HACETTEPE JOURNAL OF BIOLOGY AND CHEMISTRY Research Article
Hacettepe J. Biol. & Chem., 2008, 36 (2), 83-98

Bioengineering Functional Copolymers. VIII. Stimuli-Responsive Boron-Containing Graft Copolymers and Their Poly(ethylene imine) Macrocomplexes and DNA Conjugates

Zakir M. O. Rzaev,* Dönsel Erdoğan, Mustafa Türk, Erhan Pişkin

Hacettepe University, Faculty of Engineering, Department of Chemical Engineering, Ankara, Turkey

Abstract

New boron-containing graft copolymers and their poly(ethylene imine) (PEI) macrocomplexes were synthesized by amidolysis of poly(*N*-isopropylacrylamide-co-maleic anhydride)s [poly(NIPA-*co*-MA)s] with ethanolamine ester of diphenylboronic acid (EAPB), and following complexation of graft copolymers with poly(ethylene imine) (PEI), respectively. The structure, compositions and properties (stimuli-responsive, polyelectrolyte and thermal behavior) of synthesized copolymers were characterized by FTIR, ^{1}H {^{13}C} NMR and UV spectroscopy, viscometry, DSC and TGA analyses. It was shown that under given conditions (pH, temperature and grafting degree), these H-bonding macrocomplexes with hydrophobic diphenylborate side chain groups in aqueous solutions undergo a reversible cooperative conformational transition which is provided a promising possibility of adjusting the hydophobic–hydrophilic balance in the studied graft copolymer systems. Fluorescence measurements indicated that poly[(NIPA-*co*-MA)-*g*-EAPB]/PEI macrocomplex easily transferred to the HELA tumor cells, and showed low cytotoxicity against a normal cell line. Obtained results, especially stimuli-responsive and very high transferable behavior allow use these boron-containing graft copolymer systems as non-viral vectors in gene- and bioengineering processes and drug delivery systems, as well as in boron neutron capture therapy.

Key Words: boron-containing graft copolymers, poly(ethylene imine), stimuli-responsivity, DNA conjugates.

INTRODUCTION

In the last decade, growing interest and much effort have been focused on the synthesis of boron-containing low molecular weight functional compounds, biopolymers and drugs with boron ligands and evaluation of their suitability for the bioengineering applications. On the other hand, considerable progress has been demonstrated in the synthesis of bioengineering polymer systems on the base of *N*-isopropylacrylamide homo- and copolymers. However, a wide range of chances of the synthetic polymer chemistry can serve as more effective synthetic routes for the new boron functional compounds, especially copolymerization of boron-containing monomers and chemical modification of biocompatible polymers with organoboron reactive compounds and monomers. Unfortunately, this very important chances are not effective used at this time by many reseachers for the further development of new bioengineering *N*- and *B*-containing polymer systems.

Several aminoboron compounds have found some utilities in boron neutron capture therapy (BNCT) [1]

* Correspondence to: Zakir M. O. Rzaev,
Hacettepe University, Department of Chemical Engineering, 06800 Ankara-Turkey

Tel: +90312 297 64 39 Fax: +90312 312 299 21 24

E-mail: zmo@hacettepe.edu.tr

and other forms of cancer therapy [2]; as a result, much effort has focused on the synthesis of boron-containing amino acid and peptide derivatives for possible applications as enzyme inhibitors [3]. Organoboronic acids, $RB(OH)_2$, and boronate esters have received considerable attention as glucose sensors for diabetes therapy [4]. They have been also found to facilitate the transport of various ribonucleosides in and out of liposomes, an important attribute in the field of drug design [5]. An important clinical trial of BNCT, using 4-hydroxyborylphenyl alanine, was carried out by researchers of Massachusetts Institute of Technology [6].

A variety of boron-containing amino acid derivatives that may carry boron to tumors by becoming incorporated in protein synthesis or metabolism within the rapidly growing tumor cell and boron delivery mechanism have been described by Hawthorne [7]. Chen et al. from Radiosynthesis Laboratory, University of North Carolina and Boron Biological Inc. were synthesized trimethylamine-[^{14}C]carboxyborane and trimethylamine-[^{14}C] carboxy- methoxyborane and L1210 leukemia cell uptake. The anti-neoplastic agents trimethyl- amine-carboxyborane and its methyl ester have successfully been radiolabeled with carbon-14 in the carboxylic group. Using the radiolabeled agents authors have demonstrated that their leukemia cell uptake appeared to be by a passive process and binding of these agents to DNA, RNA and protein over 24 h was minimal [8].

The synthesis aspect of different type *B*-containing organic and inorganic compounds including B-containing derivatives of nucleic acid precursors, amino acids and related peptides, were described and discussed in recently published reviewed article [9] and Book titled “Contemporary Boron Chemistry” [10] including very broad area of synthesis, reactions and applications, especially in the medical field (boron-rich clusters and polymers to tumor-targeting species). Some organic boron compounds having a monomer structure and their iodized derivatives were used as X-ray contrasting agents and for the pharmaceutical preparations, as well as in the selective therapy of tumors [11].

Yang et al. [12] reviewed the recent developments in the field of boronic acid compounds-potential pharmaceutical agents. As noted authors, these compounds having unique structural features can be used for the development of potent enzyme inhibitors, and as antibody mimics that recognize biologically important saccharides. A general synthetic method has also been developed for the preparation of boron-rich macromolecular structures for conjugation with or inclusion in receptor-mediated delivery systems [13]. This method was used to yield precisely ordered soluble and hydrophilic oligophosphates which may by prepared with a variety of functional groups. Synthesis of boron-containing enzyme-analogue built polymers by the introduction of amino- and boronic acid groups into chiral polymer cavities has been reported by Sarhan and Wulff [14].

Recently, Tuncel et al. [15] reported synthesis of thermosensitive aminophenylboronic acid modified NIPA copolymers and their stimuli-responsive properties against RNA. These copolymers were prepared by (1) solution radical copolymerization of NIPA with *N*-acryloxysuccinimide (ASI) in toluene/THF mixture at 65^{o}C and (2) reaction of synthesized poly(NIPA-co-ASI)s with *m*-amino-phenylboronic acid (APBA). It was observed that the LCST value of copolymer increased with increasing RNA concentration, while LCST decreased with increasing RNA concentration at both neutral and alkaline pH values. According to the authors, the produced boron-containing reactive copolymer can be also utilized for the recognition of the other amine or vicinal diol-carrying biomolecules [16].

Today, organic boronic acid and esters are widely used in industrial synthesis for production of complex specially chemicals, such as building blocks for active pharmaceutical ingradients, electronic materials or high-performance metallocene catalysts [16]. Some *B*-containing aromatic compounds, such as *p*-carboxyphenyl boronic acid and its mono- and di-substituted derivatives, *p*-hydroxyborylphenyl-, *L*-carboranyl- and *L*-*p*-dihydroxyborylphenyl alanines, various substituted benzeneboronic acids and some carborane derivatives are effective tumor targeting agents possessing low toxicities and very suitable for brain tumor [17-20].

Yang et al. [21] developed a highly efficient method for the introduction of Lewis acidic boron centers into the side chain of organic polymers. This method involved three steps: (1) the controlled polymerization of a functional monomer, (2) the exchange of the functional groups for boronic acid centers, and (3) the finetuning of the Lewis acidity of the individual centers through substituent exchange reactions with nucleophiles. This approach allowed to synthesize new well defined Lewis acidic poly(arylboronate)s.

Binding of nucleophiles to organoboron polymers can be exploited for the design of new supported reagents and immobilized catalysts [22,23] and of highly selective sensor materials [21,24,25]. Boron-containing reactive polymers play a major role as intermediates in the synthesis of new functionalized polymers by the various chemical modifications and macromolecular reactions [26,27]. These polymers can also be used as polymeric electrolytes for batteries [28,29], sophisticated flame retardants [30-32], and as preceramic [33-36] and photoluminescent materials [37,38]. Through functionalization at the carbon centers, carboranes have been also incorporated in a wide range array of polymeric and coordination compounds, catalysts and phamaceuticals [39]. Synthesis of synthrons for carborane containing macromolecules have been reported by Parrott et al [40]. In order to increase water solublity and biocompatibility of carborane-containing polymers, the modification of *para*-carborane with appropriate functionalities was accomplished by functionalizing the carbon centers with protected hydroxyl and carboxylic reactive fragments. This principle was also adapted to the preparation of polymerizable carborane-containing acrylate monomer.

Synthesis and characterization of bioengineering boron-containing functional copolymers, which are prepared by radical-initiated copolymerization of donor-acceptor monomer systems such as p-vinylphenylboronic acid–maleic and citraconic anhydrides, p-vinylphenylboronic acid–maleimide, p-vinylphenylboronic acid–*N*-isopropylacrylamide, as well as studies, their stimuli-responsive, polyelectrolyte and thermal behavior were a subject of our recently published works [41-43].

This work presents synthesis and characterization of new generation of bioengineering boron-containing graft copolymers and their macrocomplexes, which are synthesized (1) by amidolysis of poly(*N*-isopropylacrylamide-*co*-maleic anhydride)s [poly(NIPA-*co*-MA)]s having different compositions with ethanolamine ester of diphenylboronic acid (EAPB), and (2) by complexing reactions of poly[(NIPA-*co*-MA)-*g*-EAPB] with poly(ethylene imine) (PEI). The determination of low critical solution temperature (LCST), polyelectrolyte and thermal behavior, as well as the study of graft copolymer macrocomplexes as temperature sensitive non-viral vectors for transfection of HeLa cells is another aspect of this work.

EXPERIMENTAL

Materials

NIPA monomer (Aldrich) was purified before use by distillation under vacuum and recrystallization from diethyl ether solution: b.p. 91.5°C/2 mm, m.p. 61.6°C; ^{1}H NMR spectra, ppm (in THF with trace of DMSO-d_6): NH, 1H 7.75, CH=, 1H multiplet 6.19-6.25, CH_2=, 2H two doublets 6.11-6.16 and 5.45-5.48, CH, 1H multiplet 4.02-4.03 and 2CH_3, 6H 1.11-1.13 in –$CH(CH_3)_2$, respectively. MA monomer (Fluka) was purified by recrystallization from anhydrous benzene and through sublimation in vacuum; m. p. 52.8°C. EAPB (Aldrich) was purified by recrystallization from anhydrous ethanol: m.p.193.5°C (by DSC). ^{1}H NMR spectrum, ppm (in $CHCl_3$-d_1): CH_2O 1.49, CH_2-NH_2 2.96, 7.38-7.40 (4H), 7.19-7.24 (4H) and 7.13-7.16 (2H) in *p*-, *o*- and *m*-CH= in phenyl groups, respectively. 2,2'-Azobisisobutyronitrile (AIBN) (Fluka) was twice recrystallized from methanol: m.p. 102.5°C. Poly(ethylene imine) (PEI) (Fluka) was obtained with M_n 25000 g/mol.

Human cervix epithelioid carcinoma cell line (HeLa) was obtained from the tissue culture collection of the Şap Institute (Turkey). Cell culture flasks and other plastic materials were purchased from Corning (USA). The growth medium, wthich is Dulbecco Modified Medium (DMEM) without L-glutamine supplemented fetal calf serum (FCS), and trypsin-EDTA were purchased from Biological Industries (Israel).

Copolymerization Procedure

The copolymers of NIPA and MA with different compositions were made by changing the molar ratio of the monomers (NIPA:MA = 95:5, 90:10 and 80:20 mol %) in the feed under similar reaction conditions as described in our previously published works [44,45].

Grafting (Amidolysis) Procedure

The reaction mixtures of poly(NIPA-*co*-MA) with given compositions and EAPB were dissolved in 1,4-dioxane and placed in a preheated oil bath at 50°C for 2 h. The reaction mixture was diluted with dioxane and precipitated by diethyl ether. Purification of graft copolymers was done by dissolving in dioxane and reprecipitating in diethyl ether and washing benzene. Drying of the graft copolymers was done in vacuum at 40°C until constant weight. The graft copolymer compositions were determined by elemental analysis, results of which are given in Table 1.

Table 1. Composition of poly[(NIPA-*co*-MA)-*g*-EAPB]s

Copolymer/EAPB Mixture (mol. %)			*B* (%)		Graft copolymer composition (mol. %)[b]	
[Copolymer]	[EAPB]	*N* (%)	found	calcul.[a]	m_1	m_2
95	5	9.52	1.23	0.77	87.93	12.07
90	10	8.17	1.56	1.14	78.98	21.02
80	20	6.07	2.38	1.71	64.87	35.13

[a]These values are calculated using corresponded nitrogen amounts in graft copolymers.

[b]m_1 is NIPA unit and m_2 is grafted linkage in graft copolymers (mol. %).

Measurements

Fourier transform infrared (FTIR) spectra of the copolymers (KBr pellet) were recorded with FTIR Nicolet 510 spectrometer in the 4000-400 cm^{-1} range, where 30 scans were taken at 4 cm^{-1} resolution. $^{1}H\{^{13}C\}$ NMR spectra were recorded on a JEOL 6X-400 (400 MHz) spectrometer with $CHCl_3$-d_1 as the solvent at 27°C. Differential scanning calorimetry (DSC) and differential (DTG) and gravimetrical (TGA) thermal analyses of copolymers were performed on a DuPont TA 2000 calorimeter and Setaram Labsys TG-DTA 12 Thermal Analyzer, respectively, under nitrogen atmosphere at a heating rate of 10°C/min. Before these analyses, all the polymer samples (10 mg) were thermotreated at 110°C during 30 min to remove their thermal history, followed cooling to 20°C.

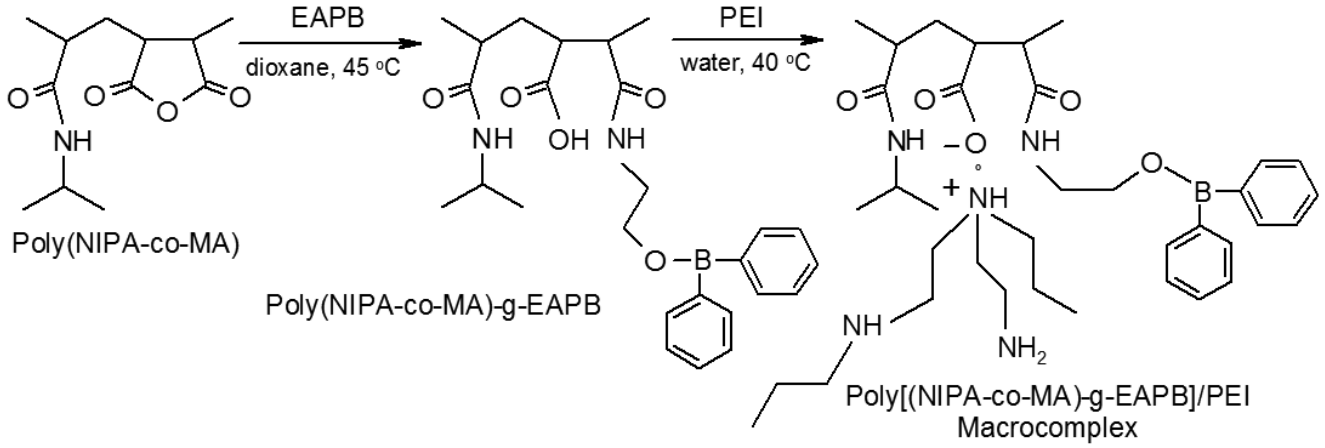
The lower critical solution temperature (LCST) measurements were performed in a UV spectrophotometer (UV 1602, Shimadzi, Japan) equipped with a heating system and temperature control unit. The temperature of the polymer solutions (1.0 wt.%) at pH 4.0 (an acetic acid/acetate buffer) or 7.4 (a phosphate buffer) was increased at a rate of 1.0°C/min starting from room temperature, and the absorbance of the solutions was periodically recorded at a wavelength of 500 nm. The LCST value as a coil-globule transition temperature of studied polymer in aqueous solution was calculated from the absorbance-temperature curve.

For *in vitro* DNA uptake experiments, a HeLa cell line was used. Transfections were followed by fluorescence microscopy (Fluorescence Inverted Microscopy, Olympus IX70, Japan), 4 h after uptake of labelled copolymer. Details of used experimental conditions were described in recently published work [46].

RESULTS AND DISCUSSION

Synthesis of Graft Copolymers and Their Macrocomplexes

Poly(NIPA-*co*-MA)s, containing 9.52, 16.65 and 30.82 mol % of MA unit were synthesized by radical-initiated random copolymerization in 1,4-dioxane at 65°C under nitrogen atmosphere. Then these copolymers with given compositions undergo amidolysis by ethanolamine ester of diphenyl boronic acid (EAPB) in aqueous medium. Prepared boron-containing copolymers with free carboxylic groups easily formed macrocomplexes with poly(ethylene imine) (PEI, M_n = 25000 g/mol). General scheme of synthesis routes of these copolymer systems can be presented as follows (Scheme 1).



Scheme 1. Grafting through amidolysis reaction of poly(NIPA-co-MA) with AEPB and complexation of graft copolymer with PEI.

Structure and composition of synthesized copolymers were determined by FTIR, ^{1}H {^{13}C} NMR spectroscopy and elemental analysis (Table 1), respectively.

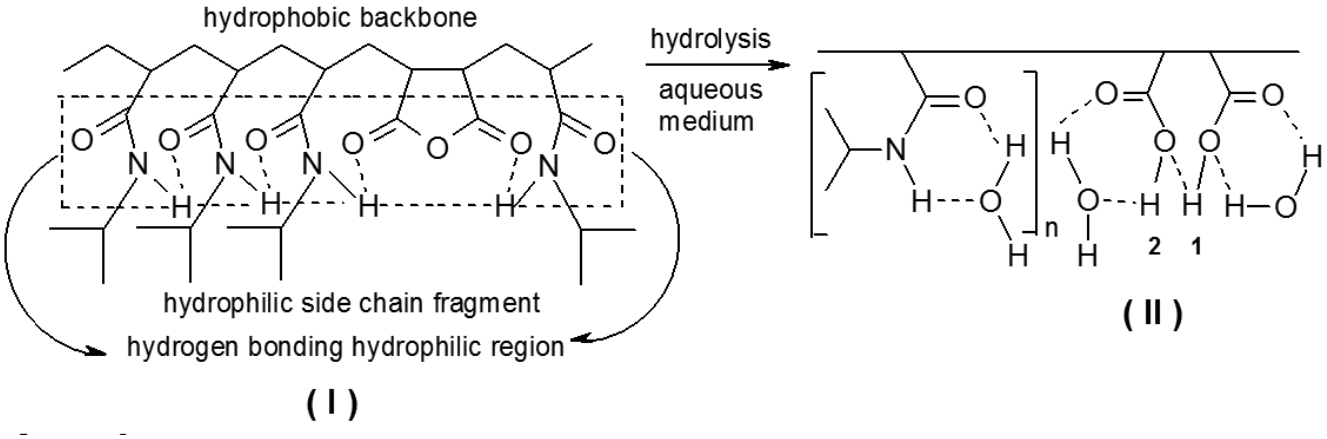
The comparative analysis has been made for FTIR spectra of EAPB grafting agent, poly(NIPA-*co*-MA) and poly[(NIPA-*co*-MA)-*g*-EAPB], which are illustrated in Figure 1. The formation of B-containing graft copolymer is confirmed by (1) disappearance of anhydride unit carbonyl bands at 1850 and 1775 cm^{-1} and primary amine bands at 3325, 1590 and 1065 cm^{-1}, (2) formation of characteristic bands for diphenylborate fragments: 1600 (C=C benzene ring),1430 (benzene ring vibration in B-Ph) and two 725 and 690 cm^{-1} bands (mono-substituted benzene ring).

^{1}H NMR and ^{13}C NMR spectra of N- and B-containing graft copolymers are illustrated in Figures 2 and 3, respectively, which are confirmed proposed structure of synthesized graft copolymers. These spectra contain the following characteristic signals from monomer units and grafted linkages: ^{1}H NMR spectra (in $CHCl_3$-d_1 at 27°C), d (ppm): 2.67–2.19 (H^1, CH), 1.84–1.34 (H^2, CH_2), 6.75–6.12 (H^5, NH), 3.87 (H^6, CH) and 1.15 (H^7 and H^8, CH_3) for NIPA unit; 4.30 (H^6, CH) for maleamide unit; 6.86–7.02 (H^9, NH), 4.01 (H^{11}, CH_2), 3.16 (H^{12}, CH_2) and 7.84–7.23 (*p*-H^{13}, *o*-H^{14} and *m*-H^{15}, phenyl) for grafted linkage. ^{13}C NMR spectra (in $CHCl_3$-d_1 at 27°C), d (ppm): 174.4 (C^5, C^9 and C^{10}), 134.7-126.6 (B-Ph for *p*- *o*- and *m*-positions of carbon atoms; C^{13}, C^{14}, C^{15} and C^{16}), 77.7 (CH, C^1), 42.2 (CHCO, C^3 and C^4), 41.1 (CH-NH, C^6), 37.3-32.5 (CH_2, C^2 and CH_2O-C^{11}), 22.6 (CH_3, C^7 and C^8) and 19.1 (OCH_2, C^{12}).

Hydrolysis and Phase Transition Behavior

The synthesized poly(NIPA-*co*-MA)s with random structure containing different amount of anhydride units (9.5, 16.5 and 26.7 mol %) easily undergo hydrolysis in aqueous solutions with formation of H-bonded diprotonic carboxylic groups (Scheme 2).

Aqueous solutions (2.0 wt.%) of these acidic copolymers of NIPA (II), poly[NIPA-*co*-MAc (maleic acid)]s are showed pH and temperature responsive and phase transition (formation of physically crosslinked structure) behavior at low critical solution temperature (LCST) (Table 3), which are differ from those for poly(NIPA) synthesized in the similar conditions. Polar acidic functionalization of poly(NIPA) by radical copolymerization of NIPA with MA and further hydrolysis anhydride units increases LSCT values of poly(NIPA-*co*-MAc)s at pH 4.0 and



Scheme 2. Schematic representation of hydrolysis of poly(NIPA-co-MA): (I) structure of copolymer in organic medium or solid state and (II) structure of hydrolyzed copolymer in aqueous medium containing diprotonic –COOH groups with different constants of dissociation: pK1 (1) < pK2 (2).

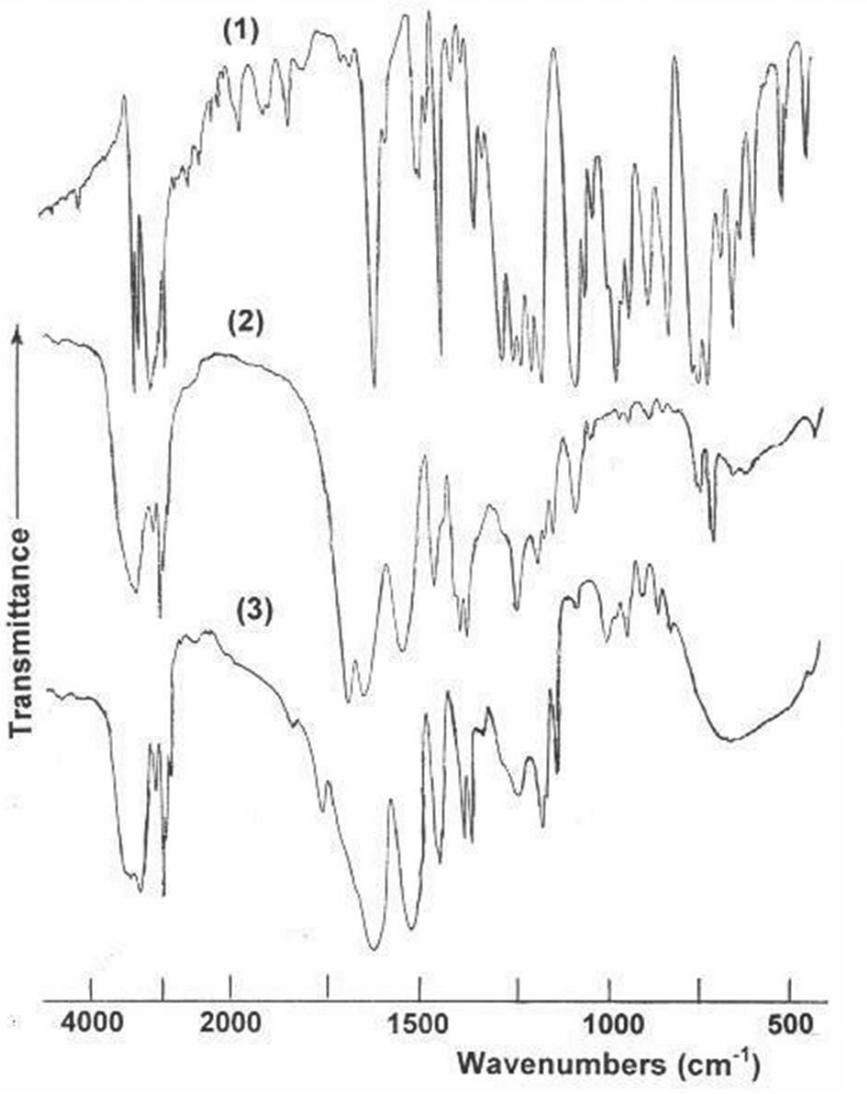


Figure 1. FTIR spectra of (1) EAPB, (2) poly[(NIPA-co-MA)-g-EAPB] and (3) poly(NIPA-co-MA).

5.0. For these copolymers, phase transition in water solution at pH 7.4 is not observed.

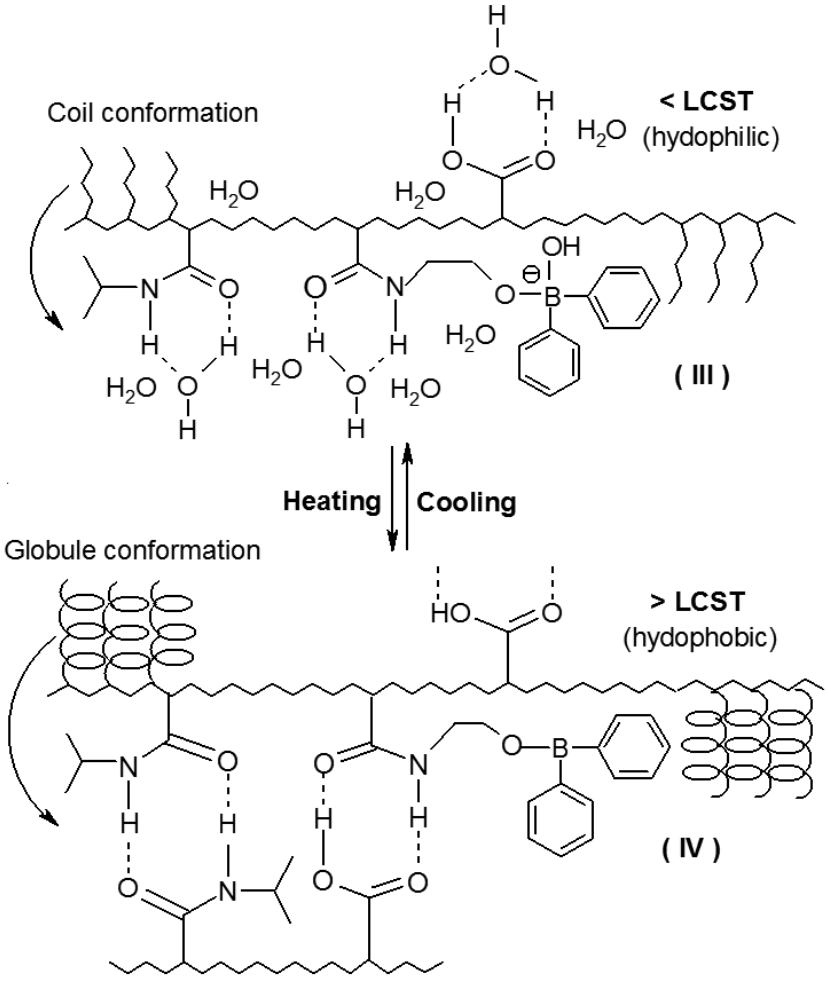
The phase transition behavior of poly(NIPA-*co*-MAc)s and their AEPB grafted derivatives, poly[(NIPA-*co*-MA)-g-EAPB]s in aqueous solutions was studied by monitoring the change in UV transmittance in the isothermal conditions around 20-50°C at heating rate of 0.1°C/min. The low critical solution temperature (LCST) values as a function of coil-glouble transition temperature of studied copolymers was calculated from the absorbance-temperature curves (Figure 4) and summarized in Table 2. The resulting copolymer composition–LCST relationship at various pH of medium showed that an increase of acidic strength (acid number), e.g., amount of maleic acid units is significantly increased the LCST values at both pH values (4.0 and 5.0). However, introduction of diphenylboronate grafted linkage in copolymers decreased the LCST values. Moreover, the grafted copolymers show relatively lower values of LCST as comparison with non-grafting poly(NIPA-*co*-maleic acid)s, and oven with poly(NIPA) (26.92°C against 30.5°C for NIPA homopolymer) (Table 2). Thus, amidolysis of poly(NIPA-*co*-MA) with AEPB significantly changes the hydrophobicity of the side chain without affecting the character of the backbone structure and seems to stabilize the globular form of graft copolymer macromolecules. It can be suggested that the H-bonding between hyrophilic fragments of side chain through amide–amide, amide–carboxylic, and carboxylic–carboxylic interactions, as well as donor-acceptor incorporation through phenylborate and

Table 2. LCST behavior of homopolymer, copolymers and boron-containing graft copolymers of NIPA.

Polymers	LCST values at different pH:		
	4.0	5.0	7.4
Poly(NIPA)	30.50	31.05	32.25
Poly[(NIPA-*co*-MA (9.51 mol%)]-I	30.96	32.32	Not observed
Poly[(NIPA-*co*-MA (16.5 mol%)]-II	37.05	48.61	"–"
Poly[(NIPA-*co*-MA (26.7 mol%)]-III	>50	>50	"–"
Poly[(NIPA-*co*-MA)-*g*-EAPB]-I	26.92	30.27	"–"
Poly[(NIPA-*co*-MA)-*g*-EAPB]-II	29.90	38.85	"–"
Poly[(NIPA-*co*-MA)-*g*-EAPB]-III	33.25	41.73	"–"
Poly[(NIPA-*co*-MA)-*g*-EAPB]-II/PEI	22.10	34.52	"–"

carbonyl groups also play important role in observed coil-globule conformational transition. This observed transition can be schematically presented as follows (Scheme 3).

In the case of PEI complexed *B*-containing copolymers, free carboxylic acid proton have been transferred onto the nitrogens of secondary and tertiary (preferely) amine groups of PEI and this



Scheme 3. Schematic representation of H-bonding effect in coil-globule conformational transition of poly[(NIPA-co-MA)-g-EAPB]s in the middle acidic aqueous solutions.

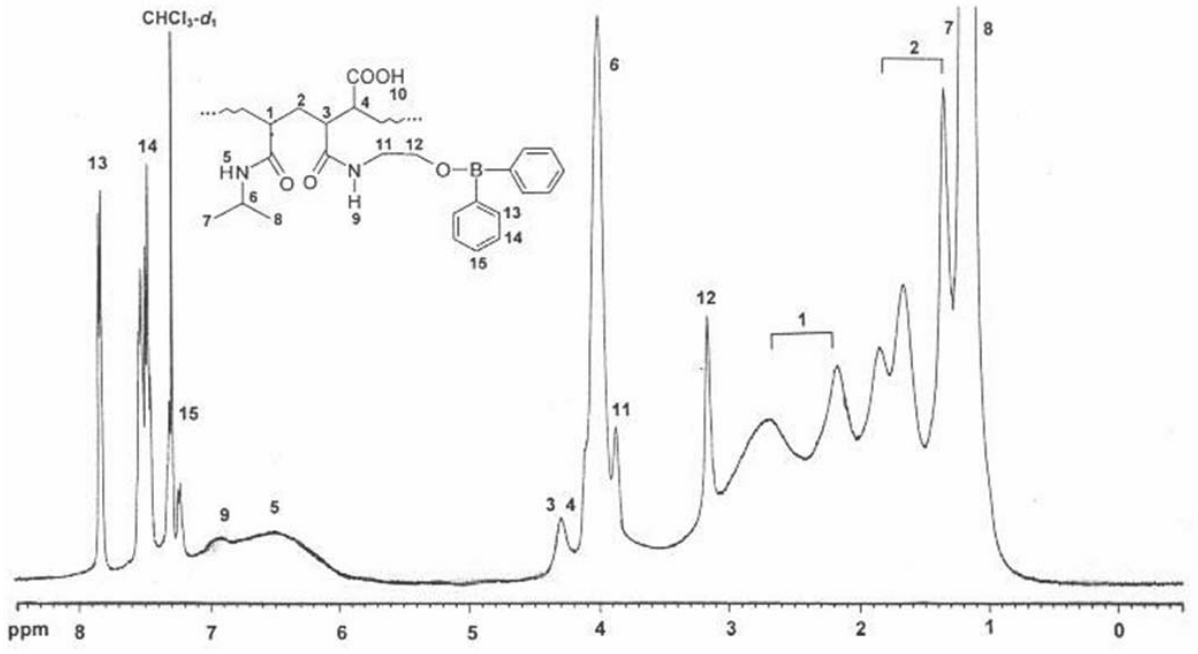


Figure 2. [1]H NMR spectra of poly[(NIPA-co-MA)-g-EAPB]-II synthsized at molar ratio of poly(NIPA-co-MA(21.0 mol. %): EAPB = 90:10.

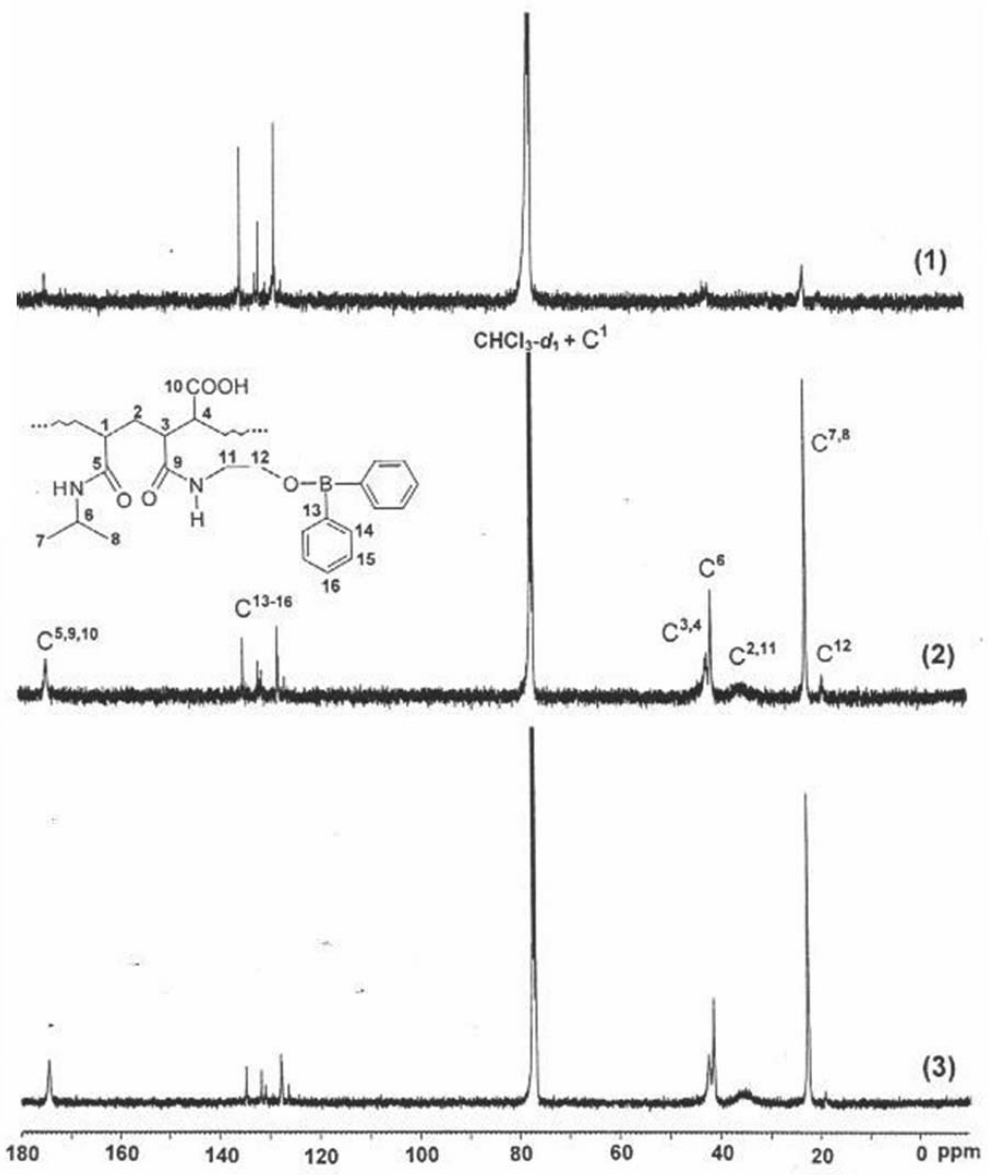
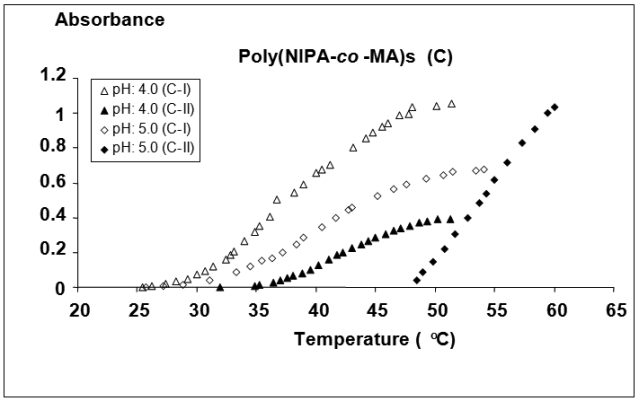


Figure 3. [13]C NMR spectra of graft copolymers I (1), II (2) and (3) III in $CHCl_3$-d_6.

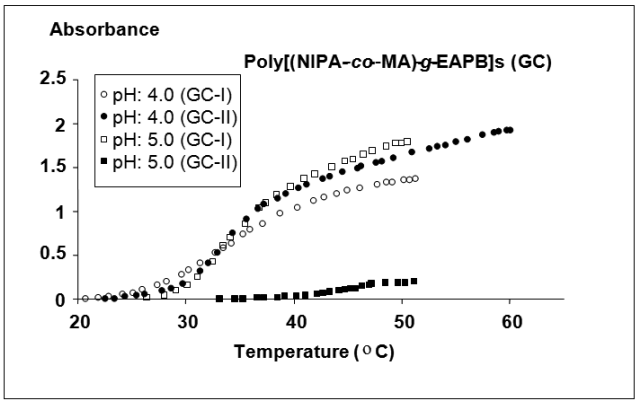
charge-separation of hydrogen bonds to carboxylic oxygen atom provided the formation of positive charges on the branched fragments as shown in Scheme 1.

It can be noted that amidolysis reaction between primary amine group of PEI and anhydride unit of copolymer occurred spontaneously without use of any catalyst. Probability, complexes of tertiary or secondary amines with MA unit (or trace of free carbıxylic group) are executed role of catalyst in this reaction. Thus, it can be concluded that in the organic medium, interaction of poly(NIPA-*co*-MA) with PEI is accompanied by amidation of anhydride units while in aqueous medium, where this copolymer transferred to acidic form, poly(NPA-*co*-maleic acid), this interaction is only limited by formation of macrocomplexes. One the other hand, the chain entanglement and the association of collapsed chains (IV) via H-bonding between side-chain amide and carboxylic groups are predominantly formed at relatively low temperature (around 27-39°C) in copolymer aqueous solutions with middle acidity (pH 4) (Table 2).

It was found that the poly(NIPA-*co*-MA)s and their organoboron-grafting derivatives and PEI macro-complexes showed pH- and temperature sensitivity; low critical solution temperature (LCST) of these copolymers depends on the pH medium and content of organoboron linkage in copolymers, and changes from 28.1 to 36.9°C with increasing hydrophobicity of their macromolecules. UV spectroscopic measurements in dilute aqueous solutions of poly(NIPA-*co*-MA)s (Figure 4a) and their B-containing graft (Figure 4b) and PEI macrocomplexes (Figure 4c) showed that macroscopic phase separation takes place when the temperature is raised above the LCST of poly(NIPA) and poly(NIPA-*co*-MA) at pH 4.0 and 5.0. Thi observed stimuli-responsive behavior can be attributed to the interconnection of the hydrophilic



(b)



(c)

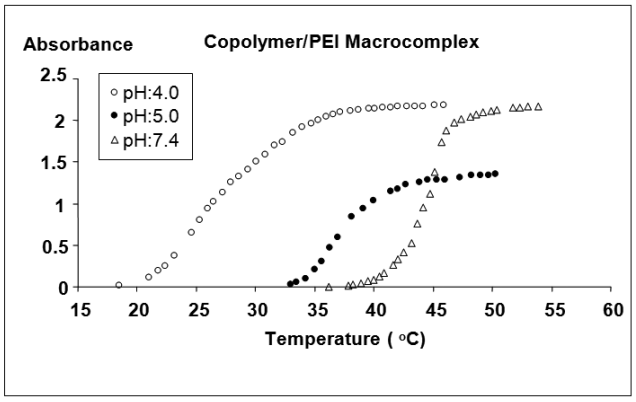


Figure 4. Temperature–absorbance (UV) plots for the aqueous solutions (0.2 g/dL) of:

(a) poly[NIPA-co-MA (9.5 mol.%)] and poly[NIPA-co-MA (16.5 mol.%)];

(b) poly[(NIPA-co-MA)-g-EAPB- -I and poly[(NIPA-co-MA)-g-EAPB]-II and;

(c) poly[(NIPA-co-MA)-g-EAPB- -II/PEI macrocomplex at different pHs.

fragments of copolymer (amide and free carboxylic groups) by means of hydrophobic diphenylborate side chain aggregates formed as the temperature increases above the LCST of this graft copolymer. This behavior of the graft copolymer should also be related to the formation of hydrogen-bonding

intramolecular complexes between NH of amide group and OH of carboxylic group in NIPA and maleic acid units, respectively. It is known that poly(NIPA-*co*-MA) and its hydrolyzed derivative, i. e., poly(NIPA-*co*-maleic acid) form strong hydrogen-bonding intra- or intermolecular complexes [44]. On the other hand, phenylborate fragments as a moderate Lewis acidity can also take placed in the donor-acceptor interactions of macromolecules. The incorporation of electron-deficient phenylboron centers into polymer structures provides an opportunity to further manipulate the polymers through donor-acceptor binding.

Under neutral conditions (pH = 7.0) no hydrogen-bonding association is effective and the stimuli-responsive behavior of the graft copolymer should be governed only by the thermosensitive behavior of NIPA segments. LCST value of graft copolymers increase in increasing grafted diphenylborate content in copolymers from 6.1 mol % to 17.8 mol % which can be explained by increasing hydrophobicity of copolymer macromolecules and by decreasing of hydrogen-bonding effect. On the other hand, LCST decreases at relatively high content of diphenylborate hydrophobic groups in graft copolymer, which can be explained by stabilization of gloubular form because hydrophobic interaction between two diphenylborate fragments. It is known that copolymer of maleic acid with styrene or deutered styrene in aqueous solutions also undergo the similar coil-globule transition and hyrophobic interaction between phenyl groups plays an important role in stabilizing the compact form. According to authors, in the compact form these groups are buried in the interior of the molecule [47,48]. It can be suggested that H-bonding macrocomplexes with amphiphilic boron-containing linkage are able to form a compact structure in aqueous solutions and provide a promising possibility of adjusting the hydophobic–hydrophilic balance in the studied copolymer systems.

Polyelectrolyte and Thermal Behavior

Polyelectrolyte and thermal behavior of copolymer systems was evaluated by viscosity and DSC-TGA methods, respectively. A tendency of copolymers observed for the formation of macromolecular complexed linkages allows one to propose that these copolymers must as well show some polyelectrolyte behavior. This is confirmed by the observed dilution effect, i. e., increase in viscosity values with the dilution of dioxane solution of copolymer (Figure 5). This phenomenon can be explained by specific behavior of complexed macromolecules and their conformational changes resulting in the expansion of polymer coil in the diluted solution. This fact relates the polyelectrolyte behavior of the studied graft copolymers and their temperature- and pH-sensitivities.

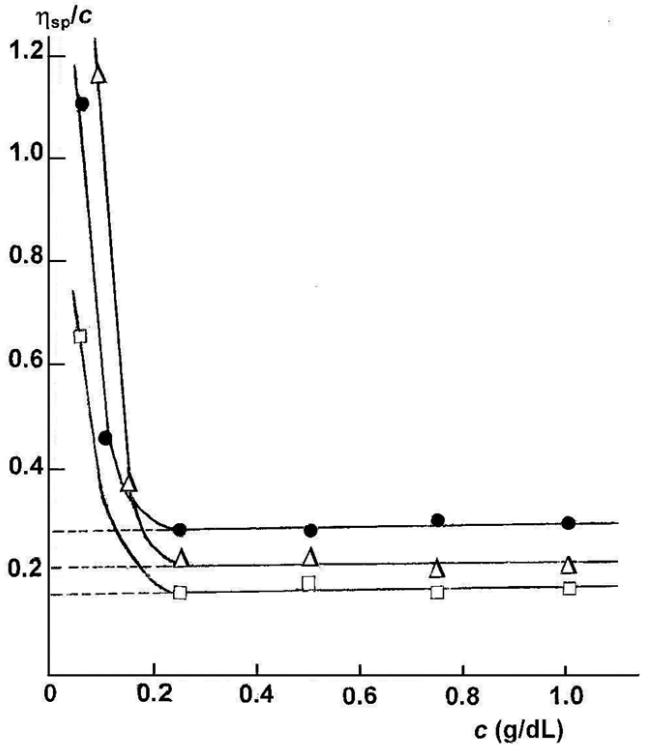


Figure 5. The plots of $\eta_{sp/c}$ vs. c (graft copolymer concentration) for the determination of intrinsic viscosity and evaluation of copolymer composition–viscosity relationships (dilution effect and polyelectrolyte behavior in THF solutions): (-●-) graft copolymer I, (-- -) II and (-□-) III.

The results of DSC and TGA-DTG studies of poly(NIPA), poly(NIPA-*co*-MA)s, poly[(NIPA-*co*-MA)-*g*-AEPB]s and poly[(NIPA-*co*-MA)-*g*-AEPB]/PEI are summarized in Table 3. Two endo-effects for all the studied systems are observed on the DSC curves. It can be proposed that the first transitions in the form of endo-peaks around 42.5-64.7°C are glass-transition temperatures (T_g) which can be related to 'aging' process of intermolecular H-bonded linkages. The second endo-effects associated with the melting points (T_m) of the studied copolymer systems. The comparative analysis of these results indicated that introduction diphenyborate fragments in copolymers and incorporation of *B*-containing graft copolymers with PEI decreased T_g and T_m values while thermal stability (T_d decomposition temperature) increased. In this case, a visible increase of intrinsic viscosity was also observed (Table 3).

Copolymer–DNA Conjugates
(*in vitro* transfection)

One of the most important phenomena for successful gene therapy is the non-viral vector for effectively delivering genes into cells. In this study, synthesized temperature and pH-sensitive *N*- and *B*-containing graft copolymer systems were investigated as non-viral vectors for transfection of HeLa cells in cell culture media.

The fluorescence micrographs taken in the cell culture studies are presented in Fig. 6 (a and b). For *in vitro* DNA uptake experiments, a HeLa cell line was used. Fluorescence measurements indicated that the boron-containing graft copolymers were not able to enter the cells. However, PEI macro-complexes of these copolymers, poly[(NIPA-*co*-MA)-*g*-EAPB]/PEI, which have positive charges, easily transferred to the HeLa tumor cells. The preliminary transfection studies showed that efficiency of the macrocomplex conjugates was very high, up to 98 % of the cells were transfected. The graft copolymers not are showed a toxicity in the range of used concentrations (Figure 7). However, the complexed copolymers showed lower cytotoxicity against a normal cell line (Figure 8). The increase of polymer concentration was increased its toxicity and provided the formation of more dead cells. Using copolymer/PEI macrocomplexes

Table 3. Thermal behavior (glass transition temperature T_g, melting point T_m and decomposition temperature (T_d) and intrinsic viscosity ($[\eta]_{in}$) of homopolymer, copolymers and boron-containing graft copolymers of NIPA.

Polymers	DSC analysis T_g (°C)	DSC analysis T_m (°C)	DSC analysis T_d (°C)	$[\eta]_{in}$ in THF at 25 °C
Poly(NIPA)	57.4	143	295	0.26
Poly[(NIPA-*co*-MA(9.51 mol%)]-I	55.6	155	337	0.21
Poly[(NIPA-*co*-MA(16.5 mol%)]-II	63.0	177	346	0.14
Poly[(NIPA-*co*-MA(26.7 mol%)]-III	64.7	182	347	0.10
Poly[(NIPA-*co*-MA)-*g*-EAPB]-I	42.5	149	342	0.29
Poly[(NIPA-*co*-MA)-*g*-EAPB]-II	45.0	156	365	0.16
Poly[(NIPA-*co*-MA)-*g*-EAPB]-III	49.6	161	376	0.18
Poly[(NIPA-*co*-MA)-*g*-EAPB]-II/PEI	46.5	124	310	0.35

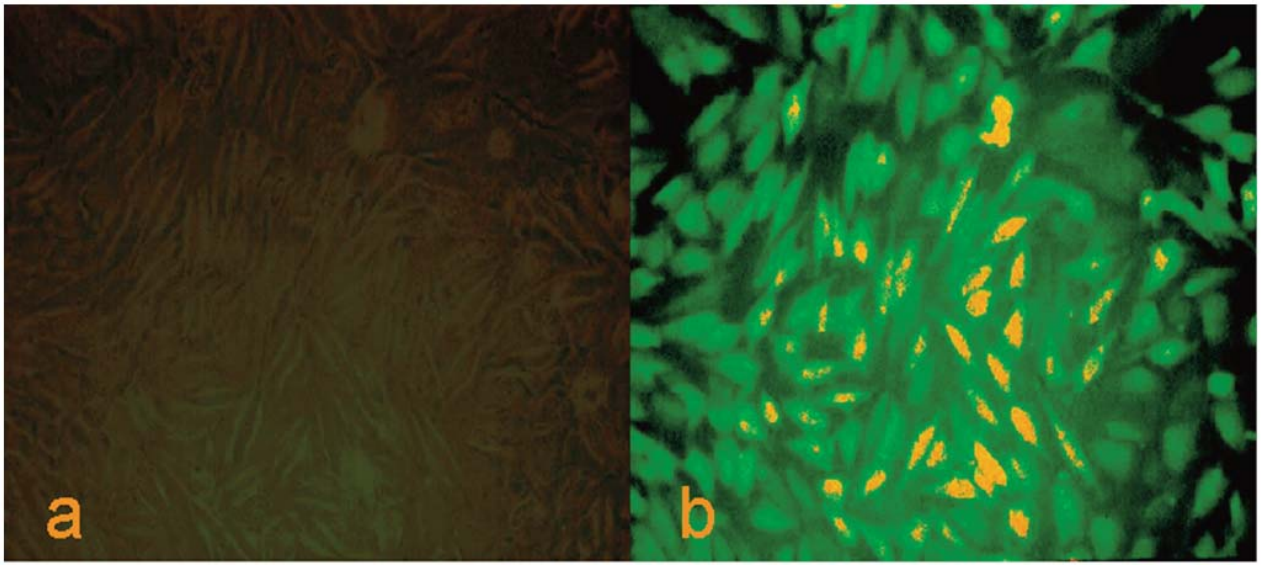


Figure 6. Fluorescence microscopy image of (a) non labelled polymers in HeLa cell culture and (b) fluorescein labelled copolymers in HeLa cell culture. Magnification 10 x 20; green dots showed that the labelled polymers were uptaken by cells.

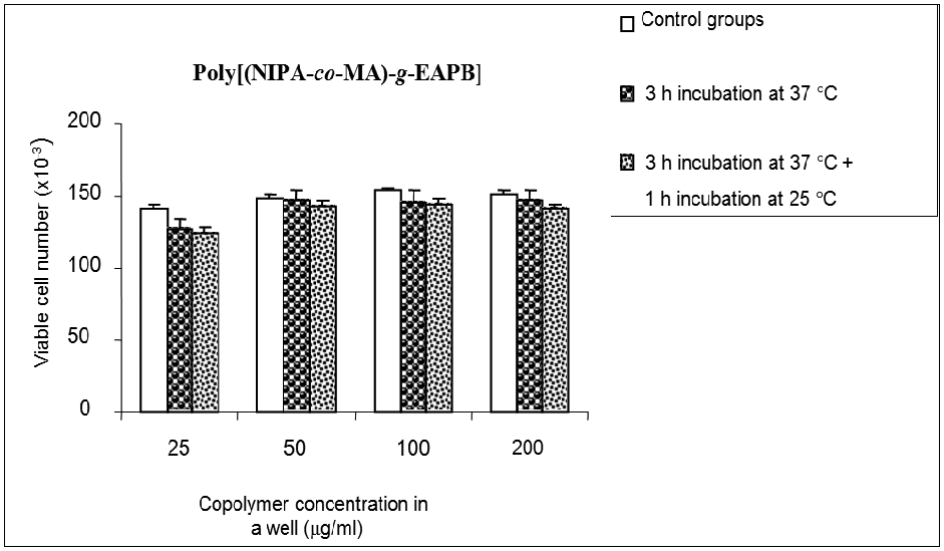


Figure 7. In vitro cytotoxicity (HeLa cell line) of poly[(NIPA-co-MA)-g-AEPB]s at different polymer concentrations and temperatures. The blocks show the average numbers and the bars give the standard deviations.

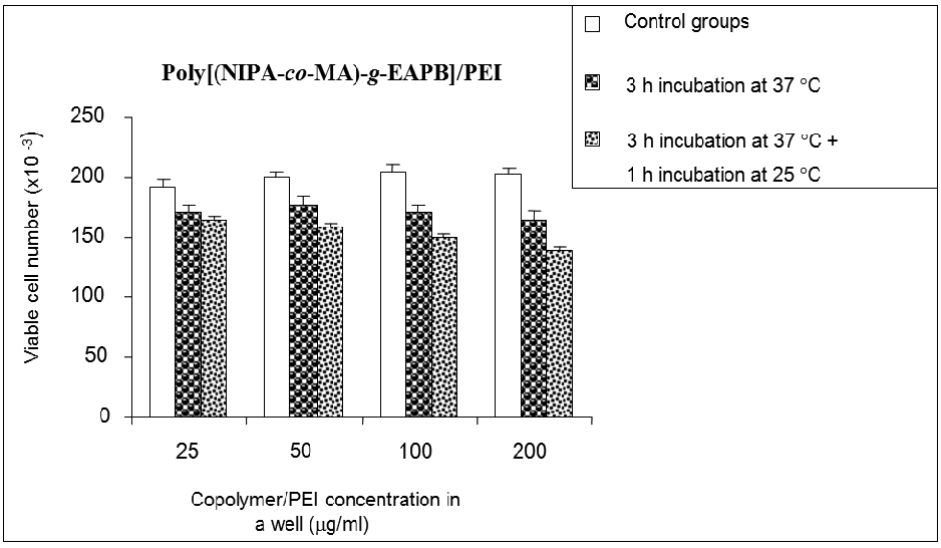


Figure 8. In vitro cytotoxicities (HeLa cell line) of poly(NIPA-co-MA)-g-AEPB/PEI with different polymer concentrations and temperatures.

reduced the cytotoxicity profoundly, and this was actually one of the main targets of this study when it was initiated. This fact can be explained by incorporation of NIPA fragments in copolymer which reduced the charge density on each polymer chain [46]. In addition, the globular form of temperature sensitive boron-containing macrocomplexes at body temperature can be served as an important fact making the total copolymer chain less cytotoxic.

CONCLUSIONS

New stimuli-responsive boron-containing co-polymers and macrocomplexes with very high transfection behavior and lower cytotoxicity toward HeLa tumor cells were generated by the grafting of AEPB into the side-chain of the random copolymers of NIPA and MA and incorporation with PEI, respectively. The formation of hydrophilic/ hydrophobic balance after grafting onto side-chain of poly(NIPA-*co*-MA) strong hydrophobic diphenylborate groups is provided favourable condition for intermolecular H-bonding between hydrophilic fragments of macromolecules, and therefore, for their coil-globule conformational transition at relatively low temperature. The grafted copolymers show relatively lower values of LCST as comparison with non-grafting poly(NIPA-*co*-maleic acid)s, and oven with poly(NIPA). Obtained results, especially stimuli-responsive and very high transferable behavior of synthesized copolymers allow us used these new water-soluble boron-containing copolymer systems as non-viral vectors in gene- and bioengineering processes and drug delivery systems, as well as in BNCT.

ACKNOWLEDGEMENTS

This study was carried out in accordance with Polymer Science and Engineering Program of Chemical Engineering Department and Bioengineering Division, Hacettepe University. The financial support of the TÜBİTAK (Turkish National Scientific and Technical Research Council) through TBAG-2386 Project is kindly acknowledged.

REFERENCES

1. Shull BK, Spielvogel DE, Gopalaswamy R, Sankar S, Boyle PD, Head G and Detivo, K, J Chem Soc Perkin Trans 2:557 (2000).
2. Hall IH, Henry JR, Peaty, NJ, Barnes BJ and Pawelke G, Appl Organomet Chem 14:86 (2000).
3. Carter CAG, Vogels CM, Harrison DJ, Gagnon MKJ, Norman DW, Langler RF, Baker RT and Westcott SA, Organometallics 20:2130 (2001).
4. Stoll VS, Eger DT, Hynes RC, Martichonok V, Jones JB and Pai, EF, Biochemistry 37:451 (1998).
5. Westmark PR, Gardiner SJ and Smith BD, J Am Chem Soc 118:11093 (1996).
6. Busse PM, Zamenhof RG, Harling OK, Solares FR,Tishler R, Stevenson R, Coleman N, Shafman T, Kaplan, I and Norregaard T, The Clinical State of Boron Neutron Capture Therapy Workshop, Department of Energy, Chasrlotte, NC; Nov. 3-5 (1997).
7. Hawhorne MF, Angew Chem Int Ed Engl 32:950 (1993).
8. Chen SY, Burnham BS, Spielvogel BF, Sood A, Wyrich SD and Hall IH, Appl Organomet Chem 10:279 (1998).
9. Soloway AH, Tjark W, Barnum BA, Rong F-G, Barth RF, Codogni IM and Wilson JG, Chem Rev (ACS) 98:1515 (1998).
10. Davidson MG, Hughes AK, Marder TB and Wade K (Eds.), Contemporary Boron Chemistry, The Royal Society of Chemistry, Cambridge (2000).

11. Martin-Jimenez JL, Carretero-Colon JM, Martinez-Sanz A and Krause W, ES Pat. 9400034 (1995).

12. Yang W, Gao X and Wang B, Med Res Rev 23:346 (2003).

13. Kne RR and Hawthorne MF, US Pat. 9403272 (1995).

14. Sarhan A and Wulff G, Makromol Chem 183:85 (1982).

15. Uzundoğan E, Kayı H, Denkbaş EB, Patır S and Tuncel A, Polym Int 52:649 (2003).

16. Zenk R and Partzch S, Chem Orggi 70:70 (2003).

17. Advances in Boron Chemistry, ed by Siebert W, The Royal Society of Chemistry, Cambrige, UK (1987).

18. Cancer Neutron Capture Therapy, ed by Mishima Y, Plenum Press, New York (1996).

19. Ichihashi M, Nakanishi T and Mishima Y, J Invest Dermatol 78:215 (1982).

20. Schwyzer R, Do KQ,Eberle AN and Fauchere L, Helv Chim Acta 64:2078 (1981).

21. Yang Q, Cheng G, Parab K, Sundararaman A and Jakle F, Macromol Symp196:337 (2003).

22. De Vos DE, Vankelecom IFJ and Jacobs, PA, Chiral Catalyst Immobilization and Recycling, Wiley-VCH, New York (2000).

23. Sherrington DC and Kybetti AC, Supported Catalysts and Their Applications, The Royal Society of Chemistry, Cambridge (2001).

24. Appleton B and Gibson TD, Sensors and Actuators 65B:302 (2000).

25. Nicolas M, Fabre B and Simonet J. J Electroanal Chem 509:73 (2001).

26. Boffa LS and Novak BM, Chem Rev 100:1479 (2000).

27. Kondo Y, Garcia-Cuadrado D, Hartwig JF, Boaen NK, Wagner NL and Hillmyer MA, J Am Chem Soc 124:1164 (2002).

28. Metha MA, Fujinami T and Inıue T, J Power Sources 81-82:724 (1999).

29. Matsumi N, Sugai K and Ohno H, Macromolecules 35:5731 (2001).

30. Sun X and Angell CA, Electrochimica Acta 46:1467 (2001).

31. Armitage, P, Ebdon JR, Hunt BJ, Jones MS and Thorpe FG, Polym Degrad Stab 54:387 (1996).

32. Gao J, Liu Y and Wang F, Eur Polym J 37:207 (2001).

33. Seyferth D, Adv Chem Ser 245:131 (1995).

34. Riedel R, Kroke E, Greiner A, Gabriel AO, Ruwisch L, Nicolich J and Kroll P, Chem Mater 10:2964 (1998)..

35. Weinmann M, KamphoweTW, Schulmacher J, Müller K and Aldinger F, Chem Mater 2:2112 (2000).

36. Kho J-G, Moon K-T, Nouet G, Ruterana P and Kim D-P, Thin Solid Films 389:78 (2001).

37. Matsumi N, Naka K and Chujo Y, J Am Chem Soc 120:3112 (1998).

38. Brunner AR, Bujalski DR, Moyer ES,Su K and Aldinger F, Chem Mater 12:2770 (2000).

39. Valliant JF,Guenther AS,King P, Morel P, Schaffer OO and Sogbein KAS, Coordination Chem Rev 232:173 (2000).

40. Parrott MC, Marchington EB, Valliani JF and Adronov A, Macromol Symp 196:201 (2003).

41. Kahraman G, Beşkardeş O, Rzaev ZMO and Pişkin E, Polymer 44:2897 (2003).

42. Rzaev ZMO, Kircı B, Çimen EK and Pişkin E, 40th IUPAC World Polymer Congress, Paris, France, 2004, Congress Proc. # 959.

43. Çimen, EK, Rzaev ZMO and Pişkin E, J Appl Polym Sci 95:573 (2005).

44. Dinçer S, Kesim H, Rzaev ZMO and Pişkin E, Eur Polym J 38:43 (2002).

45. Köseli V, Rzaev ZMO and Pişkin E, J Polym Sci Part A: Polym Chem 41:1580 (2003).

46. Türk M, Dinçer S, Yuluğ IG and Pişkin E, J Control Rel 96:325 (2004).

47. Sugai S, Nitta K, and Ohno N, Polymer 23:238 (1982).

48. Ohno N and Sugai S, Macromolecules 18:1287 (1985).