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Bioengineering Functional Copolymers. XX. Synthesis of Novel Anticancer Active Poly(maleic anhydride-*alt*-2-vinyl-1,3-dioxolane) and its Organoboron Amide-Ester Branched Derivatives

Biyomühendislik Fonksiyonel Kopolimerleri. XX. Yeni Antikanser Aktif Poli(maleik anhidrid-alt-2-vinil-1,3-dioxolan) ve Organoboron Amid-Ester Dallanmış Türevleri

Research Article

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ABSTRACT

Novel bioengineering alternating copolymer and its organoboron amide and α-hydroxy-ω-methoxypoly(ethylene oxide) (PEO) branched derivatives were synthesized by (1) complex-radical alternating copolymerization of two non-homopolymerizable in chosen reaction conditions monomers such as maleic anhydride (MA) and 2-vinyl-1,3-dioxolane (VDO), (2) amidolysis of synthesized poly(MA-***alt***-VDO) with 2-aminoethyldiphenylboronate and (3) esterification-grafting of organoboron copolymer with PEO. The complex-formation (K_c) and the monomer reactivity ratios (r_1 and r_2 copolymerization constants), structure and composition of the synthesized copolymers were characterized by chemical (alkali titration), FTIR-ATR and NMR spectroscopy, TGA-DSC thermal analysis methods. Cytotoxicity found as having an order as organoboron branched PEO copolymer < poly(MA-alt-VDO) < organoboron copolymer of these novel copolymers containing a combination of ionizable, hydrophilic/hydrophobic, organoboron, carboxyl-amide-ester-ether groups, toward HeLa cells was investigated.

Key Words

Synthesis, Alternating copolymer, Amidolysis, Esterification, Organoboron copolymer, Citotoxicity, HeLa cells.

ÖZET

Yeni biyomühendislik alternatif kopolimerinin ve organobor amid ve α-hidroksi-ω-metoksi-poli(etilen oksit) (PEO) ile dallanmış türevlerinin sentezi, (1) Seçilen reaksiyon şartlarında homopolimerleşmeyen maleik anhidriti (MA) ve 2-vinil-1,3-dioksolan (VDO) monomerlerinin kompleks-radikal alternatif kopolimerizasyonu, (2) sentezlenen poli(MA-*alt*-VDO) makromoleküllerinin 2-amino-etildifenilboronat ile amidolizi ve (3) PEO ile esterleşmesi yöntemleri kulllanarak gerçekleştirilmiştir. Monomerlerin kompleks oluşumu (K_c) ve kopolimerleşme sabitleri (r_1 ve r_2), çok fonksiyonel gruplar içeren kopolimerlerin yapısı, içeriği ve ısısal özellikleri kimyasal analiz (alkali titrasyon), FTIR-ATR and NMR spektroskopisi ve TGA-DSC ile karakterize edilmiştir. İyonize olabilen, hidrofilik/hidrofobik, organobor, anhidrit-karboksil-amid-ester-eter gibi gruplar içeren bu kopolimerlerin HeLa hücrelerine sitotoksik etkisi araştırılmış ve organobor-PEO kopolimer < poli(MA-*alt*-VDO) < organobor kopolimer sırasında olduğu belirlenmiştir.

Anahtar Kelimeler

Sentez, Alternatif kopolimer, Amidoliz, Esterleşme, Organobor kopolimer, Sitotoksisite, HeLa hücreleri.

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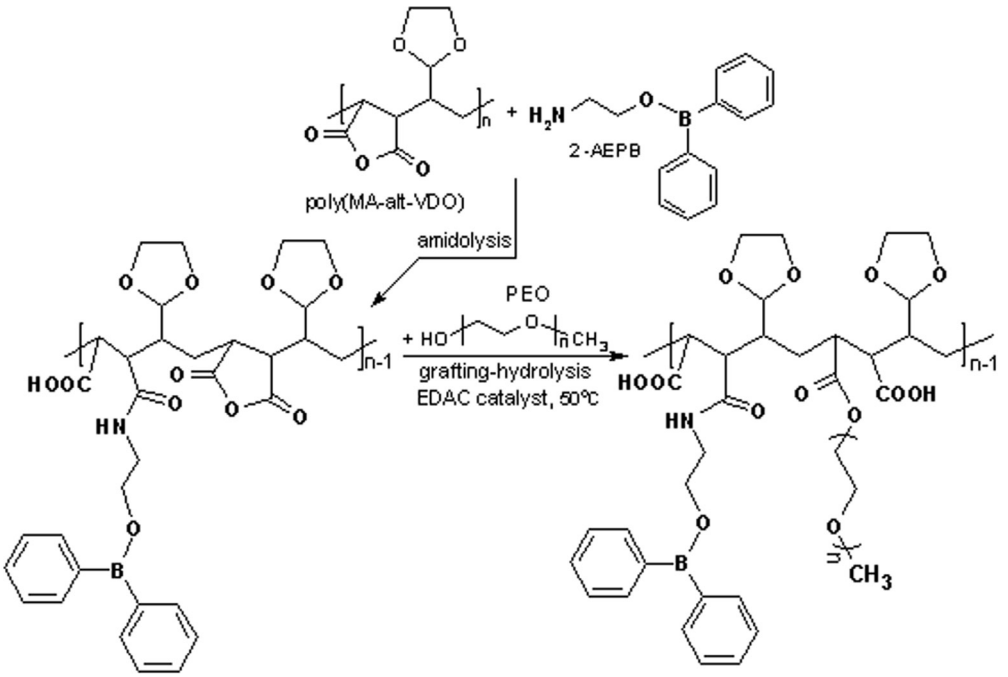
INTRODUCTION

Alternating copolymers of alkyl vinyl ethers with maleic anhydride (MA) have received significant attention because of their unique properties and wide range of applications such as adhesives, photocrosslinkable and photosetting resin compositions, photographic films, electrophotographic recording and glass fiber coatings, reversible shear thinning gels, detergents, viscosity improvers, flocculants, and cellular plastics (only *tert*-butyl derivative), as well as controlled-release coatings, medicinal tablet coatings, animal antidiarrhea capsules, and so forth. Methyl vinyl and divinyl ether copolymers, show antitumor activity and the property of induced production of interferon in animals, which is potentially useful for inhibiting the growth of Friends leukemia virus [1,2] The alternating copolymerization of unsaturated cyclic ethers, such as *p*-dioxene [3-5], 2,3-dihydro-2H-pyran (DHP) [6] and 4-glycidyl-2,3-dihydro-2H-pyran [7] as the electron-donor monomers with MA as the electron-acceptor monomer was a subject of early publications of Iwatsuki and Yamashita, Fujimori and Rzaev et al., respectively. Han et al. [8,9] have reported the synthesis, characterization and bioactivity of poly(DHP-*alt*-MA) and its derivatives, with different substituents (e,g., acetoxy, methoxy, ethoxy, methoxycarbonyl, formyl, acetoxymethyl, and tosyloxymethyl groups, as well as guanine derivatives) in the 2-position of pyran ring of the copolymer backbone. These pyran-containing copolymers with a high density of carboxylic acid functionality as the polynucleotide analogues exhibit antitumor activities in *in vitro*, which were found to have higher activites against the tumor cells (B16 and 3LL) than those of their acyclic analogues, especially alternating cyclocopolymer of divinyl ether with MA (M_n = 5500 g/mol and M_w/M_n = 1.3), whose biological activities have well been reported [2,10]. Radical-initiated binary and ternary polymerization of MA-DHP, DHP-MA-vinyl acetate (VA) and MA-DHP-N-isopropyl acrylamide acceptor-donor and donor-acceptor-donor monomer systems, respectively, also reported [11-13]. The synthesis and characterization of this copolymers, some kinetic parameters of rections, the coplymer-thermal behavior relationship, and their antitumor activity were examined. The formation of charge-transfer complexes (CTC) in the MA-donor monomer systems and complex-radical copolymerizations of MA with various electron-donor functional monomers such as *p*-dioxene [14], *p*-oxathiene [3], 2,3-dihydropyrane [6], phenylvinyl alkyl ethers and thioethers [15], alkyl vinyl ethers [16] and 2-vinyl-1,3-dioxane [17] have been reperted by many researchers. MA is an excellent monomer which can provide reactive anhydride or carboxylic groups, and amphiphilic molecules can be obtained easily via the copolymerization of MA and hydrophobic monomer. The basic concepts of complex-radical alternating copolymerization of maleic monomers were described by Rzayev [18]. Recently, extensive studies have been performed on the radical copolymerization of MA with other monomer and the self-assembly of their copolymers [19-27]. Blenvins [28] reported synthetic procedure for the alternating copolymers (oligomers with M_n 1330 and 1790 g/mol) through radical copolymerization of 2,2-dimethyl- and 2-methyl-2-ethyl-4-vinyl-1,3-dioxolanes with maleic anhydride in toluene. According to this patent publication, the reactive anhydride groups in the copolymers can be utilized to crosslink the copolymers with a variety of materials to produce useful coatings, films, binders, and dispersing agents for particulate materials.

Biomaterials synthesized from synthetic polymers having biocompatible and bioactive properties have been developed for biomedical and tissue engineering applications [29-31]. Many researchers have investigated the degradable cyclic acetal biomedical polymers that produce less toxic degradation products, therefore decreasing the inflammatory response of the surrounding tissue [32-35]. Shi et al. [36] studied cyclic acetals that used as hydrogen donors for bimolecular photoinitiating systems and benzodioxole derivatives as a substitute for commercial aromatic tertiary amine in dental applications. Pate et al. [37] reported synthesis of the hydroxyapatite nanoparticle/cyclic acetal hydrogel systems to create nanocomposites that could be used to repair surgically created orbital floor defects in a rabbit animal model.

On the other hand, growing interest and much effort has been focused on the synthesis of boron-containing low molecular weight functional



Sheme 1. Synthetic pathway of the organoboron amide-ester branched derivatives of alternating copolymer of maleic anhydride and 2-vinyl-1,3-dioxolane.

compounds, biopolymers and drugs with boron ligands and evaluation of their suitability for the bioengineering applications, especially for the Boron Neutron Capture Therapy (BNCT) [38,39]. Synthesis of novel organoboron functionalized copolymers and investigation of their interaction with various cancer cells were a subject of our recent publications [13,40,41]. However, synthesis of alternating copolymer of MA with 2-vinyl-1,3-dioxolane (VDO) as a cyclic analogue of methyl vinyl ether (antitumor activity of alternating copolymers of methyl vinyl (or divinyl) ethers with MA is well known [2]) by complex-radical bulk copolymerization and its organoboron and PEO functionalized derivatives as anticancer agents are not reported.

The goal of this work is synthesis and characterization of novel organoboron amide derivatives by amidolysis of poly(MA-*alt*-VDO) with 2-aminoethyldiphenyl borinate (2-AEPB) and their α,ω-hydroxyl-methoxy-poly(ethylene oxide) (PEO) macrobranches by grafting of synthesized organoboron copolymer with PEO to improve biocompatibility and degree of conjugation with biomacromolecules. An important aspect of this work is comparative investigations of the interactions of pristine alternating copolymer and its organoboron and PEO branched derivatives with HeLa (human cervix carcinoma cell) cancer cells, and investigation of copolymer structure–composition–antitumor activity relationships. Synthetic pathway of the side-chain amide-ester-carboxyl functionalized organoboron copolymers can be represented as follows (Scheme 1).

EXPERIMENTAL

Materials

MA monomer (Fluka, Switzerland) was purified by recrystallization from anhydrous benzene solution and sublimation in vacuum. 2-Vinyl-1,3-dioxolane (VDO) was purchased from Aldrich: FTIR-ATR spectra (cm^{-1}): 2994-2866 (m) antisym. Stretching C-H in R-CH=CH_2, 1437 (m) and 1347 (m) CH_2 and C-H bending in R-CH=CH_2, respectively, 1147-1028 C-O vibration bands. 2-Aminoethyldiphenyl borinate (2-AEPB) (Sigma-Aldrich, Germany) was purified by recrystallization from anhydrous ethanol: m.p. 193.5°C (by DSC); FTIR-ATR spectra (cm^{-1}): 3284 (vs) and 3220 (s) N-H stretching in NH_2, 3066-2870 (s) C-H stretching, C=C stretching in phenyl groups, 1491(m) and 1334 (m) B-O band, 1432 (vs) fairly strong, sharp band due to benzene ring vibration in phenyl-boronic acid linkage, 1263-1154 (s) fairly strong, sharp bands due to C-N stretching

in C-NH_2, 1061 (vs) C-O bending and 750-710 (s) sharp bands from boron-phenyl linkage; ^{1}H NMR spectra (δ, ppm), in $CHCl_3$-d_1: CH_2-O 1.49, CH_2-NH_2 2.96, and 7.38-7.40 (1H), 7.19-7.24 (2H) and 7.13-7.16 (2H) for protons of *p*-, *o*- and *m*-positions in benzene ring, respectively. α-Hydroxy-ω-methoxy-PEO (M_n 2000 g.mol^{-1}) (Fluka): ^{1}H NMR spectra (δ, ppm) in $CHCl_3$-d_1: CH_2-O 3.75-3.45, OH end group 2.61 and O-CH_3 end group 2.16. α, α'-Azoisobutyronitrile (AIBN) (Fluka) as a initiator was purified by recrystallization from anhydrous ethanol. *N*-Ethyl-*N*-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) as a catalyst and folic acid (FA) as a targeting agent were supported from Aldrıch-Sigma (Germany). All solvents and reagents were analytical grade and used without purification. HeLa (human cervix carcinoma cell) cancer cells were obtained from the tissue culture collection of the SAP Institute (Ankara, Turkey). Cell culture flasks and other plastic material were purchased from Corning (NY, USA). The growth medium, which is Dulbecco Modified Medium (DMEM) without L-glutamine supplemented fetal calf serum (FCS), Trypsin-EDTA were purchased from Biological Industries (Kibbutz Beit Haemek, Israel). The primary antibody, caspase-3 was purchased from Lab Vision (Germany), cell proliferation reagent WST (Roche, Germany).

Synthesis of poly(maleic anhydride-*alt*-2-vinyl-1,3-dioxolane)

Poly(maleic anhydride-*alt*-2-vinyl-1,3-dioxolane) were synthesized by bulk polymerization technique. Appropriate quantities of solid MA were dissolved in liquid VDO with AIBN initiator under the nitrogen atmosphere. Reaction conditions: mole ratios of (VDO/MA) = 1:1 and AIBN = 1.0 wt.%]. The reaction medium was mixed at 70ºC for 6 h under a nitrogen atmosphere. Then, the synthesized copolymers were isolated from reaction mixture by precipitation with diethyl ether and dried at 60ºC under vacuum. FTIR-ATR spectra (cm^{-1}): 2962 (w) for backbone CH stretching band, 1781 (w) and 1724 (m) bands for antisymmetrical and symmetrical C=O band of MA unit, 1258 (w-m) and 1159 (m, broad) anhydride C–O–C stretching; ^{1}H NMR spectra (in DMSO-d_6 at 25ºC) (δ, ppm): 1.38, 1.76 and 3.62 for the backbone CH, CH_2 (VDO unit) and CH-CH (MA unit), respectively, 3.92 and 6.5-6.1 for CH and CH_2-O of VDO ring, respectively and broad peak around 13.21-12.15 for partially hydrolyzed MA unit (-COOH); ^{13}C NMR spectra (δ, ppm): 66.2, 62.3-64.1 and 58.2 for the backbone carbon atoms in CH, CH_2 (VDO unit) and CH-CH (MA unit), respectively, 109 and around 60-58 for OCO and O-CH_2 of dioxolane ring, respectively, 167-164 for anhydride C=O group.

Synthesis of 2-amidoethyldiphenylborinate-poly(MA-*alt*-VDO)

Amidolysis of poly(MA-*alt*-VDO) with 2-AEPB using various [copolymer]/[2-AEPB] molar ratios was carried out in *N,N'*-dimethylformamide (DMF) at 60ºC with EDAC catalyst under a nitrogen atmosphere using a standard Pyrex-glass reactor supplied by a mixer, temperature control unit and condenser. Reaction conditions: [2-AEPB] = 0.066 mol.L^{-1}, mole ratios of [(MA-*co*-VDO)/[2-AEPB] = 1:1, 3:1, 5:1 and EDAC = 1.0 wt.%. Appropriate quantities of poly(MA-*co*-VDO), 2-AEPB, DMF and EDAC were placed in a reactor and the reaction mixture was flushed with dried nitrogen gas for at least 2 min, then sealed and placed in a thermo stated silicon oil bath at 60ºC to intensive mixing for 5 h. The organoboron amide copolymer was isolated from reaction mixture by precipitation with diethyl ether and dried under vacuum. FTIR-ATR spectra (KBr pellet), cm^{-1}: 1734 (vs) C=O stretching (amide I band), 1651 (m) and 1558 (m, broad) N-H deformation (amide II band), 1437(w) and 1369 (m) C-N stretching (amide III band); ^{1}H NMR spectra (in DMSO-d_6 at 25ºC), δ ppm: protons of phenyl groups 7.2-7.7, weak 2H from CH_2 in –CH_2-CO-NH- fragment 5.9, very weak 2H from B-O-CH_2 group 3.25, 2H from NH-CH_2 2.75 and 2H from backbone –CH-CH- group around 3.60-353; ^{13}C NMR spectra (*δ*, ppm): C=O of amide and carboxylic groups 165, CH= in phenyl groups around 135-127 (CH=), 163 (C-B), backbone CH_2 17.1 and CH 10.2, CH_2-O 36.5-32.3 (organoboron linkage), CH-CH 41-42, CH_2-O and CH-O around 65-58 (dioxolane ring).

Synthesis of 2-amidoethyldiphenylborinate-PEO-ester-poly(MA-*alt*-VDO)

The esterification (grafting) of organoboron amide polymer, containing 19.24 mol % of organoboron linkages, with PEO (M_n 2000 g.mol^{-1}) at polymer/PEO feed molar ratio 1:0.01 was carried out in DMF at 60ºC for 1 h. PEO branched copolymer was

isolated from reaction mixture by precipitation with diethyl ether and dried 40°C under vacuum. Prepared PEO ester of organoboron copolymer has the following average characteristics: Boron (B) content 0.86 % (by ICP-MS) and 0.92 % (by TGA), and T_g 146.8°C (by DSC); ^{1}H NMR spectra (in DMSO-d_6 at 25°C), δ ppm: protons of CH-CH backbone around 4.15-3.92 for maleamide group, CH_2-O and CH-O of dioxolane ring around 4.65-4.58, CH_2-O and CH_3-O (end group) of PEO branch around 4.42-4.18 and 2.94, respectively, phenyl groups 7.92-7.70 (CH=) and 5.86 HN-CO amide group for organoboron linkage; ^{13}C NMR spectra (d, ppm): 42.2 and 163 backbone CH-CH and C=O of maleamide unit, around 70-65 for CH_2-O and CH-O groups in dioxolane ring, 62 and 58 CH_2-O, 52 CH_3-O (end group) in PEO branch, around 136-127 CH= and 162.9 (C-B) in phenyl groups, 36.8 and 32.2 CH_2 of organoboron linkage.

Characterization

Fourier transform infrared (FTIR-ATR) spectra were recorded with FTIR Nicolet 8700 spectrometer in the 3700–600 cm^{-1} range. ^{1}H and ^{13}C NMR spectra were performed on a Bruker Avance (300 MHz) spectrometer with DMSO-d_6 as a solvent at 25°C. Thermogravimetric (TGA) and differential scanning calorimetric (DSC) analyses were performed in a TGA-DTA (Perkin Elmer TG/DTA6300) and a DSC2010 Thermal Analyzers, respectively, under nitrogen atmosphere at a heating rate of 10°C/min. Boron amount in organoboron copolymers and PEO branched derivative were determined by TGA and High Resolution Inductively Coupled Plasma Mass Spectrometry (HR-ICP-MS) (Thermo Element XR) with microwave digestion technique, respectively. WST assay for cytotoxicity, HeLa cells (5x10^3 cells per well) were placed in DMEM by using 96-well plates. The plates were kept in the CO_2 incubator (37 °C in 5% CO_2) for 72 h; the medium was replaced with fresh medium. Then, Different amounts of Copolymer (C), C-B, C-B-PEO (about 0-200 μg.mL^{-1} in medium) were put into wells containing cells, respectively, and incubated at the same conditions for 72 h. Following of this incubation, WST reagent (15μl) was added into each well, and the cells were cultured for further 4h incubation. After that, plates were read in Elisa Microplate Reader at 440 nm and reference wave length at 630 nm.

RESULTS AND DISCUSSION

The monomer reactivity ratios

Both the MA and VDO monomers are not homopolymerize with free radical initiation and related to the electron-acceptor–electron-donor system which are form a charge-transfer complex (CTC) and easily undergo to alternating copolymerization. Experimental results were summarized in Table 1. The compositions of copolymers were calculated using acid number values (AN) (by alkali titration) for the copolymers prepared from the various monomer feed ratios in the lower conversion conditions (around 5.5-9.6 %) according to the following our equation:

$$m_1 = \frac{W_2 . 100}{\frac{2M(KOH)}{AN} - (W_1 - W_2)} \quad (1)$$

Table 1. Copolymer Compositions for the Various Monomer Feeds and 1H NMR Analysis; Results for MA/VDO Acceptor-Donor Monomer Mixtures at [MA] ≪ [VDO] in $CHCl_3$-d_1 solution at 35°C.

Monomer feed (mol %) [MA] (VDO)	Acid Number (mgKOH/g)	Copolymer composition (mol %) m_1	m_2	[MA] (mol L^{-1})	[VDO]$^{-1}$ (L mol)	^{1}H NMR analysis d_c^{MA*} (ppm)	D_{obs} (ppm)	$(D_{obs})^{-1}$ (ppm)$^{-1}$
30	554	48.95		0.1	0.25	7.010		0.110
70		51.05						9.09
40	557	49.18		0.1	0.50	7.035		0.085
60		50.82						11.76
50	568	50.16		0.1	1.00	7.045		0.075
50		49.84						13.33
70	572	50.50		0.1	2.00	7.052		0.068
30		49.50						14.71

*δ_f^{MA} = 7.12 ppm for the protons of free MA.

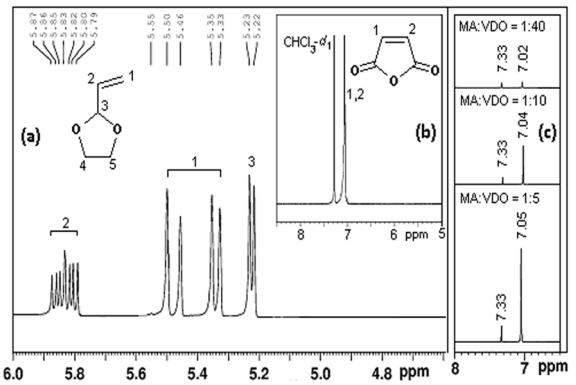


Figure 1. ^{1}H NMR spectra of (a) vinyl-1,3-dioxalane, (b) maleic anhydride and (c) various MA/VDO monomer mixtures in $CHCl_3$-d_1 at 35°C.

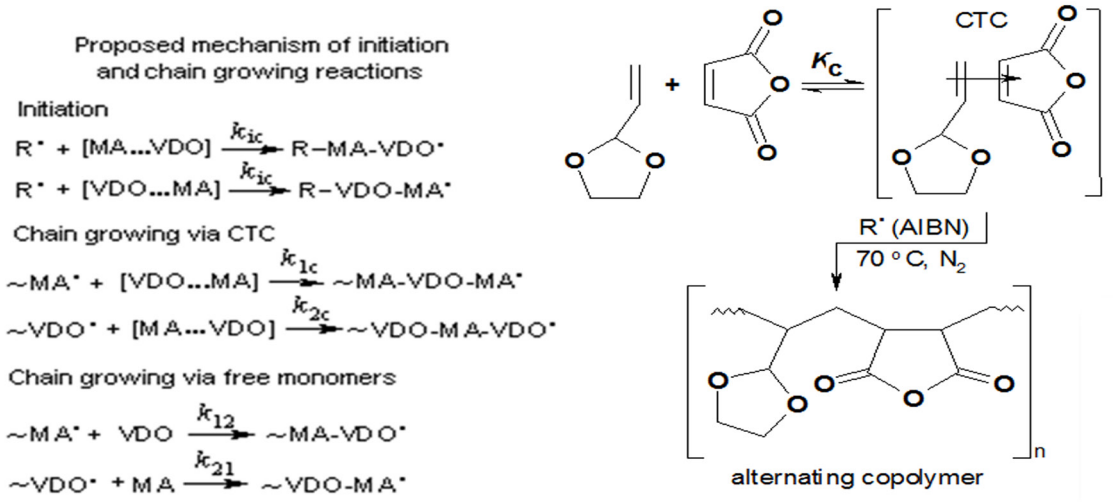
where W_1 and W_2 molecular weights of MA(m_1) and VDO (m_2) monomer units (mol %), respectively. As seen from the monomer feed ratio–copolymer composition relationships, copolymerization of leads to the formation of copolymers of constant composition (close to a 1:1 molar one), irrespective of the composition of the monomer feed ratios. Since neither MA nor VDO homopolymerized under chosen binary copolymerization conditions, the monomer pair exhibits a strong tendency to alternating copolymerization ($r_1.r_2 \ll 1$) according to known concept of 'homopolymerization' monomeric CTCs [18]. The formation of CTCs in this monomer system was verified by an analysis of the ^{1}H NMR spectra of the pristine monomers and their various mixtures at [VDO] >>[MA] (Figure 1), the results of which were also summarized in Table 1. It can be seen from these Figures that the MA (acceptor) singlet has a high upfield shift ($\Delta_{obs} = \delta_f^{MA} - \delta_{obs}$ = 7.12 – 7.01 = 0.11 ppm for MA/VDO =1/40), while chemical shift of VDO (donor) double bond protons was almost not changed. The obtained values of chemical shifts (Δ_{obs}) allow us calculate the equilibrium constant (K_c = 0.029 L.mol^{-1} in $CHCl_3$-d_1 at 35°C) for MA...VDO complex from plots of $[VDO]^{-1}$ (L mol^{-1}) versus $(\Delta_{obs})^{-1}$ (ppm^{-1}) using the following known equation [42]:

$$[VDO]^{-1} = (\Delta_c K_c (\Delta obs)^{-1} - K_c \qquad (2)$$

where Δ_{obs} is the difference in chemical shifts of free MA protons and those in MA/VDO mixtures at [VDO] >>[MA]; $\Delta_{obs} = \delta^f_{MA}$ (chemical shift of free MA at 7.12 ppm) – δ^c_{MA} (chemical shifts of complexed MA in the different MA/VDO monomer mixtures); $(\Delta_{obs})^{-1}$ is the intercept on the ordinate axis and Tgα = $(\Delta_{obs})^{-1}K_c$.

The monomer reactivity ratios (r_1 and r_2) of MA-VDO acceptor-donor monomer pair were calculated using obtained values of copolymer compositions (Table 1) and modified Kelen-Tüdos equation [43] from plot of $(F^2/f)/(F^2/f + \alpha)$ versus $F(f\text{-}1)/f + \alpha$: r_1 (MA) = 0.007 and r_2 (VDO) = 0.002; r_1K_c = 0.00026 (~0) and $r_2K_c^{-1}$ = 0.054.

$$\frac{F(f-1)}{f} + \alpha = (r_1 K_c + \frac{r_2 K_c^{-1}}{\alpha})(\frac{F^2}{f})/(\frac{F^2}{f} + \alpha) \qquad (3)$$



Scheme 2. Schematic representation of radical alternating copolymerization of MA (acceptor) and 2-vinyl-1,3-dioxolane (donor) via charge transfer complex formation and mechanism of initiation and chain growing reactions.

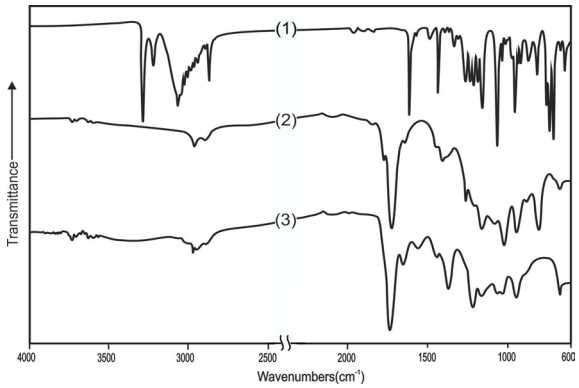


Figure 2. FTIR spectra of (1) 2-AEPB (2) poly(MA-co-VDO) and (3) poly(MA-co-VDO)-2-AEPB-1 organoboron copolymer.

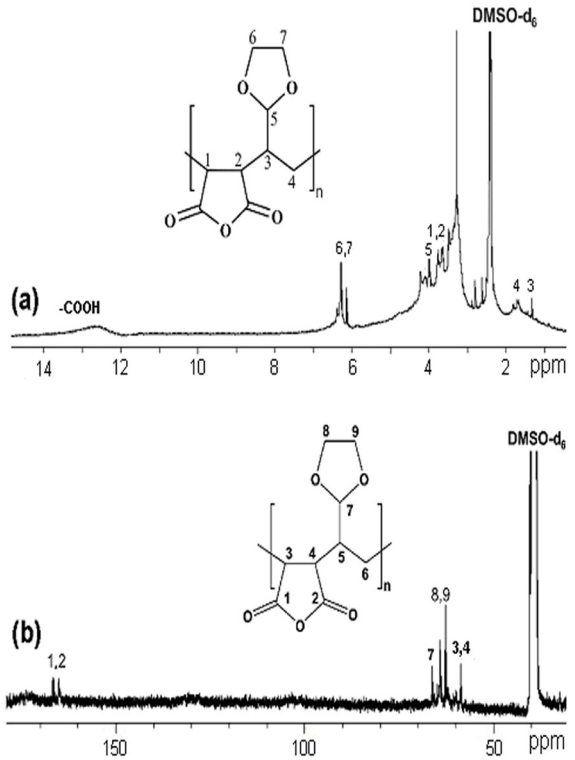


Figure 3. (a) ^{1}H NMR and (b) ^{13}C NMR spectra of poly(MA-alt-VDO) in DMSO-d_6.

where α (arbitrary constant) ~1, F = [MA]/[VDO] (monomer feed ratio) and $f = m_1/m_2$ (monomer unit ratio in copolymer).

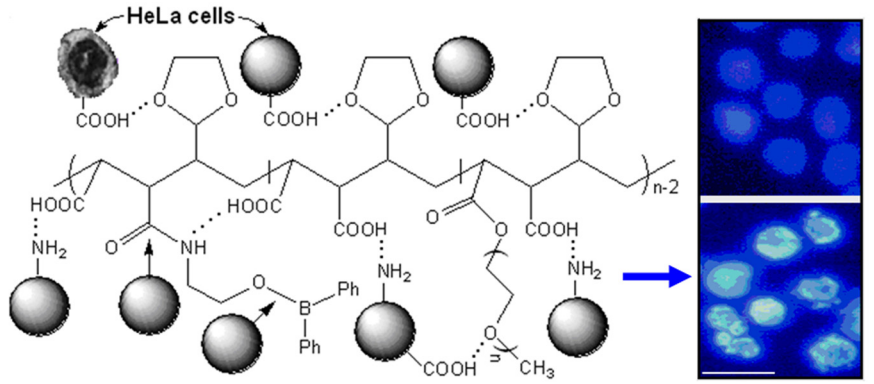
Thus, VDO monomer as cyclic analogue of alkyl vinyl ethers copolymerize with MA through formation CTC (1:1) and copolymer with strong alternating structure that reasonable agreement with classical theory of complex-radical alternating copolymerization of two non-homopolymerizable monomers [18]. Proposed mechanism of initiation and chain propagation reactions can be represented as follows (Scheme 2).

Structure of Organoboron Derivatives of Poly(MA-*alt*-VDO)

The structures of synthesized copolymers, organoboron copolymers and their PEO branches were confirmed by FTIR-ATR and ^{1}H (^{13}C) NMR analysis. The characteristic C=C stretching bands of vinylene and vinyl groups of MA and VDO monomers (around 1631-1563 cm^{-1}), respectively, are not observed in the FTIR-ATR spectra of copolymer (Figure 2). But, characteristic bands of anhydride and dioxalane units (1781, 1723, 1258, and 1018 cm^{-1}) are appeared in spectra of poly(MA-alt-VDO). Comparative spectral analysis of 2-AEPB, poly(MA-alt-VDO) and its organoboron derivative indicates that the characteristic bands of anhydride C=O groups disappearance in the spectra of poly(MA-alt-VDO)-B-1 polymer prepared from equimolar feed ratio of copolymer:2-AEPB = 1:1. The formation of amide group in this organoboron copolymer is confirmed by the appearance of new bands such as 1734 (amide I. band), 1651 and 1558 (amide II. band), 1437 and 1369 (amide III. band).

Table 2. Composition and Thermal Behavior of Alternating Copolymer and Its Organoboron Amide-Ester Derivatives.

Functional organoboron copolymers	B (%) (by TGA)	T_g (°C) (by DSC)	T_d (°C) (by TGA) first step degradation T_{d1}	T_{dmax}	T_{d2}
Poly(MA-alt-VDO)	0.0	185.5	189	195	225
Poly(MA-alt-VDO)-g-B-1	3.69	142.0	152	170	231
Poly(MA-alt-VDO)-g-B-2	3.11	140.2	148	174	243
Poly(MA-alt-VDO)-g-B-3	1.85	135.5	151	178	255
Poly(MA-alt-VDO)-g-B-2-g-PEO	0.92	146.8	138	159	245



Scheme 3. Schematic representation of copolymer/Hela cells coniugation. Images: before (from the top) and after (from the bottom) interaction. Scale: x400 magnification, 40 μm. Inner ring structure of HeLa cell, which is presented in Scheme, was adapted from [44].

The characteristic proton peaks of VDO are observed OCH_2 peaks at 4.7 ppm and 4.1 ppm in the ^1H NMR spectra of poly(MA-*alt*-VDO) (Figure 3a). In the ^{13}C NMR spectra of poly(MA-*alt*-VDO), characteristic carbon peaks of MA and VDO are also observed at 167-164 ppm for anhydride C=O, 109 ppm for OCO in dioxolane ring and 60-58 ppm for O-CH_2 in dioxolane ring, respectively (Figure 3b). Similar effect has been observed from comparative analysis of the ^1H NMR and ^{13}C NMR spectra of poly(MA-*alt*-VDO)-B-2 and its PEO branch poly(MA-*alt*-VDO)-B-PEO. The results of this analysis are illustrated in Figures 4 and 5. The formation of H-bonded amide linkages is confirmed by a presence of characteristic peaks at 5.9 and 162 ppm in the ^1H NMR and ^{13}C NMR spectra of poly(MA-*co*-VDO)-B-2, respectively (Figure 4). In addition, the presence of characteristic proton peaks of organoboron linkages such as quarter phenyl peak at 7.2 ppm, triplet B-O-CH_2 peak at 3.25 ppm and quarter NH-CH_2 peak at 2.75 ppm (Figure 4a) also confirmed that 2-AEPB is covalently bound to anhydride units. In the ^{13}C NMR spectra of poly(MA-

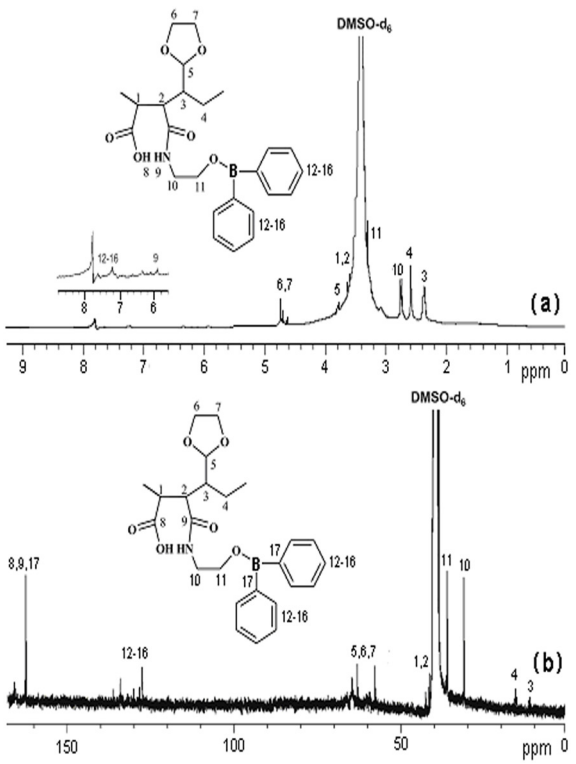


Figure 4. (a) ^1H NMR and (b)^{13}C NMR spectra of poly(MA-alt-VDO)-g-AEPB-2 in DMSO-d_6.

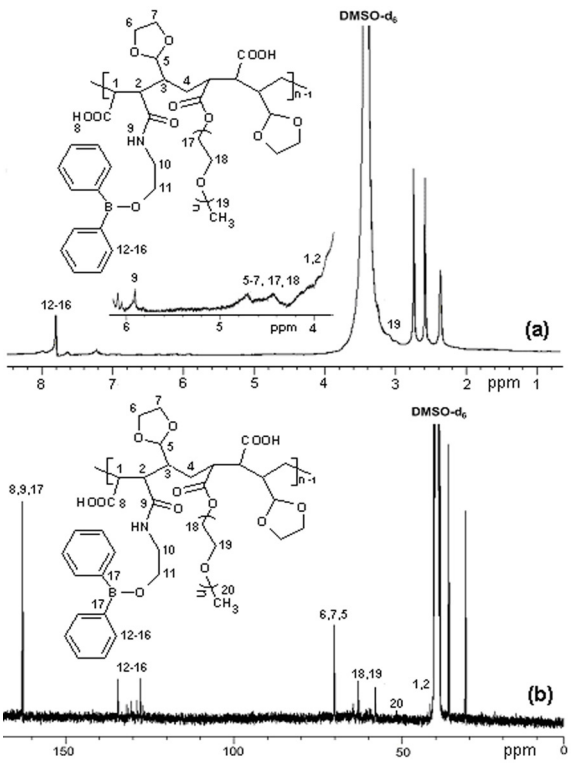


Figure 5. (a) ^1H NMR and (b) ^{13}C NMR spectra of poly(MA-alt-VDO)-g- AEPB-2-g-PEO in DMSO-d_6.

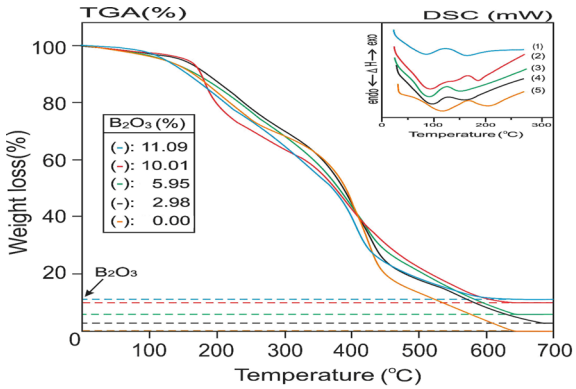


Figure 6. TGA and DSC curves of organoboron and PEO functionalized derivatives of copolymer: (1) poly(MA-alt-VDO)-g-2- AEPB-1, (2) poly(MA-alt-VDO)-g-2-AEPB-2, (3) poly(MA-alt-VDO)-g-2-AEPB-3, (4) poly(MA-alt-VDO)-g-2-AEPB-2-g-PEO and (5) pristine alternating copolymer. Heating rate10°C/min under a nitrogen atmosphere.

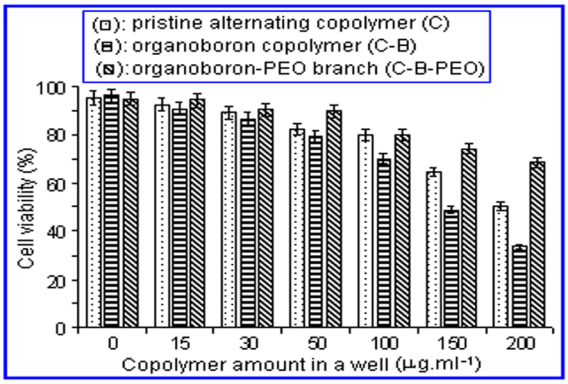


Figure 7. In vitro cytotoxicity of pristine alternating copolymer and its organoboron and PEO functionalized derivatives with different amount in a well at 72 h incubation. Results are presented as means ± SEM.

co-VDO)-B-2 polymer (Figure 4b), the characteristic carbon resonances (163, 137-134, 66-63, 41, 42, 31 and 36 ppm) from organoboron fragment are also observed. ^{1}H (^{13}C) NMR spectra of PEO grafted organoboron copolymer [poly(MA-*co*-VDO)-B-PEO] were illustrated in Figure 5. The observed weak proton signals of side-chain PEO branches at 4.2 ppm for $(CH_2\text{-}CH_2\text{-}O)_n$ units and 3.25 ppm for OCH_3 end group (Figure 5a) and carbon atom resonances (70 ppm for $O\text{-}CH_2$ and 58 ppm for OCH_3 end group) (Figure 5b) can be regarded as an additional fact to confirm the formation of side-chain macrobranced PEO linkages.

Organoboron functional copolymer composition–property relationships

Thermal behavior and phase transitions of synthesized copolymers were investigated by differential scanning calorimetric (DSC) and thermal gravimetrical analysis (TGA) methods. Last method also was utilized to determine the boron contents in organoboron copolymers. The obtained results were summarized in Figure 6. It was found that poly(MA-*alt*-VDO) and its organoboron and PEO branched derivatives exhibit amorphous structure with characteristical broad *endo*-peaks, which are associated with the glass-transition temperatures (T_g), significantly depend on the composition and content of organoboron linkages in the functional copolymers. The higher values of T_g are observed for the polymers containing relatively high organoboron linkages. Therefore, rigid H-bonded structure provides high T_g in the organoboron copolymers (curves 1-3). For the copolymer poly(MA-*alt*-VDO) (curve 5), the relatively low values of T_g are observed. The results of TGA analyses (Figure 7) indicate that the organoboron and PEO branched derivatives of poly(MA-*alt*-VDO) show higher thermal stability which increases with increasing degree of grafted organoboron linkage in the copolymer. The observed two step degradation of the poly(MA-*co*-VDO) and its functionalized derivatives indicates occurrence of some macromolecular reactions such as anhydridization-decarboxylation reactions in the first step and main chain degradation in the second step. Relatively higher thermal stability was observed for PEO branched derivative of organoboron copolymer (Figure 7, curve 5). This fact can be explained by an additional effect of H-bonded PEO branches through ether...carboxyl (>O...HOOC–) interaction. TGA analyses also allow us to determine the content of boron in studied functionalized copolymers, results of which are summarized in Table 2.

Cytotoxicity

The obtained cytotoxicity results of the pristine alternating copolymer (C) and its organoboron amide (C-B) and organoboron amide-ester (C-B-PEO) derivatives on cancer cells using a WST method were illustrated in Figure 7. As seen from plots of concentration of polymers versus percent of cell viability, the toxicity of pristine coplymer (C) against HeLa cells decreased with incresing in polymer concentration from 15 to 200 $\mu g.mL^{-1}$ for 24 h incubation at 37°C. The toxicity of C-B was more significant than other copolymers. Figure 7

shows that the number of viable cells is above 70 % for HeLa cells after incubation of the cells with C-B at concentrations around 15-50 μg.mL^{-1} for 24 h incubating time in cell culture media. The number of viable cells was lover than 50 % for HeLa in the range of 100-200 μg.mL^{-1} concentration. When organoboron linkage was introduced to the structure of copolymer via amidization, the cytotoxicity was increased. However, the toxicity for HeLa cells significantly increases for organoboron copolymer concentrations above 100 μg.mL^{-1}. When organoboron copolymer was modified with PEO (C-B-PEO), the cytotoxicity was decreased because of PEO biocompatibilization effect of PEO long branchs. The cytotoxicity of PEO containing organoboron copolymer was lower than those without PEO in 15-200 μg.mL^{-1} concentration. Viable cell ratio (%) was 41.6 ± 3% (C), 27.8 ± 3% (C-B) and 60.4 ✣ 3% (C-B-PEO) for the pristine alternating copolymer and its organoboron amide-carboxyl and amide-ester-carboxyl derivatives, respectively. The possible interactions between functional copolymer and HeLa cells can be schematically represented as follows (Scheme 3.)

As seen from this scheme, the conjugation of copolymer with DNA biomacromolecules of HeLa cell through cells/ionized amide and organoboron groups (-->), $-H_2N$...HOOC– and (ether) > O... HOOC– H-bondings can significantly influence the destruction process of supramacromolecular structure of HeLa cell biomacromolecules, and therefore, can be exhibited apoptotic and necrotic effects.

CONCLUSIONS

Novel bioengineering alternating copolymer and its organoboron amide-ester-carboxyl functionalized copolymers were synthesized and characterized. This work presents the synthesis of novel poly(MA-*alt*-VDO) alternating copolymer and its organoboron, PEO branched derivatives by complex-radical copolymerization, amidization and grafting-esterification reactions, respectively. It was found that composition of copolymer is not dependent on the monomer feed ratios and the VDO (donor) and MA (acceptor) monomers as non-homopolymerizable monomers exhibit strong tendency to the formation of charge-transfer complex (K_c = 0.021 L.mol^{-1}) and alternating copolymers (r_1 (MA) = 0.007 and r_2 (VDO) = 0.002). The synthesized organoboron and PEO functionalized copolymers contain a combination of ionizable groups (carboxylic, amide and organoboron groups), hydrophilic/hydrophobic and H-bonding fragments allow us utilize as amphiphilic bioengineering copolymers for evaluation of their antitumor activity (cytotoxicity). The functional copolymer structure–compositon–property relationships and their cytotoxicity againtst HeLa cancer cells were investigated. It was demonstrated that the synthesized organoboron copolymers exhibit high antitumor activity through complex formation (H-bonding), and therefore, significantly influence the destruction process of supramacromolecular structure of HeLa cell biomacromolecules. Evaluation of the appoptic and necrotic effects in the interaction of these maltifunctional copolymers with HeLa cells using a combination of various biochemical and microscopy analysis methods will be a subject our furture investigations.

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