR WEIGHTS OF MONOMERS (g/mol)</th>
<th>AMOUNTS OF MONOMERS</th>
<th>MOL PROPORTIONS OF MONOMERS</th>
<th>AMOUNTS OF INITIATOR (g)</th>
<th>SOLVENT NAME</th>
<th>VOLUME (mL)</th>
<th>TIME (h)</th>
<th>TEMPERATURE (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAVA</td>
<td>98.06 MA-VA 86.09 MA (g) 4.9 4.63 VA (mL) MA:VA (1:1)</td>
<td>BPO 0.05 Methyl ethyl ketone 10</td>
<td>24</td>
<td>~ 80</td>
<td></td>
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**Figure 1:** The structures of common anti-amoebic drugs used in medical practices [36].
results. It was also evidenced that at lower concentrations, 1.2-4 mg/mL, MAVA appeared to inhibit the growth of the parasite within 48 h. If the incubation time had been extended and the parasite culture had been replenished with fresh media, by taking the slope of the graph into account it could be argued that MAVA might have caused the destruction of all the trophozoites at much lower concentrations, 1-4 mg/mL (Figure 5).

Many natural and synthetic products have also been investigated for their antiamoebic activity and a large number of plant products have been shown to inhibit the growth of parasite [23,26-31,32-35]. The reported MIC and/or IC50 doses in these studies ranged from the 0.19 µg/mL to 1 mg/mL. Although these concentrations appeared to be much lower than those of MAVA, in the above-mentioned studies, cytotoxicity of the substances has not seemed to be investigated.

Conclusions

The main focus of this study was to investigate amoebicidal and growth inhibition effect of MAVA using Entamoeba histolytica trophozoites, with the aim to demonstrate such features for vinyl-based polymers that have often been used as drug conjugates or carriers.

To sum up, in this study, it was shown for the first time that MAVA, a polymer with almost no toxicity, could kill E. histolytica at 32 mg/mL for short periods of incubation time, or at much lower concentrations using longer incubation periods.

References

Benzocaine-modified maleic anhydride-cyclohexyl-1,3-dioxepin copolymer: Preparation and potential medical applications. Polymer 34: 3298-3301.