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Bioengineering Functional Copolymers. XIX. Synthesis of Anhydride-Organoboron Functionalized Copolymers and Their Interaction with Cancer Cells

Biyomühendislik Fonksiyonel Kopolimerler. XIX. Anhidrit-Organobor Fonksiyonlu Kopolimerlerin Sentezi ve Kanser Hücreleriyle Etkileşimleri

Research Article / Araştırma Makalesi

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ABSTRACT

Novel bioengineering functional organoboron homo- and copolymer of maleic anhydride (MA) were synthesized by (1) amidolysis of anhydride-containing macromolecules [poly(MA-*alt*-methyl vinyl ether (MVE) as a bioengineering polymer] with 2-aminoethyldiphenylborinate (2-AEPB), (2) esterification with α -hydroxy- ω -methoxy-poly(ethylene oxide) (PEO) as a compatibilizer and (3) conjugation of organoboron PEO branches with folic acid as a targeting agent. Structure and composition of the synthesized oligomers were characterized by FTIR-ATR and ¹H (¹³C) NMR spectroscopy. Antitumor activity of the organoboron functional (co)polymers were investigated by a combination of various physical and biochemical methods such as cytotoxicity, statistical, apoptotic and necrotic cell indexes, double staining and caspase-3 immunostaining, light and fluorescence inverted microscope analyses. It was found that cytotoxicity and apoptotic/necrotic effects of organoboron macromolecules significantly depend on the structure and composition of studied (co)polymers. Some synthesized (co)polymers at 400 μ g.mL⁻¹ concentration as a therapeutic drug exhibits minimal toxicity toward the normal cells, but influential for cancer cells.

Key Words

Organoboron polymers, structure-property relations, cytotoxicity, necrotic and apoptotic effects, cancer cells.

ÖZET

Maleik anhidritin homo- ve kopolimerlerinin yeni biyomühendislik fonksiyonel türevlerinin sentezi, (1) anhidrit-içeren makromoleküllerin [poli(MA-*alt*-methyl vinil eter (MVE) biyomühendislik polimeri] 2-aminoetildifenilborinat (2-AEPB) ile amidolizi, (2) alfa-hidroksi-omega-metoksi-polietilen oksit (PEO) uyumluluk ajanı ile esterleşmesi ve (3) organoboron PEO içeren kopolimerlerin folik asit gibi hedefleyici ajanla konjugasyonu gibi yöntemlerle gerçekleştirilmiştir. Sentezlenmiş oligomerlerin yapısı ve kompozisyonu FTIR-ATR ve ¹H (¹³C) NMR spektroskopisi ile karakterize edilmiştir. Organobor fonksiyonel kopolimerlerin antitümör aktivitesi sitotoksikite, istatistiksel, ikili boyama ve immün boyama ile apoptotik ve nekrotik hücre indeksi, ışık ve floresan inverted mikroskop analizi gibi çeşitli fiziksel ve biyokimyasal kombine yöntemler ile araştırılmıştır. Organoboron makromoleküllerinin sitotoksik ve apoptotik/nekrotik etkisi önemli ölçüde kopolimerlerin yapısına ve kompozisyonuna bağlı olduğu bulunmuştur. Sentezlenmiş bazı kopolimerlerin 400 μ g.mL⁻¹ derişim değerinde normal hücrelere düşük toksik, kanser hücrelerine ise etkili terapötik ilaç etkisi göstermiştir.

Anahtar Kelimeler

Organobor polimerleri, yapı-özellik ilişkileri, sitotoksikite, nekrotik ve apoptotik etki, kanser hücreleri.

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INTRODUCTION

The bioengineering functional polymers, such as (1) alternating and random copolymers of maleic anhydride (MA) and (2) poly(ethylene oxide) (PEO), as well as (3) PEO grafted functional macromolecules, have attracted much attention of many researchers due to their nontoxic, cell-compatible, biodegradable, stimuli-responsive properties, and therefore a wide range of biomedical and bioengineering applications as drug or enzyme carriers, biomacromolecular conjugates, in diagnostics and chemotherapy as effective antitumor agents [1-8]. It is known that these copolymers can be regarded as pre-activated polymers due to the presence of anhydride moieties susceptible to the reaction with a primary amine of a biomolecule [9]. The alternating copolymers of maleic anhydride (MA) with methyl vinyl ether (MVE) or divinyl ether (DVE) were utilized in various applications in diagnostics [10,11] and in chemotherapy as effective antitumor agents [8]. Poly(MA-*alt*-DVE), known as pyran copolymer is one of the well known bioengineering polymers having a wide range of biological activity. It processes antitumor, antiviral, antibacterial and antifungal activities, induces interferon formation, and acts as an anticoagulant and anti-inflammatory agent [8,12-17]. Chaix et al [11] described the oligonucleotide synthesis of MA copolymers covalently bound to silica spherical support and characterization of the obtained conjugates. Direct synthesis of oligonucleotides was achieved on the controlled porous glass surface grafted with poly(MA-*alt*-MVE) and poly(MA-*alt*-ethylene). Ladavière et al. [12] reported a study on the synthesis and characterization of soluble conjugates of nucleic acid probes and poly(MA-*alt*-MVE) with special attention on the kinetics of the coupling reaction and on the physico-chemical characteristics of the resulting conjugates. They also utilized this copolymer as a reactive polymer to link oligodeoxyribonucleotides containing free amine groups (ODN-NH₂) to make ODN-copolymer conjugates of potential application in diagnostics [13-15]. Recently, Sasai et al [16] reported the method to introduce a large amount of carboxyl groups onto polystyrene (PS) by the surface modification of PS petri dish with poly(MA-*alt*-

MVE) through plasma induced cross-linking reaction, followed by hydrolysis of copolymer to generate active sites for immobilization of the prostate cancer cell (LNCap). According to the authors, the microscopic images show a good attachment, adhesion and spreading behavior of cells on the PS/copolymer surface. Hirano et al [18,19] reported that the poly(MA-*alt*-DVE) conjugated with bovine erythrocyte superoxide dismutase (SOD) is resistant against the proteolytic enzymes in serum, and shows a prolonged half-life *in vivo*. They established an increase in half-life after intravenous injection, as well as its decreased immunogenicity [19]. It was demonstrated that the copolymer-SOD conjugate shows anti-inflammatory effect against rat re-expansion pulmonary edema at the first step of leukocyte adhesion [15]. Maeda [20] discussed the development and therapeutic potential of prototype macromolecular drugs for use in cancer chemotherapy an artificial bioconjugate of neocarzinostatin (NCS) and poly(maleic acid-*alt*-styrene) copolymer. The biological response-modifying effects, the mechanism of a tumor "enhanced permeability and retention" effect and the tumor-targeting mechanism of NCS-copolymer conjugate were also discussed. According to the author, a principal advantage in the use of this bioconjugate is the potential for a reduction or elimination of toxicity.

The copolymers of fumaric, citraconic and itaconic acid and their derivatives as isostructural analogues of MA, as well as copolymers of some *N*-substituted maleimides can also included to class of bioengineering polymer systems. Cam et al [21] evaluated the *in vitro* cytotoxicities of glycylmaleimide (GMI) copolymers using K-562 human leukemia cells and HeLa cells. They also evaluated the *in vitro* antitumor activities of copolymers against mice bearing sarcome 180. Monomeric GMI and its copolymers showed higher antitumor activity than well known 5-fluorouracil (5-FU) at any dosage tested.

The several researchers reported the complexes of PEO with serum albumin, pepsin, lysozyme and α -chymotrypsin [22-25] as well as mechanism of the interactions in PEO or ethylene oxide copolymer/bovine serum albumin) conjugates

[26,27]. Polymers with hydrophobic backbones and with PEO grafts are among the most studied amphiphilic graft copolymers [28]. The PEO grafted copolymers of MA were widely used as surfactants and materials for biomedical applications [29-34].

On the other hand, growing interest and much effort have been also focused on the synthesis of organoboron low molecular-weight functional compounds, biopolymer and drugs with boron ligands and evaluation of their suitability for the bioengineering applications. Aromatic boronic acid and its functional derivatives, and some functionalized carboranes have become an very important class organic compounds, which are utilized in a variety of biological and medical applications, such as carbohydrate recognition [35], neutron capture therapy for cancer treatment as effective tumour-targeting agents [36,37], especially for brain tumours [38,39], and protease enzyme inhibition [40]. Kataoka et al. [41-45] synthesized a novel water-soluble polymer with lectin-like function by introducing phenylboronates, as sugar-recognizing moieties, into the side-chain of poly(*N,N*-dimethylacrylamide) [41,42]. According to the authors, at physiological pH medium, phenylboronates form an appreciably stable complex with sialic acid (Neu5Ac), a characteristic anionic carbohydrate on the surface of the plasma membranes [43,44]. Authors suggested that boronate-containing polymer may be an effective immune-adjuvant for the induction of lymphokine-activated killer (LAK) cell [44]. They also demonstrated that the copolymers of 3-acrylamidophenylboronic acid and dimethylacrylamide with different compositions coated onto solid substrates support function as synthetic mitogens for mouse lymphocytes [45].

However, a wide range of functional polymer synthesis techniques can be utilized for the design of more effective synthetic routes to prepare new B-containing bioengineering polymers, especially copolymerization of organoboron monomer and chemical modification of biocompatible polymers with organoboron reactive compounds and monomers. Several researchers synthesized some bioengineering copolymers containing phenylboronic acid linkages by radical copolymerization and chemical modification

methods, which exhibit glucose-, RNA- and DNA-sensitive behavior [46-49]. Recently, we report the synthesis and characterization of organoboron copolymers by complex-radical copolymerization of *p*-vinylphenylboronic acid with *N*-isopropylacrylamide (NIPA), maleic and citraconic anhydrides, maleimide and chemical modification of poly(NIPA-*rand*-MA)s with organoboron amine, as well as synthesis of supramacromolecular poly(ethylene imine) macrocomplexes and PEO long branched derivatives of organoboron copolymers having stimuli-responsive and high HeLa cell transfection behavior [7,50-53].

The objective of this work is synthesis and characterization of novel bioengineering boron-containing functional copolymers with antitumor activity. In the present article, results of synthesis of a new generation of biocompatible boron-containing functional macromolecules having a combination of hydrophilic and hydrophobic segments, free carboxylic groups, positive charges and an ionized linkage as antitumor cities, along with an ability to conjugate with cancer cells were described and discussed. These organoboron copolymers were synthesized by (1) partially amidolysis of bioengineering homo- and alternating copolymer of maleic anhydride (MA) and methyl vinyl ether (MVE) with ethanolamine ester of diphenylboronic acid (2-AEPB) and (2) chemical modification (esterification) of synthesized organoboron copolymer with α -hydroxy- ω -methoxy-poly(ethylene oxide) (PEO) and complexing with folic acid (FA). Special attention was paid to the role of structural effects, especially to the influence of organoboron linkage, for the interaction of organoboron functional copolymers with HeLa cells and to the evaluation of cytotoxicity and antitumor activity by using a combination of various methods such as statistical, hematoxylin/eosin staining, apoptotic and necrotic cell indexes, and M30 immunostaining analyses.

EXPERIMENTAL

Materials

2-Aminoethyldiphenylborinate (2-AEPB) (Sigma-Aldrich, Germany) was purified by recrystallization from anhydrous ethanol: m.p. 193.5°C (by DSC); FTIR-ATR spectra of 2-AEPB, cm⁻¹: 3284 (vs) and 3220 (s) N-H stretching in NH₂, 3066(vs)-2870(s)

C-H stretching, 1611(vs) NH_2 bending and 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-NH₂, 1061(vs) N-H bending in NH₂ and 750-710(s) sharp bands due to boron-phenyl linkage; ¹H NMR spectra (δ , ppm) in CHCl₃-d₁: CH₂-O 1.49, CH₂-NH₂ 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): ¹H NMR spectra (δ , ppm) in CHCl₃-d₁: CH₂-O 3.75-3.45, OH end group 2.61 and O-CH₃ end group 2.16.

Poly(MA) with given molecular weight was synthesized by controlled/living RAFT radical polymerization method: $d = 1.24 \text{ g.cm}^{-3}$, acid number 112.5 mg KOH/g (by alkali titration), the average molecular weight (M_w) 7480 g.mol⁻¹ (by DLS), glass-transition temperature (T_g) 81.1°C (by DSC). Structure of MA oligomer was confirmed by FTIR and NMR spectroscopy: FTIR spectra (ATR), cm⁻¹: 2970 (m) for backbone CH stretching band, 1842 (w), 1774 (m) and 1703 (s) bands for antisymmetrical and symmetrical C=O band of MA unit, 1226 (w-m) and 1173 (m, broad) anhydride C-O-C stretching; ¹H NMR spectra (in DMSO-d₆ at 25°C) δ ppm: 2H around 2.5-4.0 for backbone CH-CH group in MA unit; ¹³C NMR (DEPT-135) spectra, ppm: (1) 170 for anhydride C=O 170 and 45.0 CH backbone in CH-CH group. Poly(maleic anhydride-*alt*-methyl vinyl ether), poly(MA-*alt*-MVE) (C1) (Sigma-Aldrich, Germany): M_n 80,000 g.mol⁻¹, T_g 148°C (by DSC); ¹H NMR spectra (δ , ppm) in DMSO-d₆: CH₂ 1.23, CH-O 2.11, O-CH₃ 2.08 and CH-CH 3.38. α -Hydroxy- ω -methoxy-poly(ethylene oxide) (Fluka; PEO, M_n 2000 g.mol⁻¹): ¹H NMR spectra (δ , ppm) in CHCl₃-d₁: CH₂-O 3.75-3.45, OH end group 2.61 and O-CH₃ end group 2.16.

N-Ethyl-*N*-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) as a catalyst and folic acid (FA) as a targeting agent were supported from Aldrich-Sigma (Germany). All solvents and reagents were of analytical grade and used without purification.

HeLa (human cervix carcinoma cell) cancer cells and L929 Fibroblast 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), and Trypsin-EDTA were purchased from Biological Industries (Kibbutz Beit Haemek, Israel). The primary antibody, caspase-3 was purchased from Lab Vision (Germany).

Synthesis

Boron-containing copolymer (C1-B) was synthesized by the partially amidolysis of succinic anhydride units of alternating copolymer (C1) with 2-AEPB, containing a primary amine group, in the 1,4-dioxane solution at 40°C for 3 h under nitrogen atmosphere at molar ratio of (co) polymer: 2-AEPB = 2:1. Appropriate quantities of PMA/C1 and EAPB, solvent were placed in a standard Pyrex-glass tube and flushed with dried nitrogen gas for at least 3 min, then placed in a carousel type microreactor with a thermostated heater and magnetic mixer. The resulting (co) polymer (PMA-B or C1-B) was isolated from reaction mixture by precipitating with diethyl ether. Purification of (co)polymers were done by dissolving in dioxane and reprecipitating in diethyl ether, extraction with hexane and drying under vacuum at 50°C until constant weight

Amidolysis of poly(MA) with 2-AEPB using various [MA unit]/[2-AEPB] mole ratios was carried out in *N,N'*-dimethylformamide (DMF) at 60°C with EDAC catalyst under the 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 unit]/[2-AEPB] = 1:1, 3:1, 5:1 and EDAC = 1.0 wt.%. Appropriate quantities of oligo(MA), 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 thermostated silicon oil bath at 60°C to intensive mixing for 5 h. The organoboron amide polymer was isolated from reaction mixture by precipitation with diethyl ether and dried under vacuum. Synthesized organoboron polymer has the following average parameters: M_w 7720 g.mol⁻¹ (by DLS), T_g (by DSC) 156°C, $[\eta]_m$ in deionized water at 25°C 0.02 L.g⁻¹ FTIR-ATR spectra of oligo (MA)-*g*-2-AEPB (KBr pellet), (cm⁻¹): 1693 (m) C=O stretching (amide I band), 1649

(m) and 1564 (m) N-H deformation (amide II band), 1436 (w) and 1386 (m) C-N stretching (amide III band); ¹H NMR spectra (in DMSO-d₆ at 25°C) δ ppm: protons of phenyl groups 6.9-7.18, 2H from CH₂ in -CH₂-CO-NH- fragment 5.8, 2H from B-O-CH₂ group 3.4, 2H from backbone -CH-CH- 3.1, and 2H from NH-CH₂ group 2.7; ¹³C NMR spectra, δ ppm: C=O of MA unit 177, C=O of amide linkage 173, CH= in phenyl groups 162-158, C-N 136 and 126, backbone CH 46, NH-CH₂ 41-42, CH₂-O 31-36.

PEO macrobranched (co)polymer (PMA-B-PEO and C1-B-PEO) was synthesized by the esterification of anhydride units of partially amidolysed C1-B copolymer with PEO, containing an end hydroxyl group, in the same conditions using in our previous publications [4,5]. PMA-B-PEO has the following average characteristics: M_w 8100 g.mol⁻¹ (by DLS), T_g 129°C (by DSC); ¹H NMR spectra (in DMSO-d₆ at 25°C) δ ppm: protons of phenyl groups 7.2, CH₂ in CH₂-CO-NH- amide linkage 6.0, weak and broad O-CH₂ in PEO branch 4.7, 2H in B-O-CH₂ 3.6, CH₂CH₂ in PEO branch 3.5, 3H in OCH₃ end group 3.2, backbone CH 3.1, and 2H in NH-CH₂ 2.7; ¹³C NMR spectra, ppm: C=O of MA unit 176, C=O of amide group 173, 5CH= in phenyl groups 162-158, C-N 136-126, CH₂CH₂ in PEO branch 71-72, O-CH₂ in PEO 69, (7) end OCH₃ group of PEO 58, backbone CH 46, NH-CH₂ 41-42, and CH₂-O-B 31-36.

Characterization

FTIR spectra of the organoboron copolymers (KBr pellet) were recorded with FT-IR Nicolet 510 spectrometer in the 4000-400 cm⁻¹ range, where 30 scans were taken at 4 cm⁻¹ resolution. ¹H {¹³C} NMR spectra were performed on a JEOL 6X-400 (400 MHz) spectrometer with DMSO-d₆ as a solvent at 25°C. The differential scanning calorimetry (DSC) analysis was performed on a Shimadzu calorimeter (Japan) at a heating rate of 5°C/min, under nitrogen atmosphere. The X-ray diffraction (XRD) patterns were obtained from a Rigaku D-Max 2200 powder diffractometer. The XRD diffractograms were measured at 2θ, in the range 1-50°, using a Cu-K_α incident beam (λ = 1.5406 Å), monochromated by a Ni-filter. The scanning speed was 1 °/min, and the voltage and current of the X-ray tubes were 40 kV and 30 mA, respectively.

The number of living and dead cells were counted with a haemocytometer (C.A. Hausse & Son Phluila, USA) at X200 magnification. The number of apoptotic and necrotic cells were determined by Fluorescence Inverted Microscope (Olympus IX70, Japan). The cell images were also recorded using the both above mentions microscopes. Statistical analyses were performed using Student's *t*-test for unpaired data and *P* values of less than 0.05 were considered significant. Data are presented as means ± SEM (standard errors of the mean).

Cytotoxicity

For cytotoxicity experiments, HeLa cells and L929 Fibroblast cells (25x10³ cells per well) were placed in DMEM by using 24-well plates, respectively. Different amounts of (co)polymers (about 50-500 µg.mL⁻¹ in aqueous solutions) were put into wells containing cells, respectively. The plates were kept in the CO₂ incubator (37°C in 5% CO₂) for 2-24 h; the medium was replaced with fresh medium, and incubated at the same conditions for 24 h. Following of this incubation, HeLa cells and L929 Fibroblast cells were harvested with trypsin-EDTA, and then were dyed with trypan blue [54]. The viable cells were counted with a haemocytometer (C.A. Hausse & Son Phluila, USA), using light microscope.

Hematoxylen/eosin staining

HeLa cells and L929 Fibroblast cells (25x10³ cells per well) were placed in DMEM by using 24-well plates. After treating with different amount functional (co)polymers (about 50-500 µg.mL⁻¹ in aqueous solutions) for 2-24 hours period, the medium was removed, the cells washed with distilled water and fixed in ethanol, and stained with Hematoxylen/Eosin. After staining, the cells were observed by light microscopy. By this way, cellular and nuclear morphology have been shown in cultured cells stained with Hematoxylen/Eosin.

Analysis of apoptotic and necrotic cells

Double staining were performed to quantify the number of apoptotic cells in culture on basis of scoring of apoptotic cell nuclei. HeLa cells and L929 Fibroblast cells (25x10³ cells per well) were placed in DMEM by using 24-well plates. After treating with different amount functional (co)

polymers (about 50-500 $\mu\text{g.mL}^{-1}$ in aqueous solutions) for 2-24 hours period, both attached and detached cells were collected, then washed with PBS and stained with Hoechst dye 3342 (2 $\mu\text{g.mL}^{-1}$), propodium iodide (PI) (1 $\mu\text{g.mL}^{-1}$) and DNase free-RNase (100 $\mu\text{g.mL}^{-1}$) for 15 min at room temperature. After that 10-50 μL of cell suspension was smeared on slide and coverslip for examination by fluorescence microscopy [55,56]. The nuclei of normal cells were stained light blue but apoptotic cells were stained dark blue by the hoechst dye. The apoptotic cells were identified by their nuclear morphology as a nuclear fragmentation or chromatin condensation. Necrotic cells were staining red by PI. Necrotic cells lacking plasma membrane integrity and PI dye cross cell membrane, but PI dye don't cross non necrotic cell membrane. The number of apoptotic and necrotic cells in 10 randomly chosen microscopic fields were counted and the result expressed as a ratio of apoptotic and necrotic to normal cells.

M30 immunostaining

The percentage of apoptotic cells was determined by M30 CytoDEATH antibody [57]. This is a monoclonal mouse immunoglobulin (Ig)G2b antibody (clone M30; Roche, Mannheim, Germany) that binds to a caspase-cleaved, formalin-resistant epitope of cytokeratin18 cytoskeletal protein. The immunoreactivity of the M30 antibody is confined to the cytoplasm of apoptotic cells. HeLa cells (25×10^3 cells per well), treated with C1, C1-B and, C1-B-PEO copolymers (about 50-500 $\mu\text{g. mL}^{-1}$ in aqueous solutions) for about 2-24 h, were fixed in 10% neutral-buffered formalin for 15 min, treated with 0.3% hydrogen peroxide in methanol for 10 min to block the endogenous peroxidase activity, washed in the standard phosphate buffer solution, and then incubated with M30 antibody at room temperature for 1 h. In negative controls, preimmune mouse serum instead of primary antibody was used. Immunoreactions were revealed by the avidin-biotin complex technique using diaminobenzidine (DAB) as substrate. We counted the number of M30-positive cytoplasmic staining cells in all fields found at x400 final magnification. For each image, three randomly selected microscopic fields were observed, and at least 100 cells/field were evaluated. M30

CytoDEATH antibody was not sensitive to L929 Fibroblast. On account of this reason, M30 CytoDEATH antibody did not applied to L929 Fibroblast cells.

RESULTS AND DISCUSSION

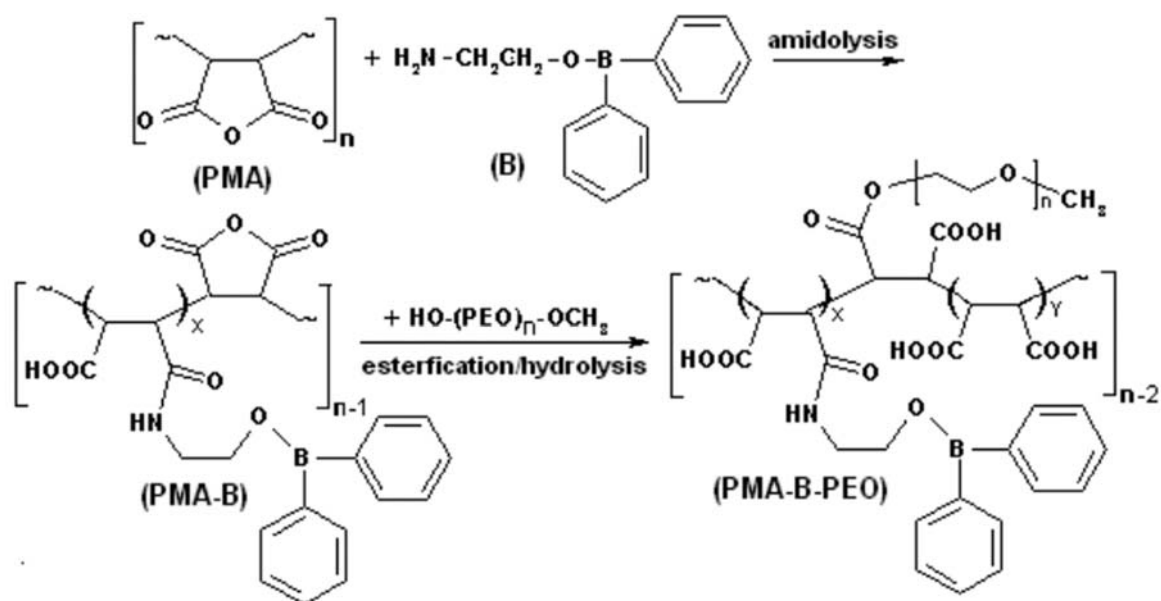
Synthesis and characterization of organoboron functional (co)polymers

Boron-containing bioengineering functional (co)polymers and its α -hydroxy- ω -methoxy-poly(ethylene oxide) (PEO) long branched derivatives were synthesized by (1) amidolysis of succinic anhydride units of water-soluble PMA and biocompatible poly(MA-*alt*-MVE) alternating copolymer (C1) with 2-AEPB containing a primary amine group, and (2) esterification (grafting) of free anhydride units of partially amidolysed C1-B copolymer with PEO, containing an end hydroxyl group, respectively.

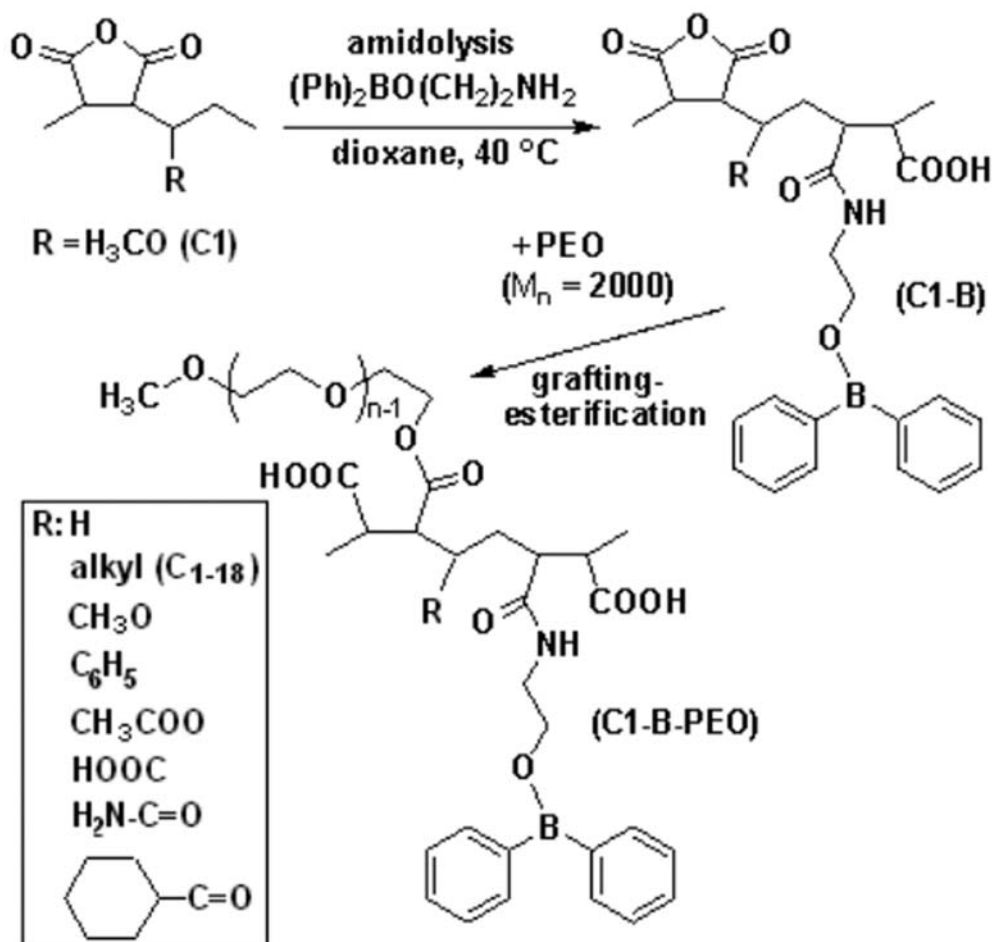
Synthetic partways of the side-chain amide-ester-carboxylic functionalized organoboron polymer can be represented in Scheme 1. General scheme of synthesis of the organoboron functional copolymers and its PEO macrobranched derivatives using various alternating copolymers of MA as matrix reactive polymers can be represented in Scheme 2.

The synthesized boron-containing copolymers contain a combination of hydrophilic/hydrophobic linkages, free carboxylic groups, positive charges and ionized organoboron linkage as antitumor sites, along with an ability to interact with cancer biomacromolecules, especially with HeLa cells. The chemical and physical structure, composition and properties (glass-transition, melting and degradation temperatures, and antitumor activity and cytotoxicity) of synthesized copolymers were characterized by spectroscopy (FTIR, ^1H and ^{13}C NMR), viscometry, DSC, and Fluorescence microscopy analyses.

The structures of synthesized organoboron polymers (PMA-B) and their PEO branches were confirmed by FTIR-ATR and ^1H (^{13}C) NMR analyses. Comparative analysis of FTIR-ATR spectra (Figure 1) of 2-AEPB, PMA and its organoboron derivative indicates that the characteristic bands of anhydride C=O groups disappearance in the spectra of



Scheme 1. Synthesis of the organoboron amide-ester-carboxylic polymers (PMA-B and PMA-B-PEO) through amidolysis and esterification/hydrolysis reactions of poly(maleic anhydride) (PMA) with 2-aminoethyldiphenylborinate (B) and α, ω -hydroxy-methoxypoly(ethylene oxide) (PEO), respectively.



Scheme 2. Schematic representation of the synthesis routes of organoboron functional copolymers (C1-B and C1-B-PEO) by the amidolysis of poly(MA-*alt*-MVE) (C1) with organoboron amine (2-AEPB) and esterification of poly(MA-*alt*-MVE)-g-AEPB (C1-B) with PEO, respectively.

PMA-B-1 polymer prepared from equimolar feed ratio of PMA:2-AEPB. The formation of amide bond in this organoboron oligomer is confirmed by the appearance of new bands such as 1693 (amide I. band), 1649 and 1564 (amide II. band), 1436 and 1386 (amide III. band). Simultaneously a very broad band between 3500 and 2800 cm^{-1} appearances in spectra due to increase hydrogen bonded fragments in organoboron oligomer (PMA-B-1). The intensities of amide bands significantly decrease in spectra of PMA-B-2 and PMA-B-3 oligomers prepared from PMA/2-AEPB mixtures enchasing with PMA (PMA \gg 2-AEPB).

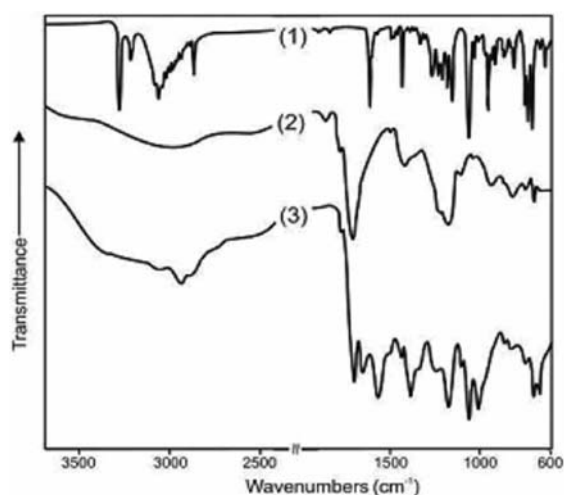


Figure 1. FTIR spectra of (1) 2-AEPB, (2) PMA and (3) PMA-g-2-AEPB.

Similar effect has been observed from comparative analysis of the ^1H NMR and ^{13}C NMR spectra of PMA-B-2 and its PEO branch (PMA-B-PEO). The results of this analysis are illustrated in Figures 2 and 3. The formation of H-bonded amide linkages is confirmed by a presence of characteristic broad peaks at 5.8 and 173 ppm in the ^1H NMR and ^{13}C NMR spectra of PMA-B-2, respectively (Figure 2). In addition, the presence of characteristic proton peaks of organoboron linkages such as quarter phenyl peak at 6.9 ppm, triplet B-O-CH₂ peak at 3.4 ppm and quarter NH-CH₂ peak at 2.7 ppm (Figure 2a) also confirmed that 2-AEPB is covalently bound to anhydride units. In the ^{13}C NMR spectra of PMA-B-2 (Figure 2b), the characteristic carbon resonances (162, 158, 136, 126, 41, 42, 31 and 36 ppm) from organoboron fragment are also observed. ^1H (^{13}C) NMR spectra of PEO grafted organoboron

oligomer (PMA-B-PEO) were illustrated in Figure 3. The observed proton signals from of side-chain PEO branches at 4.7, 3.5 and 3.2 ppm for (CH₂-CH₂-O)_n units (Figure 3a) and carbon atom resonances (71 and 72 ppm for CH₂CH₂, 69 ppm for O-CH₂ and 58 ppm for OCH₃ end group) (Figure 3b) may be served as an additional fact to confirm the formation of side-chain macrobranched PEO linkages.

The results of chemical structural analysis of the synthesized organoboron copolymers FTIR (KBr pellet) and (^1H and ^{13}C) NMR spectroscopy (in DMSO-*d*₆ solution) were summarized in Table 1 (FTIR analysis data for C1-B) and illustrated in Figure 4 (NMR spectra of C1-B). The formation of amide, carboxyl and organoboron groups in the structure of C1-B copolymer as results of amidolysis reaction was confirmed by appearance of the corresponded characteristic absorption bands for each monomer unit and diphenylboronic fragment in the spectra. Absorption bands at 1864 and 1781 cm^{-1} , relating to C=O groups of free anhydride units, indicated the partially amidolysis of these units as shown in Scheme 1.

From the comparative analysis of ^1H and ^{13}C NMR spectra of virgin alternating copolymer (C1) and its organoboron derivative (C1-B) (Figure 4a, 4b), the following changes of the characteristic signals were observed: unlike the spectra of C1 copolymer having the peaks from chemical shifts of the CH and CH₂ backbone and CH₃ (in methoxy group) protons new signals from protons of amide NH, COOH and phenyl groups (in organoboron linkage) were appeared in the spectra of organoboron copolymer (C1-B). More detailed informations about microstructure of C1-B copolymer were prepared by analysis of ^{13}C NMR spectra (Figure 4c). The following chemical shifts (δ , ppm) of carbon atoms were observed in the spectra: 174.4 (-C=O of the maleamide and anhydride units), 128-136 (-B-C₆H₅ mono-substitued benzene ring), 77.2 (-CH-NH in organoboron linkage), 58.01 (-CH-O), 49.08 (-CH-CH- chain backbone), 30.3 (-CH₃-O), and 30.15 (-CH₂).

Chemical structure of C1-B-PEO long branched copolymer was confirmed by the appearance in the FTIR spectra (Table 1) the following characteristic absorption bands (cm^{-1}): 3400 (strong broad peak

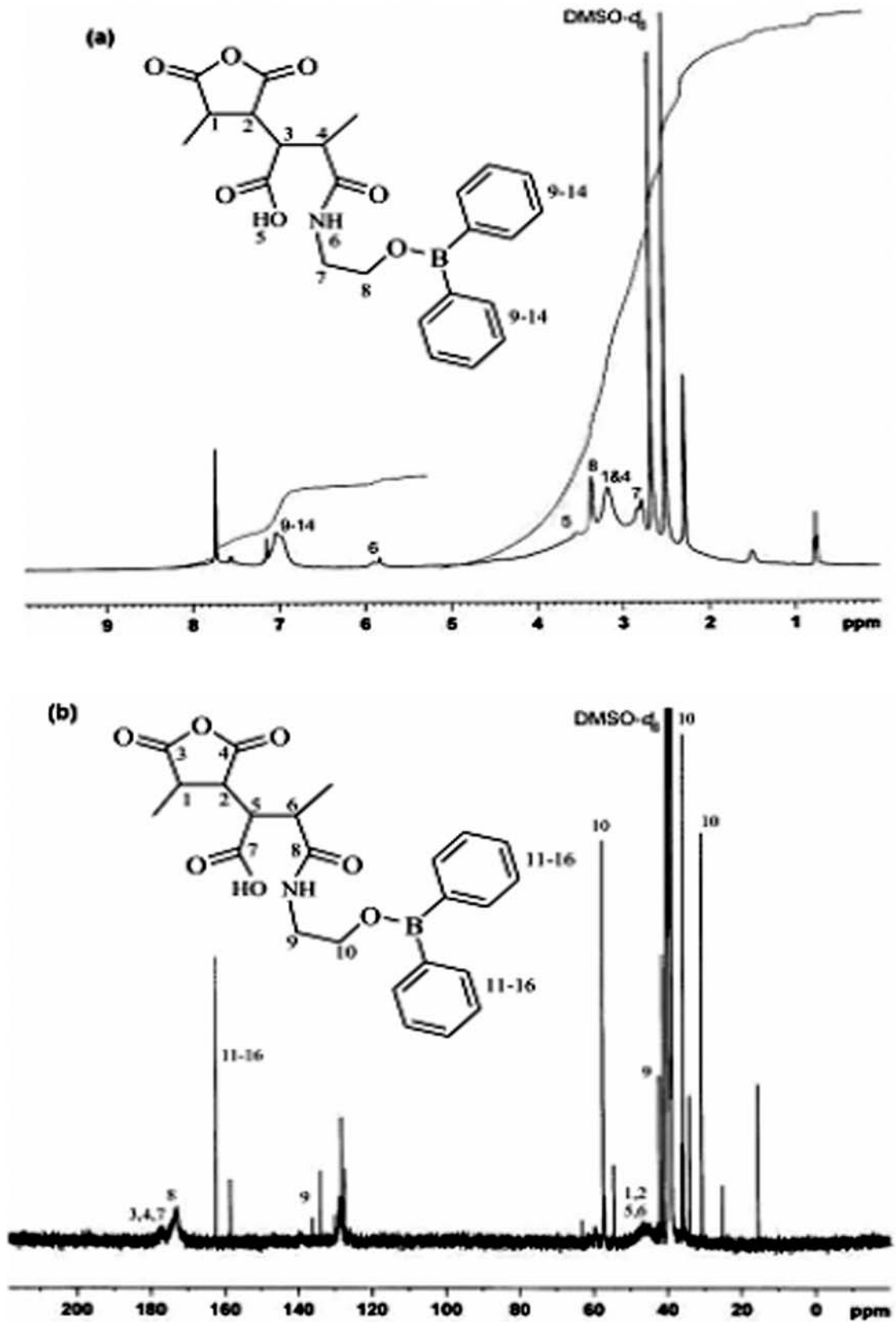


Figure 2. (a) ¹H NMR and (b) ¹³C NMR spectra of PMA-g-2-AEPB in DMSO-d₆.

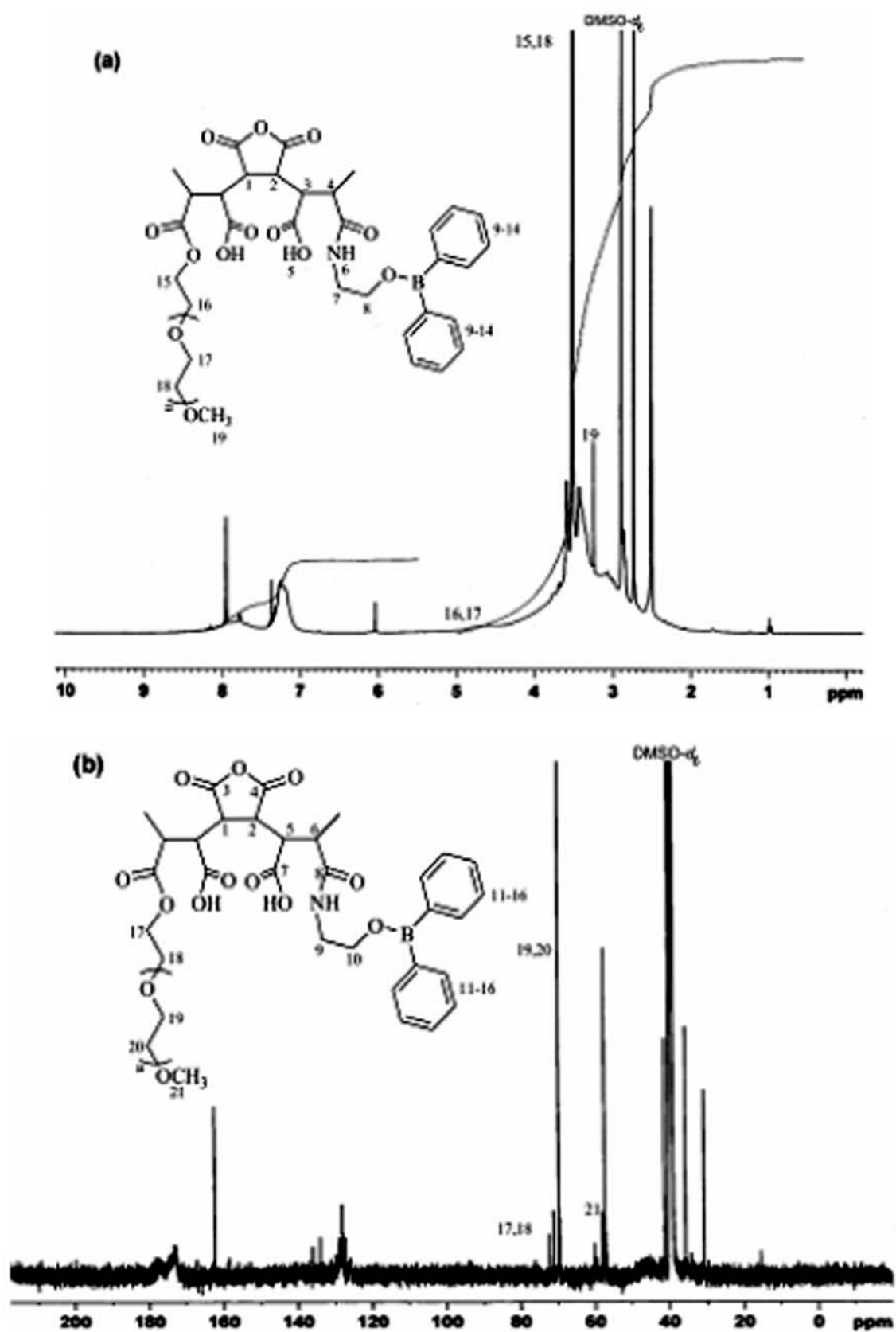


Figure 3. (a) ¹H NMR and (b) ¹³C NMR spectra of PMA-g-2-AEPB-g-PEO in DMSO-d₆.

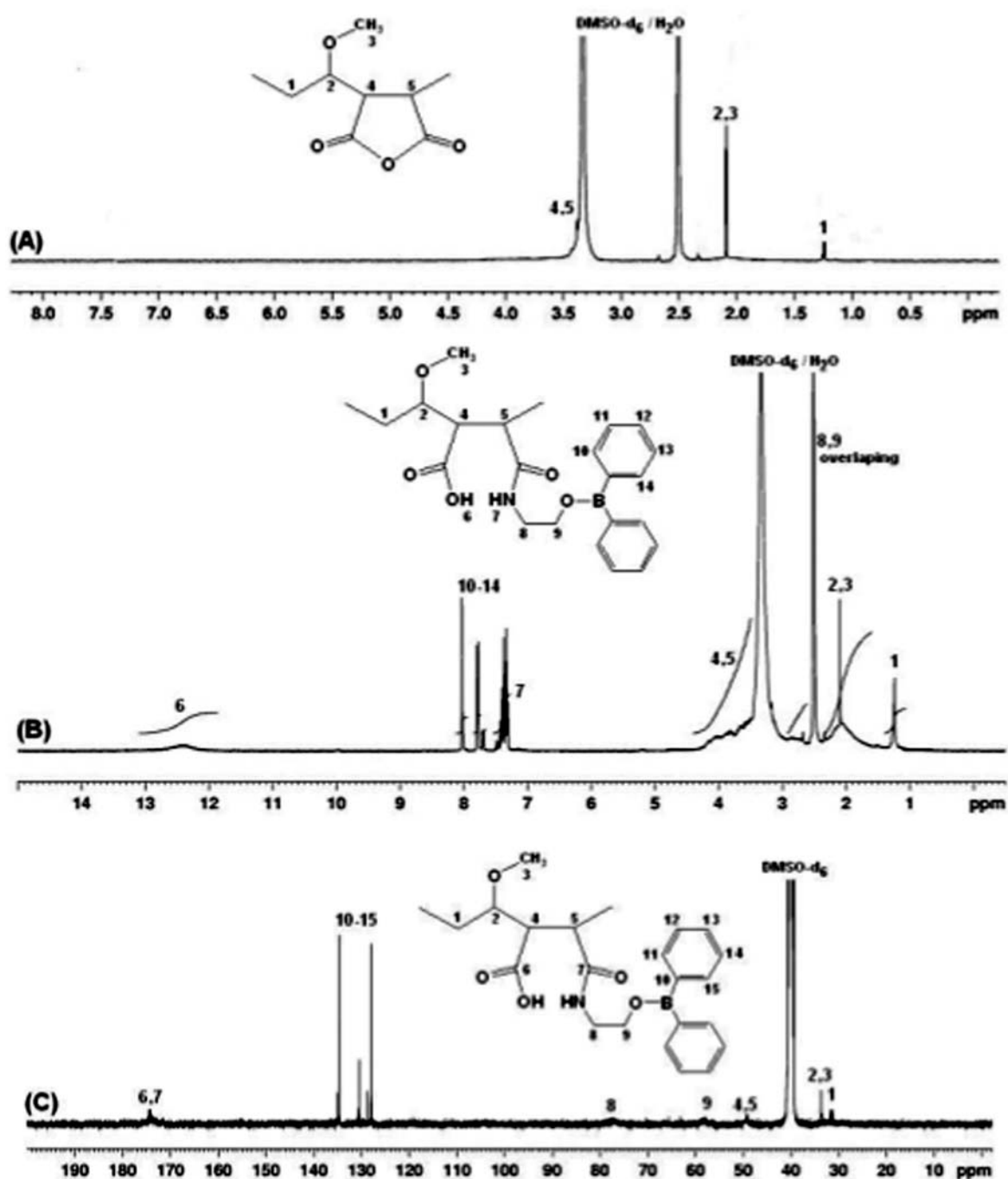


Figure 4. ¹H NMR spectra of (A) C1 copolymer and (B) C1-B organoboron copolymer; (C) ¹³C NMR spectra of C1-B copolymer.

for OH in H-bonded carboxyl groups), 2933-2735 for C-H stretching in CH₂ and CH₃), 2667 and 2600 (C-H stretching in CH₂-O of PEO branched segments), 2280 and 2135 (Fermi doublet for C-N band), 1986 and 1966 (overtones of C=O), 1746 (C=O of ester groups), 1710 (C=O of carboxyl groups), 1630 (NH-C=O amide I band), 1592 (phenyl groups), 1558 (H-bonded COO⁻ stretching), 1545 (weak peak for

B-O stretching), 1490 (C-H deformation for CH₂-O in PEO branches), 1480 and 1466 (CH₂ deformation), 1450 (B-Ph aromatic ring), 1405 (amide III band), 1372 and 1352 (CH₃ deformation in O-CH₃), 1115 (broad peak for C-O band in CH₂-O and CH₃-O of PEO and MVE units, respectively), 948 (strong peak for C-O deformation in PEO branches), and etc.

Table 1. FTIR analysis of organoboron functional copolymer: Poly(MA-*alt*-MVE)-*g*-AEPBA) (C1-B).

Absorption bands (cm ⁻¹)	Band assignments
MA unit	
1980-1925 (w)	C=O (overtones)
1864 (m-s), 1781 (vs)	C=O stretching (anhydride)
1227 (s, broad), 1094 (s)	C-O and C-O-C bands
650 (w)	CH (in chain backbone)
MVE unit	
2942 (m-s)	CH ₃ C-H stretching
2854 (m)	CH ₂ C-H (chain backbone)
1475-1416 (m)	CH ₂ and CH ₃ deformation
1372 (m)	CH ₃ deformation (in O-CH ₃)
975 (m)	CH ₃ rocking
926 (vs)	C-O deformation
735 (m-w), 720 (w)	CH ₂ and CH ₃ deformation
Maleamide unit	
1736 (m-s)	C=O stretching (in -COOH)
1575-1510 (m-w)	COO ⁻ stretching (H-bonding)
1650 (m), 1720 (m-w)	NH-C=O amide I band
1315 (w)	amide III band
Organoboron linkage	
3240 (w), 3100, 1600 (m)	CH= (in aromatic ring)
1545 (m-w)	B-O stretching
1443 (m), 1420 (w)	B-Ph aromatic ring
1180 (m)	CH in-put-bending
770 (w)	CH out-put-bending
702 (m)	O-B-Ph aromatic ring

The comparative analysis of the XRD patterns of alternating copolymer and its organoboron derivative show a significant difference between physical structures of these copolymers. C1 copolymer has an amorphous structure, while C1-B copolymer exhibits pseudo-crystallinity behavior (without re-crystallization process due to macromolecular physical interactions via H-bonding, hydrophobic-hydrophilic interactions, etc.) with degree of pseudo-crystallinity $\chi_c = 26.2\%$ (by XRD analysis), glass-transition T_g and pseudo-melt phase transition T_m at 84.2°C and 136.3°C, respectively (by DSC analysis). It can be proposed that the producing the amphiphilic organoboron linkages in side chain of copolymer causes a formation of hydrophilic/hydrophobic balance, more polar amide and carboxyl groups, which are able to form strong H-bonded segments, and therefore, self-assembled suramacromolecular structure of C1-B copolymer as in other organoboron polymer systems [7,50].

Cytotoxicity of the (co)polymer and its B-containing and PEO branched derivatives

Organoboron polymers (PMA-B): The obtained cytotoxicity results of the pristine PMA and its organoboron amide (PMA-B) and organoboron amide-ester (PMA-B-PEO) branches, and PMA-B-PEO/folic acid complex (PMA-B-PEO-F) on cancer cells using a Trypan blue staining were illustrated in Figure 5. As seen from plots of concentration of polymers versus percent of cell viability, the toxicity of pristine PMA against cancer and normal cells decreased with increasing in polymer concentration from 50 to 200 $\mu\text{g.mL}^{-1}$ for 24 h incubation at 37°C. If oligomer concentration was higher than 200 $\mu\text{g.mL}^{-1}$, its toxicity increased, especially higher toxicity exhibits for 24 h incubation. The toxicity of PMA-B (organoboron amide oligomer) was more significant than other oligomer systems.

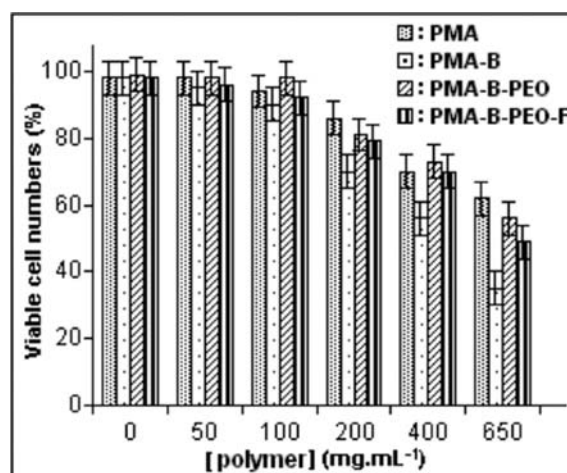


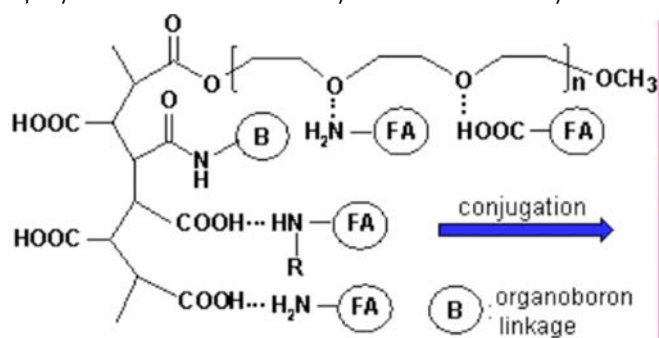
Figure 5. In vitro cytotoxicity of PMA, PMA-B and PMA-B-PEO and PMA-B-2-PEO-F functionalized oligomers with different amount at 24 h incubation. Viable of HeLa cells in wells (%). Results are presented as means \pm SEM.

Figure 5 shows that the number of viable cells is above 80 % for normal and cancer cells after incubation of the cells with OMA-B at concentrations around 50-200 $\mu\text{g.mL}^{-1}$ for 24 h incubating time in cell culture media. The number of viable cells was over 50% for normal cells in the range of 400-650 $\mu\text{g.mL}^{-1}$ concentration. However, the toxicity of cancer cells was increasing beginning from 400 $\mu\text{g.mL}^{-1}$. PMA-B and PMA-B-PEO-F complex had higher toxicity for cancer cells (35% alive cells) than normal cells (48% alive cells) in 650 $\mu\text{g.mL}^{-1}$ concentration of complex. When PEO was added to the structure (OMA-B-PEO), the cytotoxicity was

decreased because of PEO biocompatibilization effect. The cytotoxicity of PEO containing oligomer was lower than those without PEO at 400-650 $\mu\text{g.mL}^{-1}$ concentration (Figure 5). To improve the targeting of oligomer macromolecules to cancer cells, folic acid (FA) was inserted to the structure through complex-formation. The formation of oligomer...FA complex through intraction amine groups of FA with ester groups of branched PEO and free carboxylic groups and its conjugation with HeLa cells may be schematically represented in Scheme 3.

When the oligomer-FA complex was incubated, the toxicity of the HeLa cells was higher than that of the normal cells, because cancer cells had more FA receptors than normal cells. Because MA-B-PEO-F complex can be used as a therapeutic drug at 400 $\mu\text{g.mL}^{-1}$ concentration. In this concentration, the toxicity was minimal for normal cells, but influential for cancer cells.

Organoboron Copolymers (C1-B): In this study, the comparative analysis of HeLa cells (cancel cells) and L929 Fibroblast cells (normal cells) has been investigated. The cytotoxicities of C1 copolymer and corresponding C1-B, C1-B/PEO derivatives were inquired about the utility for antitumor drugs. Figures 6 and 7 give the number of viable cancer and normal cells in each group after incubation of the cells with copolymer and organoboron copolymers at their different concentrations for 24 h incubating time in cell culture media, respectively. Under the same conditions, the wells containing cells without copolymers were also studied as a control. The following important results can be drawn from this graph which is illustrated in these Figures. The C1 copolymer does not exhibit any observable toxicity



Scheme 3. Proposed supramacromolecular structure of functionalized, PEO branched and complexing with FA (folic acid) organoboron polymer and its conjugation with cancer cells (nucleus of apoptotic cells).

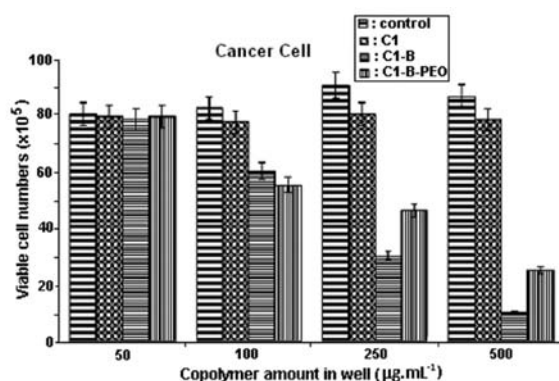


Figure 6. In vitro cytotoxicity of C1, C1-B and C1-B-PEO copolymers with different amount at 24 h incubation. Number of viable HeLa cells in wells. Results are presented as means \pm SEM. *Significant difference from control ($p < 0.05$).

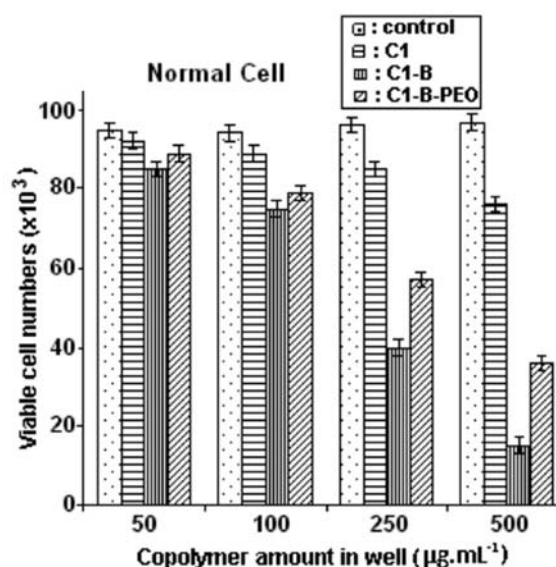
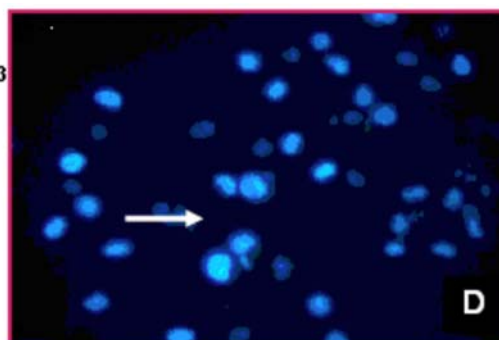


Figure 7. In vitro cytotoxicity of C1, C1-B and C1-B-PEO copolymers with different amount at 24 h incubation. Number of viable L929 Fibroblast cells in wells. Results are presented as means \pm SEM. *Significant difference from control ($p < 0.05$).



in the chosen range of copolymer concentration. The toxicity of polymers containing boron (C1-B and C1-B-PEO) was significant, most probably due to hydrogen bonding supramacromolecular structure of these copolymers containing a combination of hydrophilic/hydrophobic linkages, free carboxylic groups, which are formed after partial amidolysis of anhydride containing copolymer C1 and full hydrolysis of free anhydride units in the chosen physiological medium where positive charges and ionized organoboronyoxy groups also exist as antitumor sites along with an ability to interact with cells.

It was observed that an increase of C1-B and C1-B-PEO concentrations in each well caused higher degree of dying cells as compared to virgin C1 copolymer tested under the same conditions. C1-B copolymer exhibits relatively higher in vitro cytotoxicity than C1-B-PEO branched copolymer which can be explained by the higher content of organoboron linkages in C1-B copolymer. It is important to note that the boron containing side chain linkages, rather than the individual copolymers, increase the cytotoxicity more profoundly; an important feature which has a significant role in leading us to the present study. C1 copolymer had less toxicity compared to cultured cells at various quantities and different incubation times. On the contrary, the toxicity of C1-B and C1-B-PEO organoboron copolymers towards the HeLa cells increased by increasing their quantity from 50 to 500 $\mu\text{g.mL}^{-1}$, whereas, no significant change was observed with varying time. According to Figure 6, C1 did not show high toxicity at all although the copolymer amount was increased from 50 to 500 $\mu\text{g.mL}^{-1}$ whilst, a significant toxicity of C1-B and C1-B-PEO (100 $\mu\text{g.mL}^{-1}$ and above) started to be observed when cancer and normal cells (Figure 7) were incubated for about 4 h. As the amount of boron containing polymers and their incubation time increased, toxicity to cultured cells was increased. C1-B-PEO and especially C1-B showed higher toxicity at 500 $\mu\text{g.mL}^{-1}$. Thus, it can be concluded that virgin C1 alternating copolymer does not exhibit any toxic effect on cultured HeLa cells, whereas, its organoboron and PEO branched derivatives are definitely toxic to cells. In particular, C1-B copolymer containing relatively high amount

of organoboron linkages exhibits high toxicity toward cancer cells compared to normal cells at 500 $\mu\text{g.mL}^{-1}$ for 24 h of incubation period.

Hematoxylen-eosin staining results

Organoboron polymers: For the morphological observations in the oligomer-cell systems, the mixtures of the pristine PMA, PMA-B (amide organoboron), PMA-B-PEO (amide-ester branched organoboron) and/or PMA-B-PEO-F (amide-ester organoboron/folic acid) complex with HeLa and Fibroblast cells at 50-650 $\mu\text{g.mL}^{-1}$ concentration for 24 hours were stained by Hematoxylen-Eosin. The wells containing cells and medium without oligomer were also studied as positive control. The obtained results were illustrated in Figure 8. The results of comparative morphological analyses indicated that a significant difference between cancer cells and control group morphology does not observed at 50-400 $\mu\text{g.mL}^{-1}$ concentration for 24 hours (Figures 8A, B and C). Cell morphology is changed at 400-650 $\mu\text{g.mL}^{-1}$ for 24 hours, but remained unchanged at 50-200 $\mu\text{g.mL}^{-1}$ for 24 hours. Cell morphology of PMA-B has been changed at the 400 $\mu\text{g.mL}^{-1}$ concentration. Moreover, some of the cells have been detached from the well.

Organoboron copolymers: C-1 copolymers treated cancer and normal cells have intact nucleus of about 50-200 $\mu\text{g.mL}^{-1}$ concentration during 2-14 h incubation. Cell morphology has not been changed at the same concentration for 2-14 h (Figure 9b). While C1-B and C1-B-PEO copolymers treated HeLa cells has no morphological changes at 50-200 $\mu\text{g.mL}^{-1}$ concentration for about 2-4 h, they have vacuole formation in their cytoplasm with C1-B copolymer between 6-12 hours (Figure 9c). Vacuole formations determined rarely in normal cells (Figure 9e, f). In addition, cell membranes have lysed with C1-B copolymer around 12-24 h but, there was no change in their nuclei of cancer and normal cells. Moreover, some of the cells (30% and 15% for HeLa and fibroblast, respectively) have been detached from the well. Unaffected cells displayed similar morphological characteristics as with untreated (control) cells.

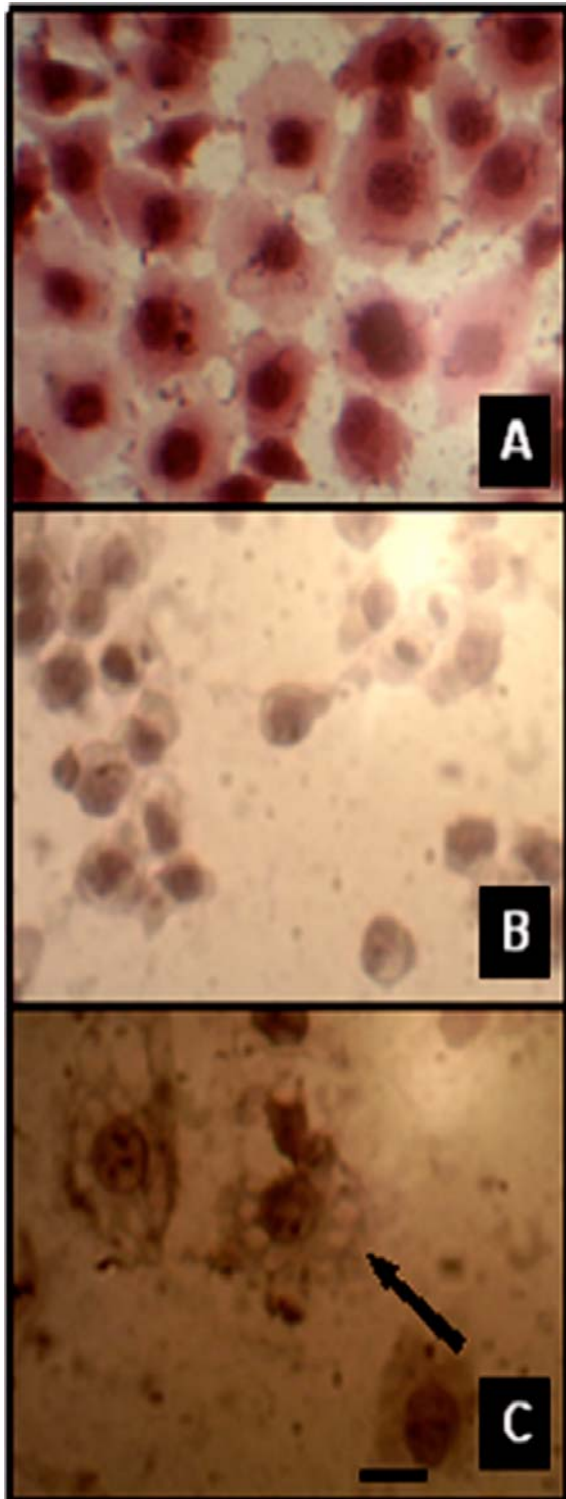


Figure 8. Light microscope image of (A) non stained HeLa cell culture as a control, (B) PMA-B-2-PEO oligomer ($650 \mu\text{g.mL}^{-1}$)/HeLa cells conjugate (stained with hematoxylin-eosin dye); dense spots were showed nucleus of cells, and distinct violet were indicated cytoplasm of cells, (C) Light microscope image of vacuole of PMA-B oligomers ($650 \mu\text{g.mL}^{-1}$)/HeLa cells cytoplasm; dense spots were showed nucleus of cells. All images were recorded under x400 magnification. Scale bar is $20 \mu\text{m}$.

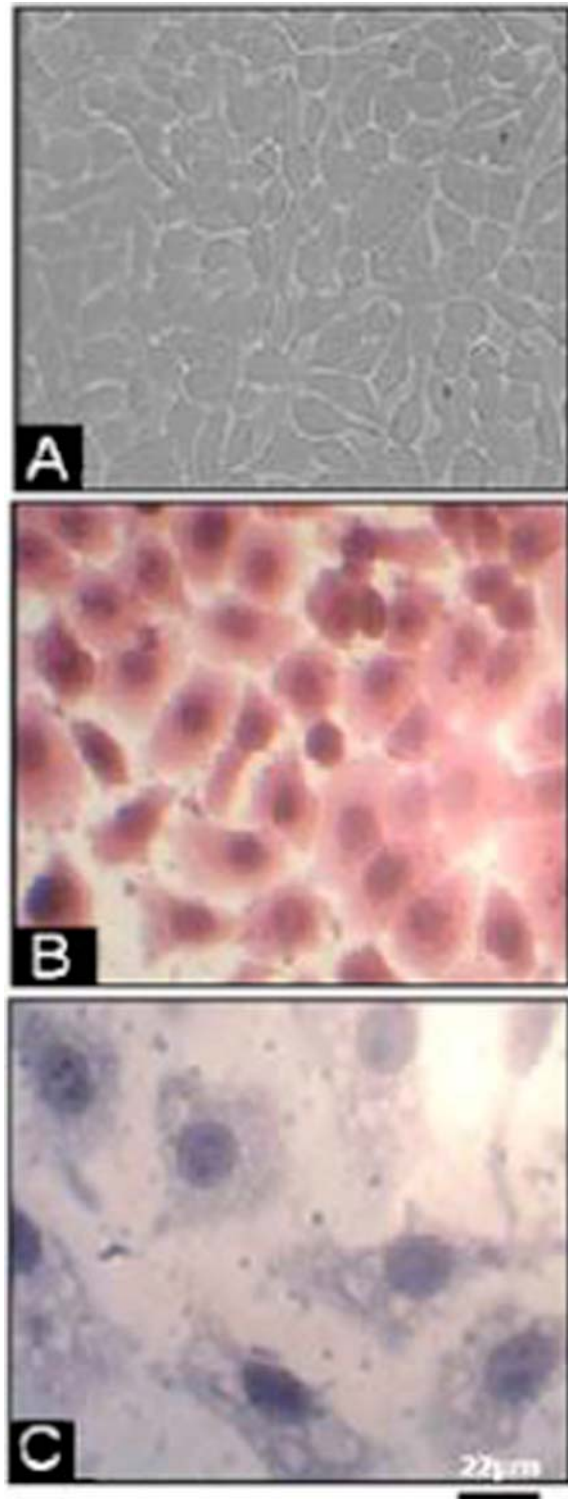


Figure 9. Light microscope image of (A) non stained HeLa cell culture as a control, (B) C1-B-PEO copolymer/HeLa cells conjugate (stained with hematoxylin-eosin dye); dense spots were showed nucleus of cells, and distinct violet were indicated cytoplasm of cells, (C) Light microscope image of vacuole of HeLa cells cytoplasm; dense spots were showed nucleus of cells. All images taken under x400 magnification.

Double staining, M30 and Caspase 3 immunostaining results

Organoboron polymers: Apoptotic index was obtained by both double staining and caspase 3 immunostaining methods. If the cells treated by PMA-B oligomer at 400-650 $\mu\text{g.mL}^{-1}$ concentration, the number of apoptotic cells was not high. While PMA-B-PEO-F complex at 400 $\mu\text{g.mL}^{-1}$ concentration had the highest apoptotic ratios on cancer cells. In addition, the number of apoptotic cells was high as well for the organoboron oligomer/folic acid complex at 400 $\mu\text{g.mL}^{-1}$ concentration. The results of Light and Fluorescent microscope investigation of the interaction of organoboron oligomers with cancer and normal cells were illustrated in Figure 10. The cytoplasm of apoptotic cells treated with complex were stained brown (Figure 10A) but, the cytoplasm of non apoptotic cells were not stained brown (Figure 10B). According to double staining results, apoptotic cells' nucleus stained bright blue and compartmentalized, but non-apoptotic cells' nuclei stained lifeless blue (Figure 10C and 10D). When the PEO-containing oligomers applied, the number of apoptotic cells was decreased. However, the number of apoptotic cells was increased as 29% when they treated by PMA-PEO-B-F complex in 650 $\mu\text{g.mL}^{-1}$ concentration. Apoptotic index for Fibroblast cells was 21% at 400 $\mu\text{g.mL}^{-1}$ concentration of PMA-PEO-B-F. Moreover, there was no significant changes on apoptotic index (21%) of Fibroblast cell targeting by folic acid. Apoptotic indexes in cancer and normal cells was estimated of caspase-3 and double staining result. The important observations can be summarized as follows: we checked for apoptosis or necrosis with double staining (Hoechst 33342 and PI) and caspase 3 immunostaining. It was observed that both the cytotoxicity and necrotic indexes of synthesized functional organoboron oligomers show approximately same values. The oligomers with lower concentrations (50-200 $\mu\text{g.mL}^{-1}$) decrease in necrosis stained with PI dye. While the necrotic indexes of normal and cancer cells increase at relatively higher concentration (400 $\mu\text{g.mL}^{-1}$) of oligomers, especially PMA-B (Figure 10F). However, when the oligomer containing PEO was incubated to cancer cells, the necrotic index was decreased in cancer and normal cells. Fluorescent microscope image of

nucleus of untreated HeLa cells (stained with PI dye) as a control was presented in Figure 10E, where formation of green spots demonstrates the nucleus of non-necrotic cells. Cancer cells exposed to oligomer-folic acid complex became highly PI-positive. This observed fact indicated that the cells were undergo to necrosis. HeLa and Fibroblast cells incorporated with PMA-B oligomer provide a lysing the cell-membrane (necrosis) and relatively higher necrotic indexes 65% and 51% for the cancer and normal cells, respectively. When both the cells treated with PMA-PEO-B-F complexes, necrotic indexes decrease for HeLa (49%) and Fibroblast cells (45%).

Organoboron copolymers: If the HeLa and L929 Fibroblast cells treated by C1, C1-B, C1-B-PEO copolymers at low concentration for a short time, the number of apoptotic and necrotic cells was not high. However, if the polymer concentration and incubation time were increased, the number of apoptotic and necrotic cells was increased as well. Especially, the number of apoptotic and necrotic cells was increased when they were treated by C1-B copolymer at 500 $\mu\text{g.mL}^{-1}$ concentration in cancer cell culture for 24 h. The number of apoptotic and necrotic Fibroblast cells was not increased according to HeLa cells at the same concentration. If cells were treated by the other copolymer under similar conditions, their apoptotic index was below 30%. Meanwhile, apoptotic HeLa cells were immunostained by M30 antibody (Figure 11a, b). The double staining and M30 immunostaining results were similar to each other in HeLa cells. Apoptotic indexes of HeLa cells for M30 immunostaining were 12% for C1, 45% for C1-B, 23% for C1-B-PEO at 500 $\mu\text{g.mL}^{-1}$ and 24 h incubation. Apoptotic L929 Fibroblast cells were stained only double staining method. In addition to these polymers, especially boron containing polymers had toxic effects towards cancer and normal cells. But toxic effect of boron containing polymers was lower to normal cells than cancer cells. After an incubation at 50-500 $\mu\text{g.mL}^{-1}$ for 24 h period, C1 resulted in less apoptosis, while incubation with C1-B and C1-B-PEO at the same concentration and incubation time led to high apoptosis of HeLa cells compared to L929 Fibroblast cells. Both C1-B and C1-B-PEO may well inhibit cell growth and viability in HeLa (Figure 11b, c d) and L929 Fibroblast cells (Figure 11e, f). One the

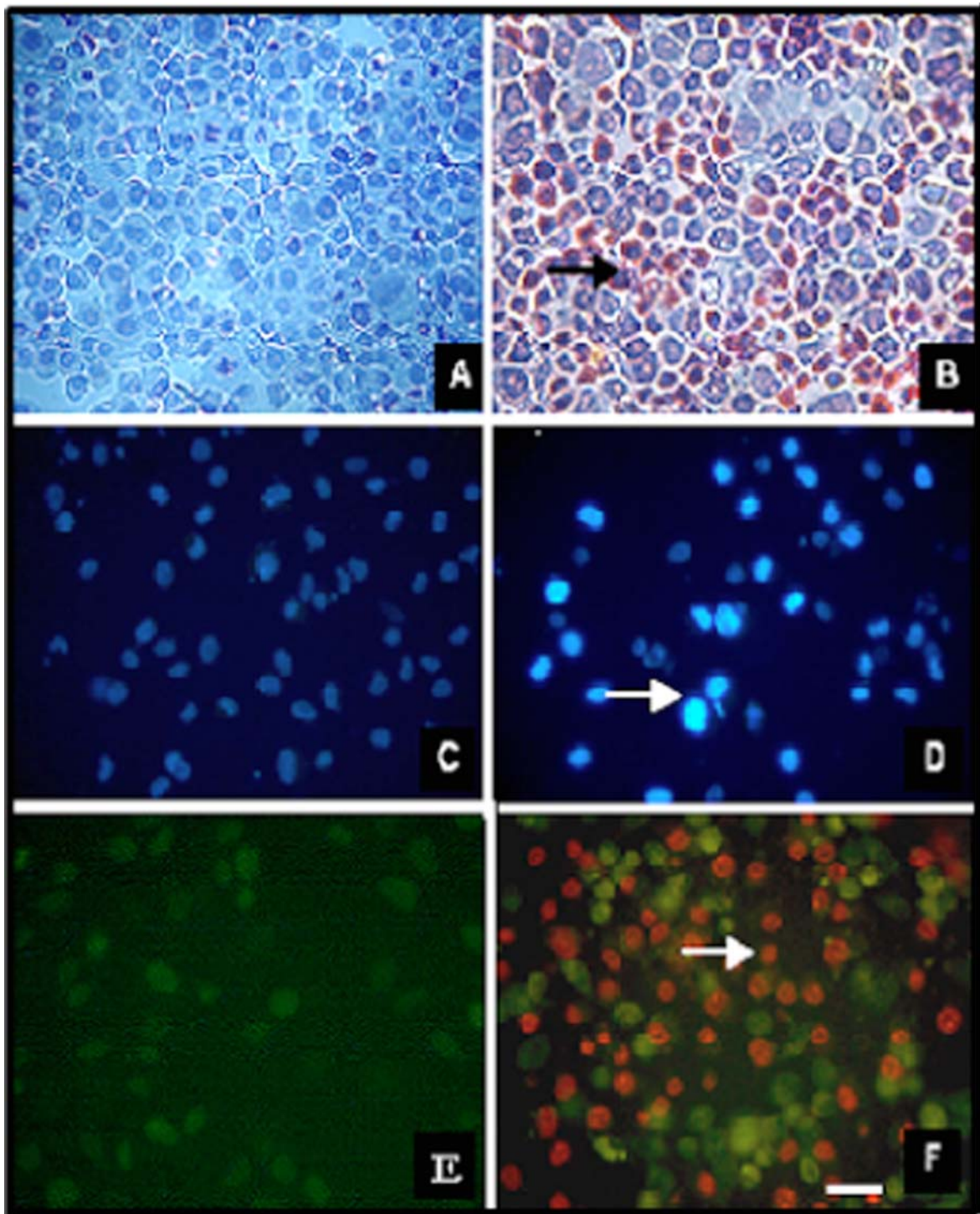


Figure 10. Light microscope images of (A) virgin (nonapoptotic) HeLa cells as a control group (stained with M30 immunostaining kit), and (B) $400 \mu\text{g.mL}^{-1}$ concentration of organoboron oligomer (PMA-B)/ HeLa cells conjugate (stained with M30 immunostaining kit), where brown cytoplasm of cells image indicates the formation of apoptotic cells; (C) Fluorescent microscope image of nucleus of untreated HeLa cells (stained with Hoechst 33342 dye) as a control, where formation of lifeless spots demonstrates nucleus of nonapoptotic cells; (D) nucleus of HeLa cells (stained with Hoescht 33342), where dense spots indicates nucleus of apoptotic cells; (E) Fluorescent microscope image of nucleus of untreated HeLa cells (stained with PI dye) as a control, where formation of green spots demonstrates nucleus of nonnecrotic cells; (F) nucleus of HeLa cells (stained with PI dye), where red spots indicates nucleus of necrotic cells and green spots indicates nucleus of nonnecrotic cells. Photos C and D taken under DAPI filter, photos E and F taken under FITC filter. All images were recorded with x400 magnification. Scale bar is $20 \mu\text{m}$.

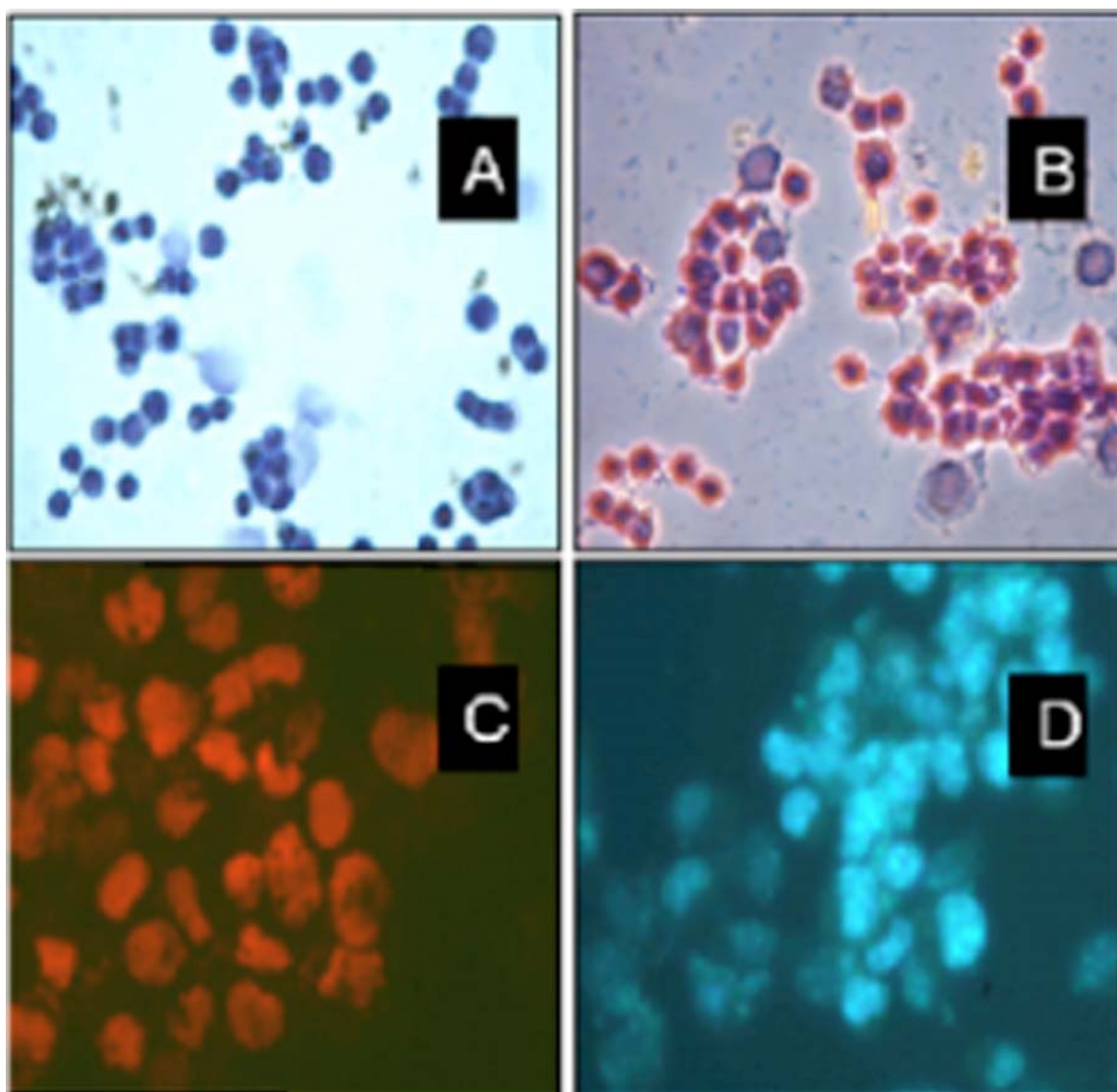


Figure 11. Light microscopy images of (A) virgin (nonapoptotic) HeLa cells as a control group (stained with M30 immunostaining kit), and (B) organoboron copolymer C1-B copolymer/HeLa cells conjugate (stained with M30 immunostaining kit), where cells image indicates the formation of apoptotic cells; Fluorescence microscopy image of (C) nucleus of HeLa cells (stained with PI), where formation of spots demonstrates nucleus of cells, and (D) nucleus of HeLa cells (stained with Hoescht 3342), where dense spots indicates nucleus of apoptotic cells. All images were recorded with x400 magnification.

other hand, around 50-500 $\mu\text{g.mL}^{-1}$ of C1-B and C1-B-PEO copolymer contents for 24 h gave rise to an increase in necrosis stained with PI dye (Figure 11c, e). It is important to note that incubation for 24 h with 500 $\mu\text{g.mL}^{-1}$ C1-B produced apoptosis supporting its high toxicity and necrotic effect. Furthermore, incubation without polymers as control cells resulted in a few PI-positive cells. Whereas, cells exposed to C1-B and C1-B-PEO became highly PI-positive, suggesting that they were in necrosis. HeLa cells incubated with a high dose of boron

containing copolymers resulted in rupture of cell membrane at around 12-24 h incubation period. Cell cytoplasm was discharged out of HeLa cells. On the other hand, great of number vacuole originated in most of HeLa cells cytoplasm. It may have given rise to metabolic changes of cells, affected by boron containing copolymers. C1-B copolymer was more toxic than virgin copolymer C1 and C1-B-PEO macrobranched copolymer. Thus we have observed the most apoptotic and necrotic effects of organoboron copolymer (C1-B) towards HeLa and

L929 Fibroblast cells whereas a minor effect relative to cancer cells was observed on L929 Fibroblast cells under the testing conditions determined by us.

CONCLUSIONS

This work has attempted to develop novel bioengineering functional organoboron (co) polymers (PMA-B, PMA-B-PEO, PMA-B-PEO-F, C1-B and C1-B-PEO), namely, amphiphilic macromolecules of which contained hydrophilic/hydrophobic fragments, ethylene amidodiphenylborinate linkages, long branched PEO segments and free carboxylic groups with an ability to conjugate with cancer HeLa cells. These (co) polymers were synthesized by amidolysis and esterification of anhydride units of PMA and poly(MA-*alt*-MVE) (C1) as a biocompatible and non toxic polymer matrix with organoboron amine and PEO, respectively. Chemical and physical structures of organoboron (co)polymers were confirmed by FTIR and ^1H (^{13}C) NMR spectroscopy and XRD parameters, respectively.

Antitumor activity (cytotoxicity, apoptotic and necrotic effects) toward HeLa and Fibroblast cells by using a combination of various biochemical, statistical and microscopy methods. It was observed that antitumor activity significantly depends on the structure, amount of ionizable free carboxylic groups, organoboron linkages and complexed fragments in the functionalized oligomers, and changes in the following row: PMA << (PMA-B) < PMA-B-PEO < PMA-B-PEO-F. Apoptotic indexes in cancer and normal cells were estimated of caspase-3 immunostaining and double staining (Hoechst 33342 and PI) results. These observations are confirmed the realization of apoptosis and necrosis processes in the interaction of organoboron functionalized polymers with normal and cancer cells. HeLa and Fibroblast cells incorporated with PMA-B polymer provide a lysing the cell-membrane (necrosis) and relatively higher necrotic indexes 65% and 51% for the cancer and normal cells, respectively. It was found that interactions of both the cells with PMA-PEO-B-F complexes were decreased the necrotic indexes for HeLa (49%) and Fibroblast cells (45%).

The comparative analysis of novel organoboron functional copolymers with antitumor activity towards cancer and normal cells was achieved. It was found that unlike the virgin amorphous C1 copolymer, organoboron copolymer (C1-B) exhibited semi-crystalline phase transition behaviour due to the formation of self-assembled supramacromolecular structures through strong intra- and intermolecular hydrogen bonding. The interactions of these copolymers with HeLa cells were investigated by using a combination of different methods such as cytotoxicity, statistical, hematoxylen/eosin staining, apoptotic and necrotic cell indexes, M30 immunostaining, double staining and M30 immunostaining, light and fluorescence microscopy analyses. *In vitro* cytotoxicities and antitumor activities of organoboron copolymers (C1-B and C1-B-PEO) against human cervix epithelioid carcinoma cell line (HeLa) was as well evaluated. It was observed that organoboron copolymers exhibited the most apoptotic and necrotic effects against HeLa cells whereas a minor effect relative to cancer cells was observed on L929 Fibroblast cells. Thus the obtained results allow us to propose that synthesized organoboron (co) polymers containing a combination of non toxic and biocompatible polymer matrix, long branched PEO segments and functional groups as antitumor sities, can be utilized as therapeutic potential functional (co)polymer drugs, which are able to form an artificial bioconjugate with HeLa cells, in cancer chemotherapy.

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