

Bioengineering functional copolymers. XII. Interaction of boron-containing and PEO branched derivatives of poly(MA-*alt*-MVE) with HeLa cells

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ABSTRACT

Novel boron-containing bioengineering copolymer and its α -hydroxy- ω -methoxy-poly(ethylene oxide (PEO) macrobranched derivatives were synthesized by (1) partially amidolysis of poly(maleic anhydride-*alt*-methyl vinyl ether) with ethanolamine ester of diphenylboronic acid and (2) esterification of synthesized B-containing copolymers with PEO. They had a combination of hydrophilic/hydrophobic linkages, free carboxylic groups, positive charges and an ionized organoboron linkage as antitumor sites, along with an ability to interact with HeLa cells. The structure, composition and properties (cytotoxicity and antitumor activity) of synthesized copolymers were investigated. *In vitro* cytotoxicity results, obtained by the fluorescence microscopy measurements indicate that unlike the virgin copolymer, boron-containing and PEO macrobranched derivatives exhibit higher antitumor activity. It was found that organoboron copolymer exhibits the most apoptotic and necrotic effects against HeLa cells whereas a minor effect relative to cancer cells was observed on L929 Fibroblast cells.

Keywords: Synthesis; Organoboron Copolymers; Structure-Property Relations; Cytotoxicity; Antitumor Activity; Apoptotic Effect

1. INTRODUCTION

The bioengineering functional polymers exhibit the characteristics of 1) alternating and random copolymers of maleic anhydride (MA) and 2) poly(ethylene oxide) (PEO), as well as 3) PEO grafted functional macromolecules. They are of great interest for many researchers

due to their nontoxic, cell-compatible, biodegradable, stimuli-responsive properties, and therefore, a wide range of biomedical and bioengineering applications exist as drug or enzyme carriers and biomacromolecular conjugates both 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]. 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 glycinylnmaleimide (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.

One 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 [22], neutron capture therapy for cancer treatment as effective tumour-targeting agents [23,24], especially for brain tumours [25,26], and protease enzyme inhibition [27]. Kataoka *et al.* [28-32] 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) [28,29]. According to the authors, at physiological pH medium, phenyl-boronates form an appreciably stable complex with sialic acid (Neu5Ac), a characteristic anionic carbohydrate on the surface of the plasma membranes [30,31]. Authors suggested that boronate-containing polymer may be an effective immune-adjuvant for the induction of lymphokine-activated killer (LAK) cell [31]. 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 [32].

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 are exhibit glucose-, RNA- and DNA-sensitive behavior [33-36]. 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) macro-complexes and PEO long branched derivatives of organoboron copolymers having stimuli-responsive and high HeLa cell transfection behavior [37-40].

The objective of this work is to develop novel boron-containing functional copolymers with antitumor activity. In the present article, results of synthesis and characterization 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 sites, along with an ability to conjugate with cancer cells were described and discussed. These organoboron copolymers were synthesized by 1) partially amidolysis of bioengineering alternating copolymer of maleic anhydride (MA) and methyl vinyl ether (MVE) with ethanolamine ester of diphenylboronic acid (EAPB) and 2) chemical modification (esterification) of synthesized organoboron copolymer with α -hydroxy- ω -methoxy-poly (ethylene oxide (PEO)). 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, hematoxylen/eosin staining, apoptotic and necrotic cell indexes, and M30 immunostaining analyses.

2. MATERIALS AND METHODS

2.1. Materials

Ethanolamine ester of diphenyl boronic acid (EAPB) (Sigma-Aldrich, Germany) was purified by recrystallization from anhydrous ethanol: m.p. 193.5°C (by DSC). 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. 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.

Human cervix epithelioid carcinoma cell line (HeLa) was obtained from the tissue culture collection of the SAP Institute (Turkey). Cell culture flasks and other plastic material were purchased from Corning (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 (Israel). M30 CytoDEATH antibody (Roche).

2.2. Synthesis

Boron-containing copolymer (C1-B) was synthesized by the partially amidolysis of succinic anhydride units of alternating copolymer C1 with EAPB, containing a primary amine group, in the 1,4-dioxane solution at 40°C for 3 h under nitrogen atmosphere at molar ratio of C1:EAPB = 2:1. Appropriate quantities of 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 copolymer C1-B was isolated from reaction mixture by precipitating with diethyl ether. Purification of copolymers was done by dissolving in dioxane and reprecipitating in diethyl ether, extraction with hexane and drying under vacuum at 50°C until constant weight.

PEO macrobranched copolymer (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 [5,37].

2.3. 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 $\{^{13}\text{C}\}$ NMR spectra were performed on a JEOL 6X-400 (400 MHz) spectrometer with DMSO- d_6 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 2 θ , in the range 1-50°, using a Cu-K α incident beam ($\lambda = 1.5406 \text{ \AA}$), 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 mentioned 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 \pm SEM (standard errors of the mean).

2.3. Cytotoxicity

For cytotoxicity experiments, HeLa cells and L929 Fibroblast cells respectively. (25×10^3 cells per well) were placed in DMEM by using 24-well plates. Different amounts of copolymers (C1, C1-B and C1-B-PEO)

(about 50-500 $\mu\text{g.mL}^{-1}$ 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 [41]. The viable cells were counted with a haemocytometer (C.A. Hausse & Son Phluila, USA), using light microscope.

2.4. Hematoxylen/Eosin Staining

HeLa cells and L929 Fibroblast cells (25×10^3 cells per well) were placed in DMEM by using 24-well plates. After treating with different amount functional copolymers (C1, C1-B and C1-B-PEO) (about 50-500 $\mu\text{g.mL}^{-1}$ 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.

2.5. 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 (25×10^3 cells per well) were placed in DMEM by using 24-well plates. After treating with different amount functional copolymers (C1, C1-B and C1-B-PEO) (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 $\mu\text{g.mL}^{-1}$), propidium 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 [42,43]. 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.

2.6. M30 Immunostaining

The percentage of apoptotic cells was determined by M30 CytoDEATH antibody [44]. This is a monoclonal mouse immunoglobulin (Ig) G2b antibody (clone M30; Roche,

Mannheim, Germany) that binds to a caspase-cleaved, formalin-resistant epitope of cytokeratin 18 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.

3. RESULTS AND DISCUSSION

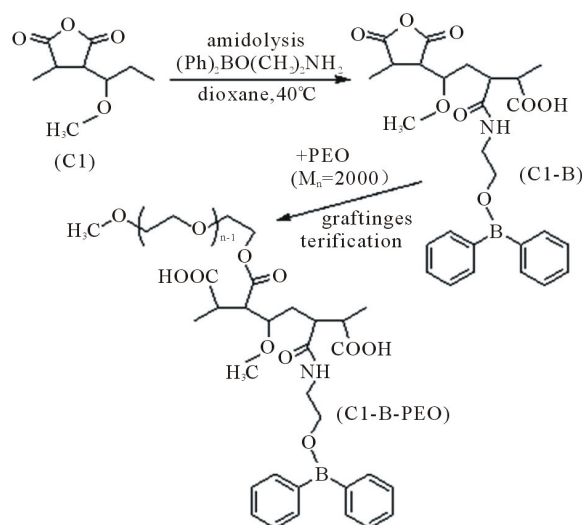
3.1. Synthesis and Characterization of Organoboron Functional Copolymers

Boron-containing bioengineering functional copolymer (C1-B) and its α -hydroxy- ω -methoxy-poly(ethylene oxide) (PEO) long branched derivatives were synthesized by (1) amidolysis of succinic anhydride units of biocompatible poly(MA-*alt*-MVE) alternating copolymer (C1) with EAPB 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. General scheme of synthesis of the organoboron functional copolymer and its PEO branched derivative can be represented as follows (Scheme 1).

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 (temperature-responsiveness, 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, X-ray diffraction and Fluorescence microscopy analyses.

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 1** (NMR spectra of C1-B) and **Figure 2** (FTIR spectra of C1-B-PEO). 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



Scheme 1. 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 (AEPB) and esterification of poly (MA-*alt*-MVE)-g-AEPB (C1-B) with PEO, respectively.

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

Absorption bands (cm^{-1})	Band assignments
MA unit	
1980-1925 (w)	C=O (overtone)
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

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 2a, 2b**), 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 CH_2 backbone and CH_3 (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 pre-pared by analysis of ^{13}C NMR spectra (**Figure 2c**). 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– C_6H_5 mono-substitued benzene ring), 77.2 (–CH–NH in organoboron linkage), 58.01 (–CH–O), 49.08 (–CH–CH-chain backbone), 30.3 (– CH_3 –O), and 30.15 (– CH_2).

Chemical structure of C1-B-PEO long branched copolymer was confirmed by the appearance in the FTIR spectra (**Figure 3**) the following characteristic absorption bands (cm^{-1}): 3400 (strong broad peak for OH in H-bonded carboxyl groups), 2933-2735 for C–H stretching in CH_2 and CH_3), 2667 and 2600 (C–H stretching in CH_2 –O of PEO branched segments), 2280 and 2135 (Fermi doublet for C–N band), 1986 and 1966 (overtone 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 CH_2 –O in PEO branches), 1480 and 1466 (CH_2 deformation), 1450 (B–Ph aromatic ring), 1405 (amide III band), 1372 and 1352 (CH_3 deformation in O– CH_3), 1115 (broad peak for C–O band in CH_2 –O and CH_3 –O of PEO and MVE units, respectively), 948 (strong peak for C–O deformation in PEO branches), and etc.

The comparative analysis of the XRD patterns of alternating copolymer and its organoboron derivative show a significant difference between physical structures of these copolymers (**Figure 3**). 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 [37].

3.2. Cytotoxicity of the Copolymer and its B-Containing and PEO Branched Derivatives

In this study, the comparative analysis of HeLa cells (cancer 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 4 and 5** 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 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 organoboronoxy 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 4**, 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 5**) were incubated for about 4 h. As the amount of boron containing polymers and their incubation

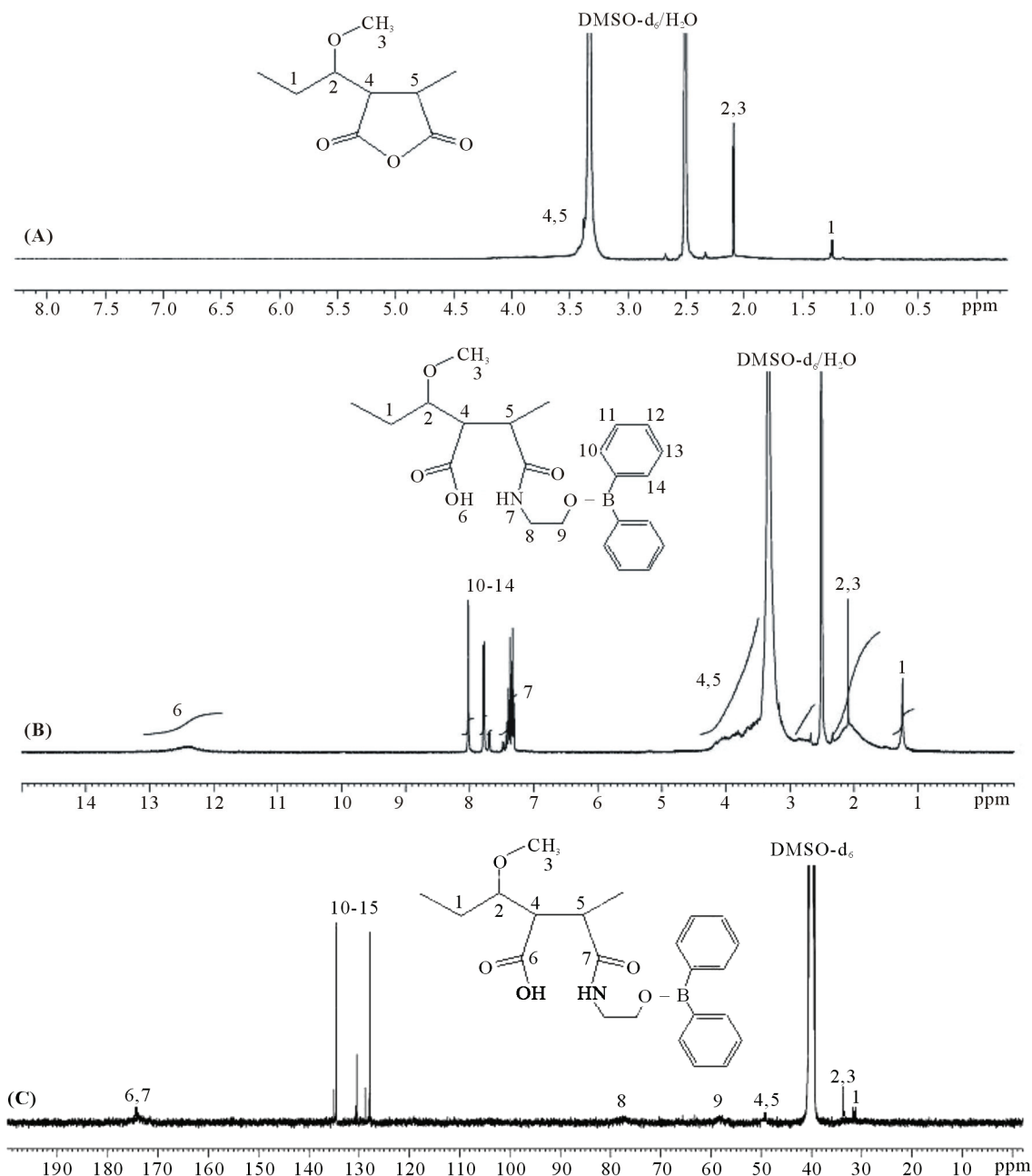


Figure 1. ^1H NMR spectra of (A) C1 copolymer and (B) C1-B organoboron copolymer; (C) ^{13}C NMR spectra of C1-B copolymer.

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.

3.3. Staining Results

The important observations can be summarized as follows: we checked for apoptosis or necrosis with double staining (Hoescht 3342 and PI), M30 immunostaining for cancer cells. For the morphological observations, cancer and normal cells were stained by hematoxylen-eosin.

3.4. Hematoxylen-Eosin Staining Results

In this study, C-1 copolymers treated cancer and normal

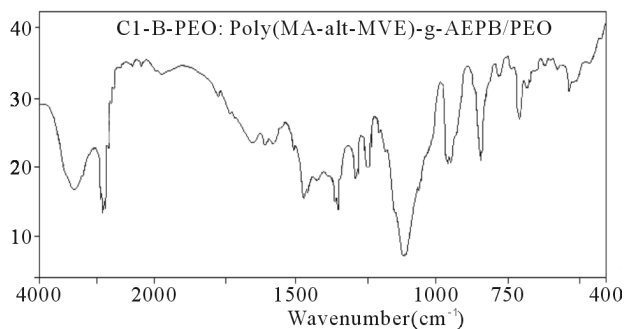


Figure 2. FTIR spectra of PEO macrobranched organoboron copolymer (C1-B-PEO).

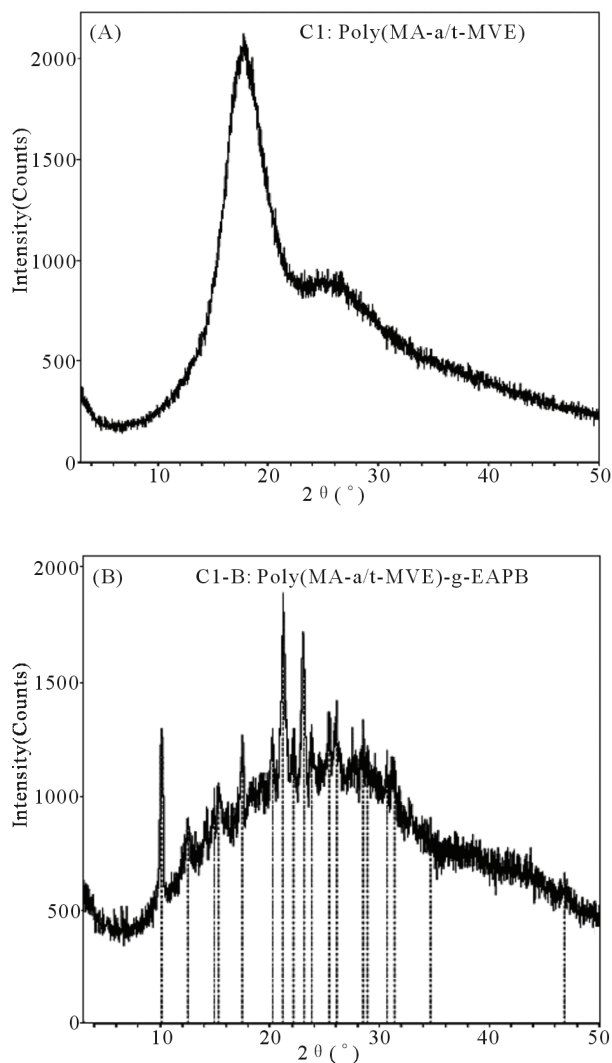


Figure 3. XRD patterns of (A) alternating copolymer (C1) and (B) its branched organoboron derivative.

cells have intact nucleus of about $50\text{--}200\ \mu\text{g.mL}^{-1}$ concentration during 2-14 h incubation. Cell morphology has not been changed at the same concentration for 2-14 h (**Figure 6b**). While C1-B and C1-B-PEO copolymers

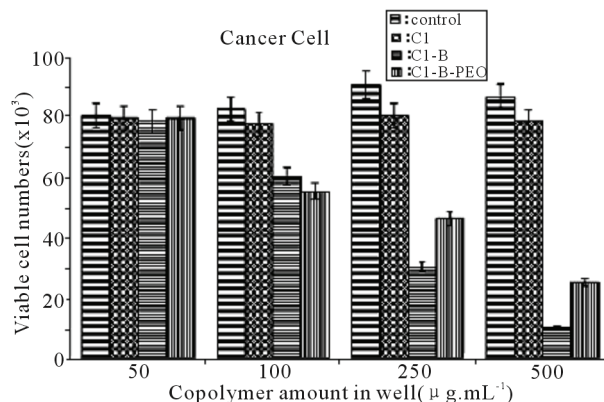


Figure 4. In vitro cytotoxicity of C1, C1-B and C1-B-PEO copolymers with different amount at 24 h incubation. Number of viable HeLa cells in wells. Results are presented as means \pm SEM. *Significant difference from control ($p < 0.05$).

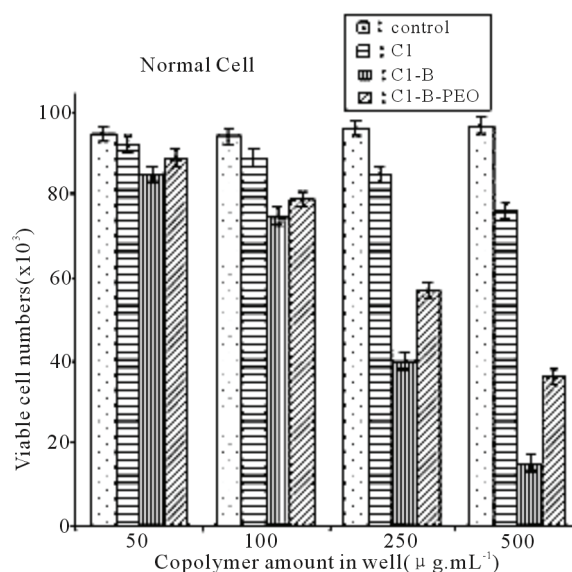


Figure 5. In vitro cytotoxicity of C1, C1-B and C1-B-PEO copolymers with different amount at 24 h incubation. Number of viable L929 Fibroblast cells in wells. Results are presented as means \pm SEM. *Significant difference from control ($p < 0.05$).

treated HeLa cells has no morphological changes at $50\text{--}200\ \mu\text{g.mL}^{-1}$ concentration for about 2-4 h, they have vacuole formation in their cytoplasm with C1-B copolymer between 6-12 hours (**Figure 6c**). Vacuole formations determined rarely in normal cells (**Figure 6e, 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.

3.5. Double Staining and M30 Immunostaining Results

In this study, 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 (Tables 2 and 3). 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 (Table 2). The number of apoptotic and necrotic Fibroblast cells was not increased according to HeLa cells at the same concentration (Table 3). If cells were treated by the other copolymer under similar conditions, their apoptotic index was below 30 %. The results obtained at $500 \mu\text{g.mL}^{-1}$ concentration for 24 h are shown in Table 2. Meanwhile, apoptotic HeLa cells were immunostained by M30 antibody (Figure 7a, 7b). 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 (Table 3). 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 $50\text{--}500 \mu\text{g.mL}^{-1}$ 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 7b, c, d) and L929 Fibroblast cells (Figure 7e, f). On the other hand, around $50\text{--}500 \mu\text{g.mL}^{-1}$ of C1-B and C1-B-PEO copolymer contents for 24 h gave rise to an increase in necrosis stained with PI dye (Figure 7c, e and Tables 2 and 3). 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 number of 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 under the testing conditions determined by us.

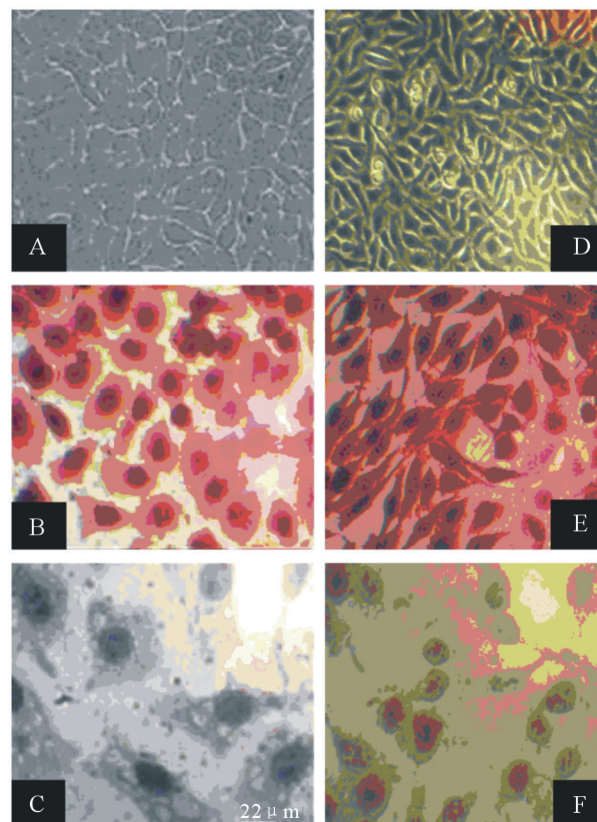


Figure 6. Light microscope image of (A) non stained HeLa cell culture as a control, (B) C1-B-PEO copolymer/HeLa cells conjugate (stained with hematoxylin-eosin dye); dense spots were showed nucleus of cells, and distinct violet were indicated cytoplasm of cells as a control, (C) Light microscope image of vacuole of HeLa cells cytoplasm; dense spots were showed nucleus of cells in C1-B copolymers ($500 \mu\text{g.mL}^{-1}$ concentration) at 24 h incubation. Light microscope image of (D) non stained L929 Fibroblast cell culture as a control, (E) C1-B-PEO copolymer/L929 Fibroblast cells conjugate (stained with hematoxylin-eosin dye); dense spots were showed nucleus of cells, and distinct violet were indicated cytoplasm of cells as a control, (F) Light microscope image of vacuole of L929 Fibroblast cells cytoplasm; dense spots were showed nucleus of cells in C1-B copolymers ($500 \mu\text{g.mL}^{-1}$ concentration) at 24 h incubation. Images (A) and (D) taken under X200 magnification, others images taken under X400 magnification.

4. CONCLUSIONS

This work has attempted to develop novel bioengineering functional organoboron copolymers (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 copolymers were synthesized by amidolysis and esterification of anhydride units of poly(MA-*alt*-MVE) (C1) as a bio-

Table 2. The comparative analysis of apoptotic and necrotic HeLa cell index for copolymer (C1), organo-boron (C1-B) and organoboron PEO branched (C1-B-PEO) copolymers at 24 h incubation.

Polymer amount ($\mu\text{g.mL}^{-1}$)	Apoptotic cells (%)	Necrotic cells (%)
C1		
control	3 ± 2	4 ± 2
50	3 ± 1	5 ± 1.5
100	6 ± 1.5	9 ± 3
250	8.5 ± 1	13.8 ± 1.5
500	15 ± 2	27 ± 3
C1-B		
control	5 ± 3	2 ± 1
50	13 ± 2	20 ± 3
100	18 ± 2.5	31 ± 4
250	32 ± 1	41 ± 2
500	43 ± 2	53 ± 3
C1-B-PEO		
control	3.5 ± 1.5	5 ± 1.5
50	9 ± 1	11 ± 1.5
100	12 ± 1	16 ± 2
250	15 ± 2	25 ± 3
500	28 ± 2.5	43.5 ± 5

Table 3. The comparative analysis of apoptotic L 929 Fibroblast cells index for copolymers (C1), organoboron (C1-B) and organoboron PEO branched (C1-B-PEO) copolymers at 24 h incubation.

Polymer amount ($\mu\text{g.mL}^{-1}$)	Apoptotic cells (%)	Necrotic cells (%)
C1		
control	2 ± 1	3 ± 1
50	3 ± 1	3 ± 1.5
100	4 ± 1	6 ± 2
250	7 ± 1	11 ± 1.5
500	9 ± 1.5	17 ± 2
C1-B		
control	2 ± 1	2 ± 1
50	7 ± 2	12 ± 2
100	12 ± 2.5	17 ± 3
250	16 ± 1	25 ± 2
500	27 ± 2	38 ± 2
C1-B-PEO		
control	3 ± 2	3 ± 1
50	4 ± 1	8 ± 1.5
100	6 ± 1	11 ± 2
250	9 ± 2	16 ± 3
500	17 ± 2.5	30 ± 1

compatible and non toxic polymer matrix with organo-boron amine and PEO, respectively. Chemical and physical structure of organoboron copolymers were confirmed by FTIR and ^1H (^{13}C) NMR spectroscopy and X-ray powder diffraction methods. 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 hy-

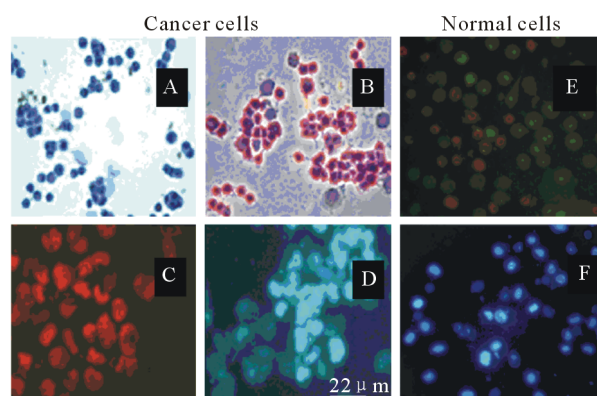


Figure 7. Light microscopy images of (A) virgin (nonapoptotic) HeLa cells as a control group (stained with M30 immunostaining kit), and (B) organoboron copolymer C1-B copolymer/HeLa cells conjugate (stained with M30 immunostaining kit), where brown cytoplasm of cells image indicates the formation of apoptotic cells; Fluorescence microscopy image of (C) nucleus of HeLa cells (stained with PI), where formation of red spots demonstrates nucleus of necrotic cells, and (D) nucleus of HeLa cells (stained with Hoescht 3342), where dense spots indicates nucleus of apoptotic cells. Fluorescence microscopy image of (E) nucleus of L929 Fibroblast cells (stained with PI), where formation of red spots demonstrates nucleus of necrotic cells and green spots demonstrates nucleus of living cells, and (F) nucleus of L929 Fibroblast cells (stained with Hoescht 3342), where dense spots indicates nucleus of apoptotic cells. Images of C and D were recorded with x400 magnification, others image were recorded with x200 magnification.

drogen bonding. The interactions of these copolymers with HeLa cells were investigated by using a combination of different methods such as cytotoxicity, statistical, hematoxylin/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 copolymers containing a combination of non toxic and biocompatible polymer matrix and long branched PEO segments with functional groups as antitumor sites, can be utilized as therapeutic potential functional copolymer drugs, which are able to form an artificial bioconjugate with HeLa cells, in cancer chemotherapy.

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