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Functional bioengineering copolymers. II. Synthesis and characterization of amphiphilic poly(*N*-isopropyl acrylamide-*co*-maleic anhydride) and its macrobranched derivatives

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Abstract

Radical-initiated copolymerization of *N*-isopropyl acrylamide (NIPA) with maleic anhydride (MA) and macromolecular reactions of synthesized poly(NIPA-*co*-MA) with polyethyleneglycol (PEG with a methoxy chain end and molecular weight of 2000 g mol^{-1}) and polyethyleneimine (PEI with molecular weight of 2000 g mol^{-1}) have been studied as a way to obtain new reactive amphiphilic water-soluble polymers potentially useful as carriers for gene delivery. Structure, composition and thermal behaviour of synthesized copolymers and their macrobranched architectures are determined by FTIR, ^{1}H and ^{13}C NMR spectroscopy, elemental (*N* content) and chemical (acid number) analysis and differential scanning calorimetry, differential thermal and thermal gravimetric methods. It is shown that synthesized copolymers with given composition have low critical solution temperature (LCST) in the range of 30.2–46.4 °C at pH values of 4.0–7.4, which suggest the possibility of their biomedical applications.

Keywords: *N*-isopropyl acrylamide; maleic anhydride; polyethyleneglycol

1. Introduction

In the last decade, many speciality functional bioengineering polymers possessing the optimum structure–property correlations [1–6] and new biomedical polymers-hybrids from synthetic macromolecules and genetically engineered protein domains [7] have been developed. It is known that the homo- and copolymers of *N*-isopropyl acrylamide (NIPA) exhibited pH and thermal sensitivity and were used in protein conjugation as cation-active polymers soluble in water and physiological medium [8–12], as well as carrier system for DNA delivery [13], affinity separation of genotoxins [14] and as reversible bioconjugates [15].

Alternating copolymers of maleic anhydride 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 [16]. Poly[(maleic anhydride)-*alt*-(methyl vinyl ether)], poly[(maleic anhydride)-*alt*-(divinyl ether)] and poly[(citraconic anhydride)-*alt*-(divinyl ether)] were used in various applications in diagnostics [17,18] and in chemotherapy as effective antitumor agents [19]. Synthesis of pH-sensitive phase separating poly[(maleic anhydride)-*co*-(methyl methacrylate)-*co*-(acrylic acid)] and poly[acrylamide-*co*-(methacrylic acid)-*co*-*N*-acryloxysuccinimide] terpolymers and their application in immunoasssay was reported by Zhou et al. [20]. A commercially available pectinase was chemically modified with poly[(maleic anhydride)-*co*-polyalkyleneoxide]. It was shown that enzymatic characteristics of the prepared biosystem were changed significantly, depending on the hydrophilicity of the copolymer-modifier and the degree of modification. The balance between repulsion and hydrophobic interactions is sensitive to environmental pH and temperature, and therefore variation of these parameters produce controllable conformational changes [21]. Acrylamide and its derivatives can undergo alternating copolymerization with maleic anhydride under the given conditions [22–26]. These copolymers are potentially useful as flocculants, for purification of industrial waste water, as coatings for microcapsule production and for paper

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dry-strength agents [26]. On the other hand, it is also known that NIPA homo- and copolymers can form thermal and pH sensitive systems in organic and water solutions [27–30]. This specific property of poly(NIPA) allows it to be used as an intelligent system for drug release [31–33]. Aqueous solutions of cationic or amphiphilic copolymers of NIPA can also be transformed into gels by changes in environmental conditions (temperature and pH). This phenomenon have recently attracted the attention of many researchers for both academic and practical interest in bioengineering, medical and pharmaceutical applications [34]. Importance of the copolymerization method for the preparation of thermosensitive polymer systems through incorporation of a more hydrophilic monomers has also been considered [35,36].

Alternating copolymers of maleic anhydride and N-substituted maleimides with different vinyl comonomers have been shown to be very useful in various diagnostics applications. Ladaviere et al. [37] reported a study on the synthesis and characterization of soluble conjugates of nucleic acid probes and poly[(maleic anhydride)-*alt*-(methyl vinyl ether)] with special attention on the kinetics of the coupling reaction and on the physico-chemical characteristics of the resulting conjugates. This copolymer was used as a reactive polymer to link oligodeoxyribonucleotides containing free amine groups (ODN–NH_2) to make ODN–copolymer conjugates of potential applications in diagnostics [38]. Covalent immobilization of protein onto this copolymer through grafting reaction and enhanced immobilization of recombinant proteins are also reported [39]. The effect of different parameters such as ionic strength and the nature of the solvent on the grafting reaction of biomolecules along with some other results are interpreted in terms of the interaction of synthetic and bioactive macromolecules [17]. Goldstein [40] used copolymers anhydride groups to covalently link biomolecules either in the solid state or in solution. The oligonucleotide synthesis on maleic anhydride copolymers covalently bound to silica spherical support and characterization of the obtained conjugates have been described by Chaix et al. [18] Direct synthesis of oligonucleotides was achieved on the controlled porous glass surface grafted with poly-[(maleic anhydride)-*alt*-(methyl vinyl ether)] and poly-[(maleic anhydride)-*alt*-ethylene]. The effect of chemical modification of enzyme pectinase with poly[(maleic anhydride)-*co*-polyalkyleneoxide] on the chitosanolytic characteristics of this enzyme, such as initial hydrolysis rate, some kinetic parameters and thermal stability, studied by Shin et al. [21]. Even though modification of the enzyme causes a reduction of initial chitosanolytic activity in any case, it shows favourable characteristics: an increase in the affinity of enzyme to chitosan, an enhancement of thermal stability, and an improvement of reducing sugar production with long-term hydrolysis.

Thus, it can be assumed that copolymers of NIPA with more hydrophilic functional comonomers can also have pH and thermal sensitivity, as well as a lower critical solution temperature (LCST). On the other hand, unlike homopolymer of NIPA, above mentioned specific behaviour of copolymers can be changed by the chemical modification of the copolymers containing reactive functional groups using different modifier agents or by varying copolymer composition using different comonomers and monomer/comonomer ratios in the copolymerization. At the same time, indicated methods will provide suitable conditions for the control of hydrophilic–hydrophobic balance in the polymer system. In this aspect, copolymers of NIPA with hydrophilic (anion active) comonomers such as maleic, citraconic and itaconic acids and their anhydride, amide and imide derivatives can be served as effective polyfunctional bioengineering polymers. Recently, radical-initiated copolymerization of NIPA with maleic and citraconic anhydrides, determination of monomer reactivity ratios and structure–composition–property relationships for both poly(NIPA-*co*-MA) and poly(NIPA-*co*-CA) with predominantly alternating structure have been reported [41]. Synthesis and characterization of cationic stimuli-responsive acrylic acid-terminated poly(NIPA) potentially useful as a carrier for gene delivery, conjugates of poly(NIPA) with amino acids as prodrugs, antitumor active binary and ternary copolymers of maleic anhydride, vinyl acetate and acrylic acid (or 2,3-dihydropyran) have also been reported [42–44].

In this work, synthesis and characterization of thermal and pH sensitive poly(NIPA-*co*-MA)s with different compositions (MA-units in copolymers ≤ 20 mol%) and their macromolecular reactions with polyethyleneglycol (PEG) and polyethyleneimine (PEI) are described. Structure–composition–properties (viscosity, pH and thermal sensitivity, LCST and thermal behaviour) relationships of the synthesized copolymers and their multibranched and macrocomplexed architectures are also presented.

2. Experimental

2.1. Materials

NIPA monomer (Aldrich) was purified before use by distillation under vacuum and recrystallization from diethyl ether solution: bp 91.5 °C/2 mm, mp 61.6 °C.

MA monomer (Fluka) was purified by recryctallization from anhydrous benzene and sublimation in vacuum: mp 52.8 °C. ^{1}H NMR spectra (in $CHCl_3$-d_1 at 27 °C): CH=, 2H singlet with 7.34 ppm.

α,α′-Azoisobutyronitrile (AIBN) (Fluka) was recrystallized twice from methanol: mp 102.5 °C.

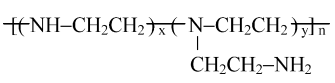
Poly(ethylene glycol) with OCH_3 end group (Fluka), $HOCH_2CH_2(OCH_2CH_2)_{n\text{-}1}OCH_3$: molecular weight 2000.

Fourier transform infrared (FTIR) spectra (KBr pellet), cm^{-1}: 4480–4400 (w) combination of CH stretching and deformation bands for CH_2 and CH_3 (end) groups, 3005 (w) aliphatic end OH group, 2880 (vs) for CH stretching band of

CH_2, 2750 (w) for CH stretching band of CH_3, 1464 (m) –CH_2– scrissor vibration or OCH_3 end group band, 1352 (s) deformation band for CH_2 group, 1285 (m) C–O–C ether stretching band, 1220 (s) band of OH bending, 1140 (s, shoulder) and 1110 (vs, broad) C–O–C bands for ether group, 1040 (m, shoulder), 951 (s) and 664 (m) bands for CH_2–OH and C–OH end groups, 843 (m.-s) C–C stretching band, 772 (vs) and 518 (w, broad) C–O bending bands for ether group.

^{1}H NMR spectra (in $CHCl_3$-d_1 at 27 °C), ppm: 3.30–3.73 for 2H CH_2, 2.42 for 1H OH end group and 2.05 for OCH_3 end group.

Poly(ethyleneimine) (Aldrich): molecular weight 2000, $d_4^{20} = 1.08$ g/cm^3.



where $x = 0.26$ and $y = 0.74$ were determined by FTIR analysis using absorption band ratio of 1650 cm^{-1} (NH deformation for primary amine group) and 1125 cm^{-1} (C–N stretching for secondary amine group.

FTIR spectra (smashed droblet), cm^{-1}: 4350–4100 4480–4400 (m) combination of CH stretching and deformation bands of CH_2 groups, 3550 (s) primary amine antisym. NH_2 stretching, 3300 (s) secondary backbone NH stretching, 3125 (s) NH amine group, 2940 (s) and 2860 (s) antisym. and sym. CH stetchnig of CH_2 groups, 2810 CH stretching of CH_2–NH_2 group, 2245 (m, broad) and 1705 (s) NH stretching, 1595 (m) and 1650 (s) primary amine deformation, 1455 (s, broad) CH_2 deformation (scissor vibration), 1370 (m) CH_2 deformation of CH_2–NH_2 fragments, 1225 (w) and 1185 (w) C–N of amine group, 1125 (m) C–N stretching of secondary amine, 1045 (m) amine C–N stretching, 935–830 (w) C–C stretching, 710 (w) secondary NH deformation, 525 (m) ternary backbone amine C–N–C bending, 455 (w) secondary amine C–N–C bending and 430 (w) overtone of torsional mode in primary amine.

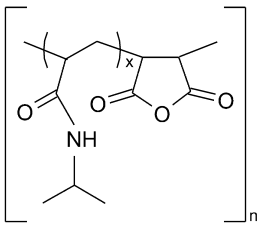
^{1}H NMR spectra (in $CHCl_3$-d_1 at 27 °C), ppm: 4.40 for 2H CH_2 [(in protonated methyleneamine groups –CH_2–N < (–NH– and –NH_2)], 2.52–2.66 for 2H CH_2 and 1.61 for 1H NH amine groups.

2.2. Copolymerization procedure

Copolymerizations of NIPA with MA using various monomer feed ratios (where [NIPA] ≫ [MA]) were carried out in 1,4-dioxane at 65 °C with AIBN radical initiator at constant total concentration of monomers under the nitrogen atmosphere. Reaction conditions for both systems: $[M]_{total} = 2.78$ mol/l, $[AIBN] = 6.5 \times 10^{-3}$ mol/l and monomer ratios of [NIPA]/[MA] = 2.33–39.0. Appropriate quantities of monomers, 1,4-dioxane and AIBN were placed in a standard pyrex-glass tube, and the reaction mixture was cooled by liquid nitrogen and flushed with dried nitrogen gas for at least 2 min, then soldered and placed in a thermostated silicon oil bath at 65 ± 0.1 °C. The NIPA–MA copolymers were isolated from the reacted mixture by precipitation with diethyl ether, then washed with several portions of benzene and dried under moderated vacuum. Copolymer compositions were found by elemental (N content for NIPA units) and chemical (acid number for anhydride units), and ^{13}C NMR spectroscopy using integral area of chemical shifts of monomer functional groups for quantitative analysis.

Copolymer prepared from 95:5 NIPA/MA molar ratio of initial monomers has the following average characterictics:

Poly(NIPA-*co*-MA)



where $x = 5.06$

Monomer unit ratio, $m_1/m_2 = 83.5 : 16.5$; content of N 10.6% (by elemental analysis); Acid number 165 mgKOH/g; $[\eta]_{in}$ 0.17 dl/g in THF at 25 ± 0.1 °C; M_n 7500 and MWD 1.67 (by GPC); T_g 55.6 °C, ΔH 1.42 mW (by Differential scanning calorimetry (DSC)) and T_m 155.2 °C, ΔH 12.7 mJ (by DSC and differential thermal (DTA)).

FTIR spectra (KBr pellet), cm^{-1}: 4480–4400 (w) bands for combination of CH stretching and deformation in CH_2 and CH_3 groups, 3300 (m) and 3120 (w) broad bands for NH secondary amide, 2975 (s), 2950 (m), 2875 (m) and 2750 (w) CH stretching in CH, CH_2 and CH_3 groups, 2540 (w, broad) band for complexed NH amide, 1850 (w) and 1775 (m) C=O stretching of anhydride unit, 1650 (s, broad) C=O stretching of amide I band, 1550 (s, broad) NH amide II band, 1460 (s, broad) CH_2 scissor vibration and CH_3 antisym. deformation, 1385 (m) and 1365 (m) doublet twist band for CH_3 deformation in isopropyl group, 1325 (w) CH_2 bending, 1240 (m) *trans*-amide III band, 1170–1080 (m) C–O–C band of anhydride unit or C–N stretching, 975–843 (m.-w) C–C stretching of main chain, 750 (w) NH deformation, 625 (w) CH bending for anhydride unit.

^{1}H NMR spectra (in CH_3COCH_3-d_6 at 27 °C), ppm: (1) 2H, CH_2 1.39–1.78, (2) 1H, CH 1.78–2.38, (3) 1H, NH 6.92–8.04, (4) 1H, CH 3.88, (5) and (6) 6H, CH_3 0.78–1.39 for NIPA unit; (7) and (8) 2H, CH 4.17 for maleic unit.

^{13}C NMR spectra (in $CHCl_3$-d_1 at 27 °C), ppm: 174.8 for C4, C9 & C10 C=O groups, 77.1–77.7 multiplet for $CHCl_3$-d_1 and C6 for backbone NIPA unit CH, 42.8 C3 for isopropyl CH, 41.7 C7 and C8 for backbone MA unit CH, 35.3–36.8 C5 for CH_2 backbone group, 23.0 C1 and C2 for isopropyl CH_3 groups.

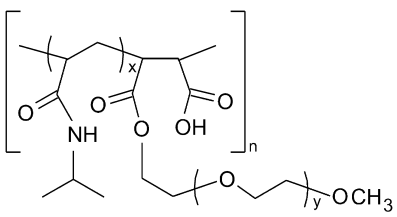
2.3. Monoesterification (grafting) procedure

Resulted copolymers with given compositions were grafted with PEG using various copolymer/PEG molar percentage ratios (97.5:2.5, 95.0:5.0, 92.5:7.5 and 90:10) in anhydrous THF at 40 °C during 30 min. Grafted copolymers were isolated from reaction mixture by precipitation with diethyl ether and centrifugation, then washed with several portions of hexane and diethyl ether and dried under moderate vacuum.

Structure and composition of obtained macrobranched copolymers were determined by elemental (content of N) and chemical (content of free –COOH groups) analyses, as well as by FTIR and quantitative ^{1}H NMR analysis.

Some characteristics of copolymer prepared from 95.0:5.0 copolymer/PEG ratio are as following:

Poly[(NIPA-*co*-MA)-*gratf*-PEG]



where $x = 5.06$ and $y = 43.7$

Monomer unit ratio, $m_1/m_2/m_3$ (grafted unit) = 83.5:11.5:5.0; Degree of grafting 37.0%; Content of N 5.6% ((by elemental analysis); Acid number 75.2 mgKOH/g; $[\eta]_{in}$ 0.14 dl/g in THF at 25 ± 0.1 °C; M_n 8200 (by GPC); T_g 50.3 °C, ΔH 17.7 mW (by DSC).

FTIR spectra (KBr pellet), cm^{-1}: 4480–4400 (w) bands for combination of CH stretching and deformation in CH_2 and CH_3 groups, 3300 (m) and 3120 (w) broad bands for NH secondary amide, 2975 (m), 2875 (s) and 2750 (w) CH stretching in CH, CH_2 and CH_3 groups, 1975 (w, broad) overtone band of C=O, 1850 (w) and 1775 (w) C=O stretching of free anhydride unit, 1735 (w) C=O band of ester group, 1650 (s) C=O stretching of amide I band, 1550 (s) NH amide II band or C=O band of –COOH group, 1470 (s) CH_2 scissor vibration and CH_3 antisym. deformation, 1345 (m) and 1335 (m.-s) bands for CH_3 deformation in isopropyl group, 1325 (w) and 1300 (m) CH_2 bending, 1285 (m.-s) C-O-C antisym. stretching band of ether group, 1248 (m) *trans*-amide III band, 1235 (m.-w) band for ether linkage, 1146(m), 1115 (s) and 1063 (m.-s) C–O–C band in grafted unit and anhydride unit, and/or C–N stretching, 958 (s, broad) band for C–OH deformation in H-bonded –COOH group, 880 (w) and 843 (s) C–C stretching of main and/or branched chain, 620 (w, broad) CH bending for anhydride unit and 525 (m) O–C=O bending band in –COOH group.

^{1}H NMR spectra (in $CHCl_3$-d_1 at 27 °C), ppm: 1H COOH 16.98, 1H NH 6.25 (broad), 1H CH 3.92 (backbone), 3.74 1H CH (isopropyl), 3.29–3.72 2H CH_2 (side-chain), 2H CH_2 2.13 (backbone), 3H OCH_3 1.75 (end methoxy group) and 6H CH_3 1.05 (isopropyl).

2.4. Measurements

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 and ^{13}C NMR spectra were recorded on a JEOL 6X-400, 400 MHz high performance digital FT-NMR spectrometer with deutered dimethylsulfoxide (DMSO-d_6) and $CHCl_3$-d_1 as solvents at 27 °C.

The compositions of the copolymers synthesized using various monomer feed ratios were determined by known NMR method [41,45] and were achieved by comparing the integrals of the isopropyl, methyne and methyl group regions in the spectra of NIPA and MA units, respectively. Molar fractions of the comonomer units (m_1 and m_2) in NIPA–MA copolymers using ^{13}C NMR analysis data were calculated according to the following equations:

$$Am_1(\text{CH of isopropyl group})/A_{\text{total}} = n_1 m_1/(a_1 m_1 + b_2 m_2) \quad (1)$$

$$Am_2(\text{CH of MA unit})/A_{\text{total}} = n_2 m_2/(a_1 m_1 + b_2 m_2) \quad (2)$$

where Am_1 and Am_2 are the normalized areas per C from the corresponding functional groups of the monomer unit regions in ^{13}C NMR spectra; A_{total} is the total area of carbon atoms in the copolymer; n_1 and n_2 are the integers of carbon atom(s) in the functional group of the monomers; a and b are the integers of carbon atoms in the monomer units (m_1 and m_2); in the case of ($m_1 + m_2 = 1$), monomer unit ratios can be calculated from Eqs. (1) and (2) using the following simplified form:

$$m_1/m_2 = f = n_2 Am_1\,(\text{CH of isopropyl group})/n_1 Am_2 \times (\text{CH of MA unit}) \quad (3)$$

DSC, DTA and thermal gravimetric (TGA) 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.

The CHNS-932 Model LECO Elemental Analyzer was used for the determination of C, H and N contents in the copolymers synthesized. Molar fractions (mol%) of comonomer units (m_1 and m_2) in NIPA–MA copolymers using elemental analysis data (content of N) were calculated according to the following equations [41]:

$$m_1 = M_2/\{(A_N/B) - \Delta M\ 10^{-2}\} \quad (4)$$

where M_2 are the molecular weight of MA unit; A_N is the atom weight of N; B is the content of N in the copolymers (%); $\Delta M = M_1 - M_2$ (M_1 is the molecular weight of the NIPA unit).

Table 1
^{13}C NMR and element analysis data for determining the composition of poly(NIPA-*co*-MA) synthesized from various initial monomer mixtures in [NIPA] ⩾ [MA] conditions

Monomer Feed (mol%)		Yield (%)	Integral area		N (%)	Copolymer composition (mol%)				Mean sequence length	
[NIPA]	[MA]		Am_1 (M_1 unit)[a]	Am_2 (M_2 unit)[a]		By ^{13}C NMR analysis		By nitrogen analysis		μ_1	μ_2
						m_1	m_2	m_1	m_2		
97.5	2.5	68.0	0.536	0.159	11.15	87.08	12.92	88.73	11.27	4.84	1.01
95.0	5.0	63.5	0.420	0.173	10.57	82.94	17.06	83.52	16.48	3.48	1.02
92.5	7.5	64.0	0.379	0.159	10.41	82.67	17.33	82.09	17.91	3.24	1.02
90.0	10.0	60.5	0.647	0.353	10.13	78.59	21.41	79.61	20.39	2.91	1.03
70.0	30.0	58.4	0.104[b]	0.050[b]	8.63	67.35	32.65	66.62	33.38	2.01	1.05

[a] Integral area for CH carbon atom chemical shift of NIPA (side chain isopropyl group) and MA anhydride units (backbone methyne group) in ^{13}C NMR spectrum.
[b] ^{1}H NMR analysis data.

Acid numbers (AN) of the anhydride-containing copolymers and their macrobranched derivatives were determined by standard titration method using a pH-meter Consort P901. Intrinsic viscosities of the copolymers with different compositions were determined in THF at 25 ± 0.1 °C in the concentration range of 0.1–1.0 g dl^{-1} using an Ubbelohde viscometer. Molecular weights of copolymers were determined by Gel-Permeation Chromatography using GPC with Shimpact 804 colunm and THF as mobile phase-eluent, relatively to PS standards. LCST values at pH 4.0, 5.0 and 7.4 were obtained spectrophotometrically (Jasco V-530 UV spectrophotometer) at 500 nm using acetic acid/sodium acetate (pH 4.0 and pH 5.0) and $Na_2HPO_4/NaH_2PO_4{\cdot}2H_2O$ (pH 7.4) buffers.

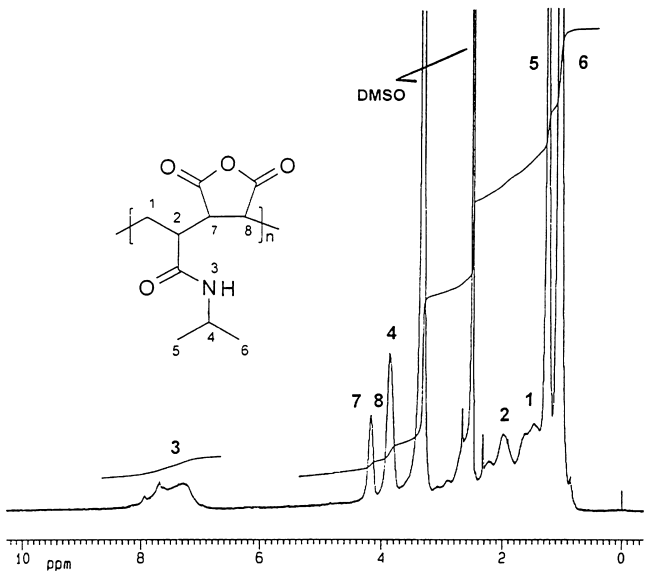


Fig. 1. ^{1}H NMR spectra of poly(NIPA-*co*-MA) in DMSO-d_6 at 27 °C.

3. Results and discussion

3.1. Synthesis and structure–composition–property relationships

Synthesis of poly(NIPA-*co*-MA)s containing different concentrations of anhydride units is carried out by radical-initiated solution copolymerization under the condition of higher concentration of NIPA in monomer feed.

Results of ^{1}H and ^{13}C NMR and elemental analyses for various initial monomer ratios of copolymers are illustrated in Figs. 1–3, and summarized in Table 1. Copolymer compositions calculated using elemental analysis data (content of N) were in reasonable agreement with those obtained from ^{13}C NMR analysis using Eq. (3). Number regarding copolymers composition provided in Table 1 are average values and represent average characteristics of the copolymer samples. While compositional heterogeneity is expected, and as seen from these date instantaneous copolymer composition is not changing drastically up to these conversions.

The monomer sequence lengths (μ_1 and μ_2) are calculated from the well known equations [46] using copolymer composition data and values of copolymerization constants $r_1 = 0.49$ and $r_2 = 0.11$ [41]:

$$\mu_1 = 1 + r_1(m_1/m_2) \quad (5)$$

$$\mu_2 = 1 + r_2(m_2/m_1) \quad (6)$$

the calculated values of μ_1 and μ_2 are presented in Table 1. As seen from the data for different copolymer compositions, the value of μ_1 (NIPA unit sequence length) changes from 4.84 to 2.01 in the NIPA–MA system with increasing NIPA feed concentration, while the values for mean unit sequence length of MA anhydride units (μ_2) do not change significantly and remain relatively low. This fact can be explained by H-bonding effect on the monomer reactivity

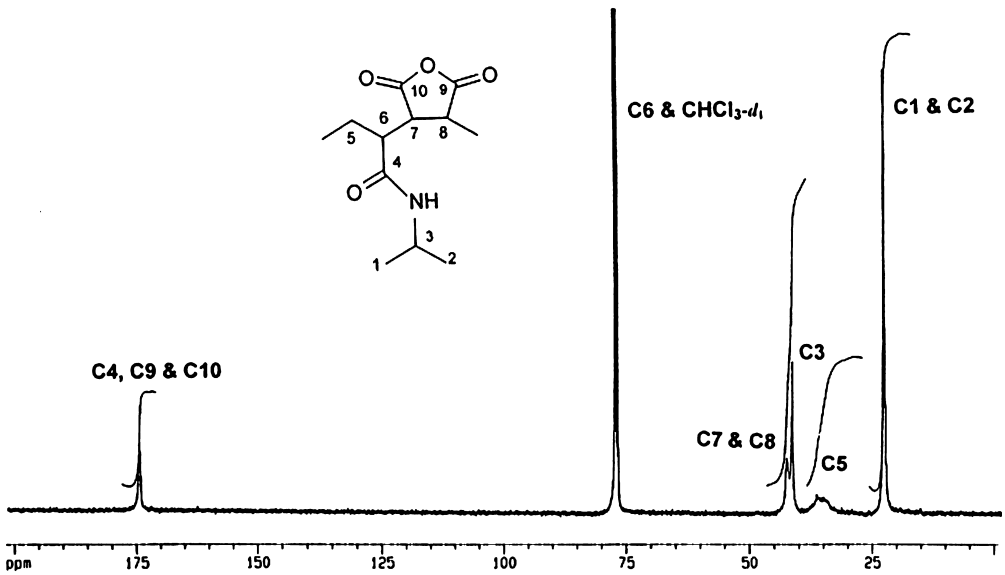


Fig. 2. ^{13}C NMR spectra of poly(NIPA-*co*-MA) in $CHCl_3$-d_1 at 27 °C.

ratios, specially on the chain growing reaction of ~NIPA$^{\cdot}$ with MA monomer.

Two characteristic peaks (3.88 ppm CH in isopropyl group of NIPA unit, 4.17 ppm CH in maleic unit) in the ^{1}H NMR spectra and 41.3 and 42.8 ppm signals (for the CH groups of MA units and isopropyl groups of NIPA units, respectively) in the ^{13}C NMR spectra are characteristic for these copolymers (Figs. 1 and 2) and can used as analytical signals for quantitative analysis of copolymer composition (Table 1). This structure–composition relationship for the copolymers is illustrated in Fig. 3.

^{13}C NMR spectra of poly(NIPA-*co*-MA)s (Fig. 2) contain a single peak at 174.8 ppm which is coming from carbon atoms of C=O groups in both NIPA and MA units. Methylene backbone carbon appears in the range of 35.3–36.8 ppm (relatively broad peak) and two methyl groups of NIPA unit are characterized by a single peak at 23.0 methyne backbone carbon peak appears in the carbon region of $CHCl_3$-d_1 (77.1–77.7 ppm), and because of that is difficult to identify.

FTIR spectra of the copolymers (Fig. 4) are characterized by the typical absorption bands for NIPA units (3300–3100 cm^{-1} broad bands for secondary NH amide or for H-bonded OH group of acid fragments in the partially hydrolysed anhydride units, 2971–2861 cm^{-1} CH bands in isopropyl group, 1650 cm^{-1} strong C=O amide band I, 1545–1516 cm^{-1} strong NH amide II band and 1260 cm^{-1} amide III band) and anhydride units (1845 and 1742 cm^{-1}

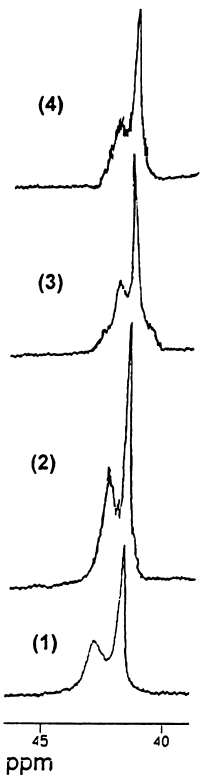


Fig. 3. Fragments of ^{13}C NMR spectra of poly[(NIPA-*co*-MA)s synthesized using various molar monomer ratios of NIPA/MA (1) 97.5:2.5, (2) 95:5, (3) 92.5:7.5 and (4) 90:10.

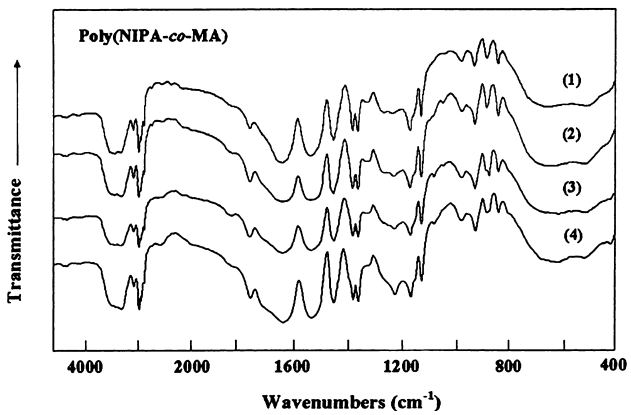
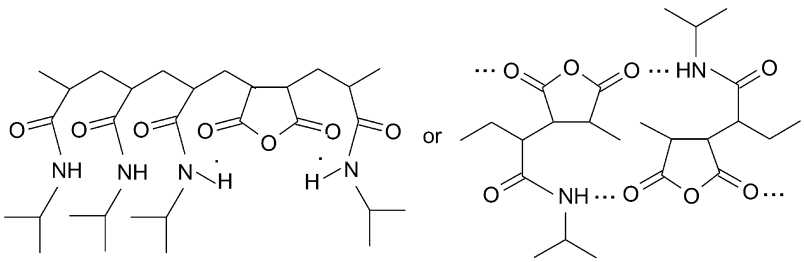


Fig. 4. FTIR spectra of poly(NIPA-*co*-MA) with different compositions: m_1/m_2 : (1) 88.7:11.3, (2) 83.5:16.5, (3) 82.1:17.9 and (4) 79.6:20.4.

symmetrical and antisymmetrical C=O bands, 1190 and 1130 cm^{-1} C–O and C–O–C anhydride bands). Spectra of these copolymers also contain characteristic bands for H-bonded C=O groups (1825–1770 cm^{-1} for anhydride C=O and 1710–1670 broad band for amide C=O) and H-bonded secondary amide NH group (1630 and 1030 cm^{-1} NH deformation of secondary H-bonded amide in –NH···O=C– complex). It can be proposed that intermolecular H-bonded fragments are formed between NIPA and MA units of macromolecules as shows in the following scheme:



noted that many water-soluble copolymers of MA, such as poly(MA-*co*-vinyl acetate), poly(MA-*co*-acrylic acid), poly(MA-*co*-methyl acrylate) and (poly(MA-*co*-vinyl acetate-*co*-acrylic acid) partially hydrolyse during the process of precipitiation, but they easily transffered to anhydride form by thermotreatment. This process of hydrolysis-anhydrization is easily controlled by FTIR spectroscopy [47]. As seen from Fig. 5, plots for the copolymers with relatively low concentration of MA-units have non-linear character. This fact indicates that the copolymers containing lower MA-unit concentrations (for m_1/m_2 values changed from 3.90 to 7.87)

The results of intrinsic viscosity measurements from η_{sp}/c (specific viscosity) vs. c (copolymer concentration in THF) plots for the copolymers having different compositions are illustrated in Fig. 5. Viscosity $[\eta]_{in}$ measurements were carried out in the anhydrous organic solvent (THF) and copolymers were thermotreated at 90–100 °C during 30 min and dried under vacuum before being used for viscosity studies, which helps to minimise the hydrolysis of anhydride units in the organic medium. Additionally, it can

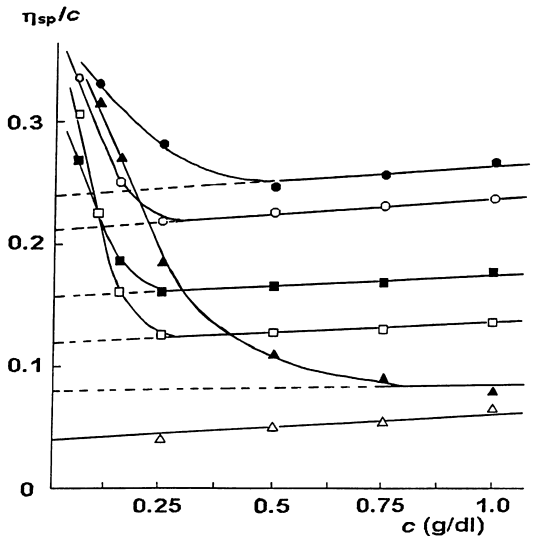
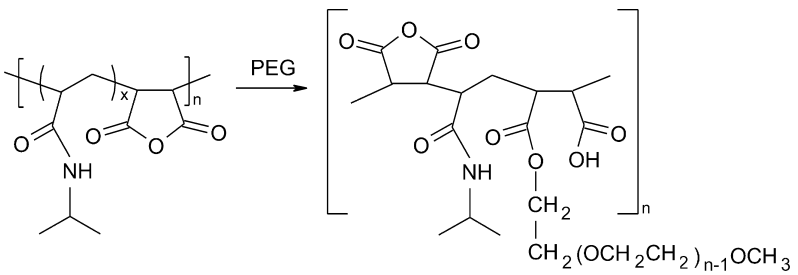


Fig. 5. The plots of η_{sp}/c vs. c (copolymer concentration) for the determination of intrinsic viscosity and evaluation of copolymer composition–viscosity relationships (Dilution effect and polyelectrolyte behaviour): –●–Poly(NIPA), –○– (copolymer with MA unit of 11.3 mol%), –■– (16.5 mol%), –□– (17.9 mol%), –▲– (20.4 mol%) and –△– (33.4 mol%).

exhibit polyelectrolytic behavior, i.e. increase in viscosity with a dilution of copolymer solution, which can be explained by specific behaviour of complexed macromolecules and their conformational changes resulting in the expansion of polymer coil in the diluted solution. It is known that carboxyl-containing polymers such as poly(acrylic acid) [48] and maleic acid copolymers [49,50] exhibit significant conformational change with changing pH, with maleic acid copolymers having more pronounced effect. Similar effect is not observed for the copolymers containing relatively high concentations of MA-units due to their rigid structure and stronger intramolecular interaction inside macromolecular coils. This phenomenon relates the polyelectrolytic behaviour of the studied copolymers and their temperature- and pH-sensivities, which exist only in the case of hydrolysed copolymers with lower maleic acid unit concentrations.

The development of new biocompatible functional polymeric gene carries and vectors with improved design, which would not elicit toxic side-effect is an important problem in the modern macromolecular engineering and gene delivery. Synthesis of stimuli-responsive macromolecular architecture on the base of carboxyl acid ended poly(NIPA), poly(NIPA-*co*-acrylic acid) and polyethyleneimine, and study its conjugation with DNA fragments were subject of our previous investigations [42,43,51]. On the other hand, it is known that the incorporation of the hydrophilic PEG in the polymeric gene carrier allows to improve the biocompatibility of self-assembling polymer···DNA complexes. PEG modified polymers have been shown to stabilize canitonic polymer···DNA complexes under physiological conditions, increase water solubility, and reduce toxicity [52,53].

LCST of hydrolysed poly(NIPA-*co*-MA), i.e. poly-(NIPA-*co*-maleic acid) can be changed by chemical modification of side-chain substituent of the anhydride-containing copolymer macromolecules, in particular by esterification (grafting) of anhydride units with alkanols, glycols or polyethyleneglycols. The similar effect can be achieved by variying copolymer composition (content of hydrophilic anhydride or carboxyl groups) and grafting degree of anhydride units. For this purpose macrobranched copolymers were synthesized by partial esterificaiton of poly(NIPA-*co*-MA) with monomethoxy-PEG. General scheme of this macromolecular reaction in a THF medium can be presented as follows:



It is important to note that it is not possible to realize this selective reaction in the aqueous solution because of a process of dissolution of copolymer in water accompanied by instantaneous hydrolysis of anhydride units before esterification. Therefore, grafting of PEG onto anhydride units of the copolymer cannot be carried out in aqueous medium because MA units present in the copolymer in low amount are easily hydrolysed under these conditions. Thus PEGylation reactions were only carried out in anhydrous organic solvent.

FTIR spectra of grafted copolymers are presented in Fig. 6. As evidenced from these spectra, partial esterification of anhydride units with PEG, containing end hydroxyl group, is accompanied by cleavage of anhydride ring and formation of free side-chain carboxyl (3750, 1970 and 1600 cm^{-1} bands for OH and C=O of COOH groups) and ester groups (1735 cm^{-1}). Intensity of characteristic bands for C=O of anhydride units (1845, 1770 and 1060 cm^{-1}) decreases and intensity of CH_2 group bands (2860, 1460 and 840 cm^{-1}) increases with increasing degree of grafting.

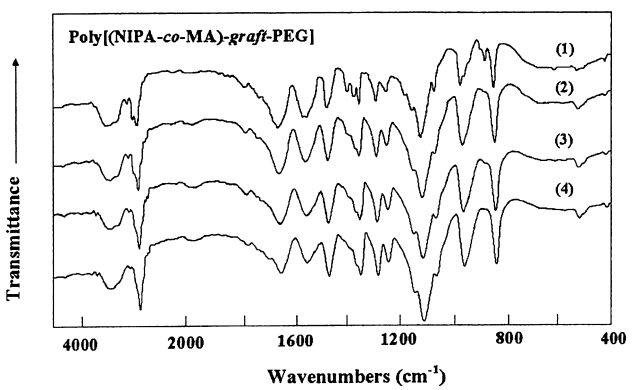


Fig. 6. FTIR spectra of poly[(NIPA-*co*-MA)-*graft*-PEG] with different degree of grafting (mol%): (1) 22.1, (2) 37.0, (3) 41.9 and (4) 49.0.

Structure of the macrobranched copolymer is also confirmed by ^1H NMR method (Fig. 7). ^1H NMR spectrum of the synthesized copolymer contains characteristic chemical shifts for a proton of free carboxylic group (16.98 ppm) and for the protons of the macrobranched fragment including end methoxy group (1.75 ppm).

For the determination of both temperature and pH sensivities of copolymers and their PEG grafted derivatives, a UV spectrophotometric method is used [43]. This method allows to measure the values of LCST at different pH of aqueous medium. Obtained results are presented in Tables 2 and 3. It is shown that relatively high LCST values are observed for the poly(NIPA-*co*-maleic acid)s having high values of acid number (Table 2). This dependence is more strongly expessed for poly[NIPA-*co*-maleic acid(≥20.4 - mol%)]s where LCST changes from 39.2 to 53.7 °C by increasing pH values from 4.0 to 7.4, respectively. Copolymers are more sensitive to the change of pH of the medium than poly(NIPA) with significant increase in LCST values with increasing pH. Experimentally obtained acid numbers perfectly agree with the expected amount of maleic acid units in the copolymers. Observed relatively higher values of LCST for polymer sample with m_1/m_2 ratio of 3.9, which are significantly changed with pH can be explained by higherst amount of acidic units and therefore by their most sensitive to changes in pH of medium. Changes in the LCST values of poly(NIPA) with pH are very minor and

Table 2
Poly(NIPA-*co*-MA) composition-LCST relationships for the different PH values of the aqueous solution

Monomer unit ratio (m_1/m_2)	Acid number (mgKOH/g)	LCST values (°C) for the different values of pH medium		
		4.0	5.0	7.4
7.87	115	32.50	32.66	30.78
5.07	165	33.40	32.47	29.98
4.58	185	34.80	35.07	30.23
3.90	210	39.21	46.43	53.73
Poly(NIPA)	–	30.50	31.05	32.25

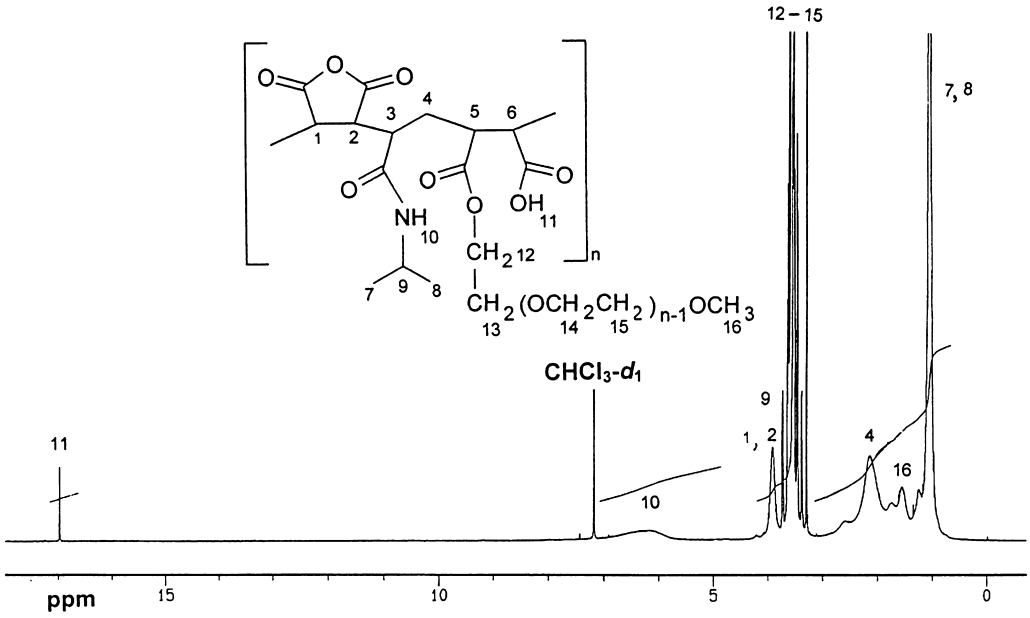


Fig. 7. ^{1}H NMR spectra of poly[(NIPA-*co*-MA)-*graft*-PEG] in $CHCl_3$-d_1 at 27 °C.

insignificant and might be related to hydrogen bonding formation between water molecules and the polymer. It is known that poly(NIPA) shows a volume phase transition in aqueous solution around its lower LCST 32–34 [54]; small increase of this temperature provided an unique coil-to-globular conformation change its macromolecules which transfered from a soluble to an non-soluble state as a result of infrigement of a balance of hydrophilic (H-bonding)-hydrophobic interactions.

Similar relationship between copolymer composition (degree of grafting or acid number) and LCST is observed for the synthesized PEG derivatives of the copolymers (Table 3). However, unlike for poly(NIPA-*co*-maleic acid)s, LCST for the poly[(NIPA-*co*-MA)-*graft*-PEG]s are not observed at the pH of 7.4. For this pH, it is possible that partial ionization of carboxylic groups together with PEG side chains prevent polymer from undergoing conformational transition.

Thus, obtained results suggest that the synthesized copolymers with lower concentrations of maleic acid units and their PEG grafted derivatives, showing relatively high sensivity toward environmental changes, can be used in the gene delivery systems as amphiphilic physiologically active macromolecular structures.

These amphiphilic macrobranched copolymers can be transformed into cation active form by complexation with poly(ethyleneimine) (PEI), which is a water-soluble polymer with primary, secondary and ternary amine groups [55]. When solutions containing PEI become more acidic, all amine groups begin to protonate [56,57]. Complexed macromolecular architectures are synthesized by the reaction of poly[(NIPA-*co*-MA)-*graft*-PEG] with PEI in methanol at 25 °C during 45 min using various grafted copolymer/PEI ratios. Complexed copolymers are isolated from the reaction medium by precipitation in diethyl ether.

Both macrobranched and complexed copolymers with given compositions show polyelectrolytic behavior (increase of the η_{sp}/c values in more diluted solution of THF and methanol at $c < 0.5$ g dl^{-1}, respectively) similar to the initial poly(NIPA-*co*-MA)s (Fig. 5). Intrinsic viscosity $\eta_{in}M$ of these copolymer systems measured in 0.75–1.5 g dl^{-1} concentration region considerably increases from 0.1 to 0.21 dl g^{-1} with the increasing degree of PEG grafting of poly(NIPA-*co*-MA) (Table 5), which can

Table 3
Poly[(NIPA-*co*-MA)-*graft*-PEG] composition (grafting degree)-LCST relationships for the different pH values of the aqueous solution

Grafted copolymer composition (mol%)			N (%)	Acid number (mgKOH/g)	Grafting degree (%)	LCST values (°C) for the different pH values of medium		
m_1	m_2	m_3				4.0	5.0	7.4
88.73	8.77	2.50	7.65	71.0	22.1	34.03	36.14	Not observed
83.52	11.48	5.00	5.58	75.2	37.0	38.42	39.06	Not observed
82.09	10.41	7.50	4.36	62.3	41.9	40.31	42.50	Not observed
79.61	10.39	10.00	3.47	56.0	49.0	46.00	51.37	Not observed

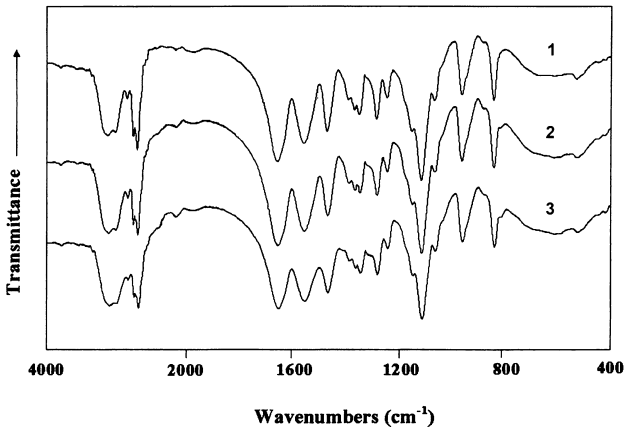


Fig. 8. FTIR spectra of poly[(NIPA-*co*-MA)-*graft*-PEG]/PEI macromolecular complexes prepared using different molar ratios of poly(NIPA-*co*-MA (16.5 mol%): PEI: (1) 1:0.25, (2) 1:0.5 and (3) 1:1.

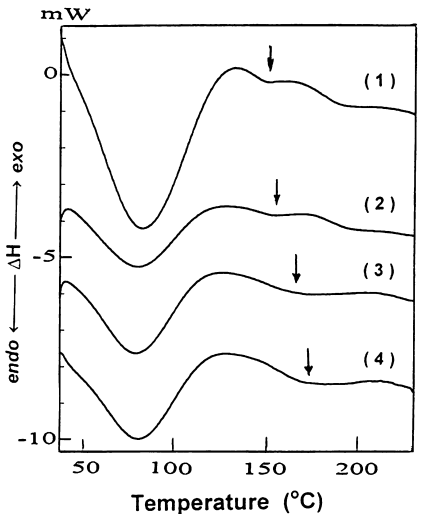
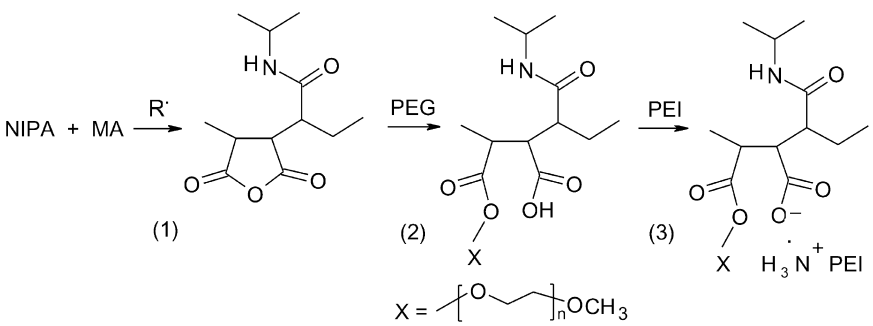


Fig. 9. DSC traces of poly[(NIPA-*co*-MA) with different monomer unit ratios of $f(m_1/m_2)$: (1) 3.51, (2) 2.40, (3) 1.40 and (4) 1.04.

be explained by the increase in the molecular weight of the polymer. While η_{in} value insignificantly decreases from 0.13 to 0.10 dl g^{-1} with the increasing degree of complexation of poly[(NIPA-*co*-MA)-*graft*-PEG] with PEI. This fact can be explained by the formation of more compact macromolecular assemblies in the methanol solution of poly[(NIPA-*co*-MA)-*graft*-PEG]/PEI system. Water solutions of these macrobranched copolymers, containing to distinct surfaces, one hydrophilic (–COOH), the other hydrophobic (macrobranched PEG fragments), can be formed complementary ionic bonds with PEI macromolecules on the hydrophilic surface through proton transferring in the $-COO^{-}\cdot{}^{+}NH-$ (from different amine groups of PEI) interaction forms. In this system, flexible PEG macrobranched hydrophilic fragments can be acted as the water release channels.

General scheme for the synthesis of the macrobranched and complexed architectures can be presented as follows:



FTIR spectra of the obtained copolymers (3) with different degree of complexation which are synthesized using macrobranched copolymer/PEI molar ratios of 1:0.25 (spectra 1), 1:0.5 (spectra 2) and 1: 1 (spectra 3) are illustrated in Fig. 8. It is seen that with increasing number of PEI branched fragments in the copolymer, intensity of NH stretching band (2150 cm^{-1}) considerably increases. At the same time a significant shift of this band from 2245 cm^{-1} (for free PEI) to 2150 cm^{-1} and disappearance of strong 1705 cm^{-1} band (NH stretching in PEI) is observed. Some C=O bands (1850, 1775 and 1735 cm^{-1}) of free and branched MA units (with the exception of 1975 cm^{-1} overtone band of C=O) also disappear or merge with amide C=O band, intensity of which increases. These observed changes in FTIR spectra of poly[(NIPA-*co*-MA)-*graft*-PEG]/PEI systems indicate the blockade of free anhydride and carboxyl groups with PEI through complex formation as shown in the scheme (3). On the other hand, intensity of most bands decreases (with the exception of 2150 cm^{-1} NH band) with increasing PEI content in the studied system. This observed phenomenon could be explained by the physical and chemical structural changes providing a decrease in flexibility and motion of the most groups and bonds, and therefore decrease of the corresponded bond vibrations in the formed self-assembled macromolecular architecture; it is a known fact the reaction of complex formation between PEI and octadecanoic acid (as a model compound for the studied copolymers) is accompanied by the formation of self-assembled lamellar mesomorphic structure [60].

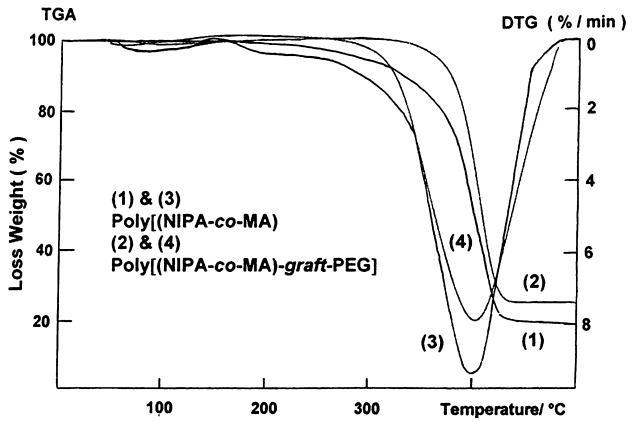


Fig. 10. TGA–DTG curves of poly[(NIPA-*co*-MA) (1 and 3) and poly[(NIPA-*co*-MA)-*graft*-PEG] (2 and 4).

3.2. Thermal behaviour of the copolymers and their grafted and complexed architectures

The results of DSC, TGA and DTA studies of copolymer composition-thermal behaviour relationship for the synthesized poly(NIPA-*co*-MA)s, poly(NIPA-*co*-MA-*graft*-PEG)s and poly(NIPA-*co*-MA-*graft*-PEG)/PEI systems are illustrated in Figs. 9–12 and summarized in Tables 4 and 5.

Fig. 9 shows DSC traces for the copolymers prepared from the different monomer feed compositions. These results indicate that the intensity and position of higher temperature broad *endo*-peaks, which are associated with the melting point (T_m), significantly depend on the monomer unit ratios in the copolymers. It is known that the high melting points of polymers are associated with

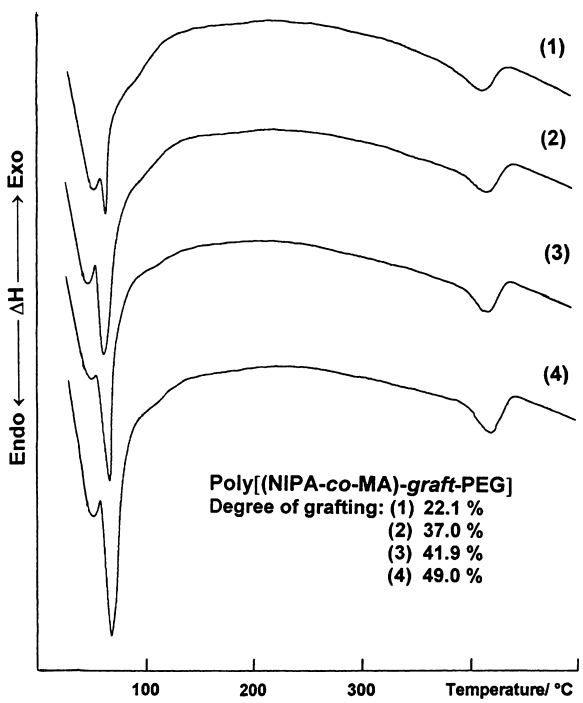


Fig. 11. DTA curves of poly[NIPA-*co*-MA(16.5 mol%)-*graft*-PEG] with different degree of grafting.

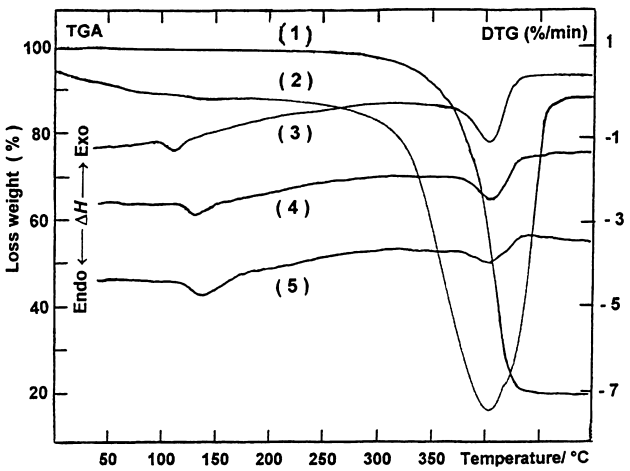


Fig. 12. TGA (1)–DTG (2)–DSC (3–5) curves of poly[NIPA-*co*-MA(16.5 mol%)-*graft*-PEG/PEI with different mol fraction of PEI: (1), (2) and (3) 0.2, (4) 0.33 and (5) 0.5.

many factors including inter- and intramolecular structural regularity and rigidity of macromolecules [58]. The lower temperature *endo*-effects on the DSC curves, associated with the glass transition temperature (T_g), change insignificantly with the appreciable increase in NIPA unit concentration in the copolymers. The values of T_g, T_m and ΔH (enthalpy) for the copolymers are presented in Table 4. It is shown that the decrease of NIPA-unit content in the copolymers increases the value of T_m from 151.8 to 195.3 °C for poly(NIPA-*co*-MA)s. This indicates that the copolymers with MA units form relatively more rigid structure than those containing only NIPA units (homopolymer of NIPA). The higher values of T_m are observed for the copolymers containing relatively high MA-units. Therefore, rigid H-bonded structure provides high T_m in the studied copolymers. The similar H-bond effect was observed for the polyamide (Nylon 11) with M_n 16.000, having lower T_g (42 °C) and higher T_m (184 °C) values [58,59]. For the poly(NIPA) synthesized under similar conditions, the relatively low value of T_m (143.3 °C) with small enthalpy is obtained (Table 4).

Results of TGA–DTG analyses of poly[(NIPA-*co*-MA)s and their PEG branched derivatives are presented in Fig. 10 and Tables 4 and 5. As evidenced from these data, macrobranched copolymers have higher thermal stability which increases with increasing degree of PEG grafting. Observed weight loss around 140–180 °C on the DTG curve of poly[(NIPA-*co*-MA)-graft-PEG] (curve 4) indicates occurrence of some macromolecular reactions, specifically, anhydridization of free carboxylic groups and loss of water, proceeding before decomposition of the copolymer.

Fig. 11 illustrates DTA curves for poly[(NIPA-*co*-MA)-graft-PEG]s with different compositions. The first weak *exo*-effects around 57–60 °C on the DTA curves might be related to a 'physical aging' of H-bonded copolymers which is accompanied by the breaking of inter- and intramolecular –NH···O=C– bonds of the macromolecules. The strong

Table 4
Viscosity and thermal behaviours of poly(NIPA-*co*-MA) with different compositions

MA-unit (mol%)	$[\eta]_{in}$ in THF[a] at 25 °C (g/dl)	Thermal behaviours							
		DSC analysis				DTA analysis	TGA analysis loss weight at		
		T_g (°C)	ΔH (mW)	T_m (°C)	ΔH (mJ)	T_d (°C)	200 °C	250 °C	300 °C
0.00	0.24	57.3	1.11	143.3	3.7	295.0	7.3	11.7	15.5
11.27	0.22	41.0	1.55	151.8	15.7	323.0	4.3	8.5	9.7
16.48	0.18	55.6	1.42	155.2	12.7	337.0	1.3	1.7	4.8
17.91	0.12	63.0	1.02	177.3	68.4	346.5	1.2	1.6	4.2
20.39	0.08	64.7	0.09	181.9	71.2	347.0	0.9	1.7	4.4
33.40	0.04	59.6	0.80	195.3	16.7	350.6	0.8	1.7	4.3

[a] at concentation range 0.1–1.0 g/dl.

Table 5
Composition-property relationships of the synthesized poly[(NIPA-*co*-MA)-*graft*-PEG]s

Monomer unit ratio (m_2/m_3)[a]	Grafting degree (%)	$[\eta]_{in}$ in THF[a] 25 °C (g/dl)	Thermal behaviour						
			T_g (°C)	ΔH (mW)	T_d (°C)	Loss weight at			
						200 °C	250 °C	300 °C	350 °C
3.51	22.1	0.10	51.3	6.7	399	3.7	5.6	5.9	6.8
2.40	37.0	0.13	50.3	17.7	400	0.0	0.5	1.0	2.2
1.40	41.9	0.17	49.8	18.6	405	0.5	0.7	1.0	2.4
1.04	49.0	0.21	47.9	27.8	405	0.9	1.1	1.4	3.6

[a] At concentation range 0.75–1.5 g/dl.

endo-effects on the curves are related to T_g as on the DSC curves (Fig. 9). Intensity of these *endo*-effects significantly depends on the degree of grafting and increases with increasing concentration of the more flexible side-chain polyethylene glycol fragment in macrobranched copolymers. All DTA curves have very broad *exo*-effects up to the decomposition region which can be explained by the occurrence of complex macromolecular transformations, including cross-linking, in the conditions of DTA. Observed relatively high thermal stability of macrobranched copolymer (Fig. 10, curves 2 and 4) can serve as an additional confirmation for the formation of network structure in the studied systems. It is necessary to note that these broad peaks disappear when copolymer samples are subjected to thermo-treatment at 150 °C for 30 min before DTA analysis.

TGA–DTG–DSC curves of poly(NIPA-*co*-MA-*graft*-PEG)/PEI containing different amounts of complexed PEI are illustrated in Fig. 12. It is shown that with increase in the concentration of PEI in copolymer/PEI system from 0.2 to 0.5 mol fraction T_g value increases from 107 to 130.2 °C (3, 4 and 5 DSC traces). Observed high thermal stability of this copolymer containing 0.2 mol fraction of PEI can be explained by possible occurrence of amidization reaction through amine (from PEI) and anhydride or carboxylic groups (from macrobranched copolymer) in the conditions of TGA–DTG analysis.

4. Conclusions

New aspects of the synthesis and characterization of anhydride and/or carboxyl containing copolymers of NIPA and their macrobranched and macrocomplexed architectures were developed and discussed. The synthesis of pH and thermo-sensitive 'intelligent' amphiphilic functional copolymers and their macrobranched and cation active macrocomplexed architectures with given structure and properties, achieved by radical-initiated copolymerization and side-chain macromolecular reactions in the non-aqueous solution, opens new perspectives in the development of bioengineering process and gene delivery. The synthesis, structural and physicochemical characterization of soluble conjugates of the resulting copolymer systems and biomolecules with special attention on the properties and behaviour of these systems in the aqueous solution will be a subject of the third part of our work.

Acknowledgements

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National Scientific and Technical Research Council) through Project MISAG-146 is acknowledged.

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