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Bioengineering functional copolymers. XIV. Synthesis and interaction of poly(*N*-isopropylacrylamide-*co*-3,4-dihydro-2*H*-pyran-*alt*-maleic anhydride)s with SCLC cancer cells

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ABSTRACT

Novel antitumor active functional polymers with supramacromolecular structures were synthesized by a complex-radical terpolymerization of *N*-isopropylacrylamide (NIPAm), 3,4-dihydro-2*H*-pyran (DHP), and maleic anhydride (MA) with 2,2′-azoisobisbutyronitrile as a radical initiator in 1,4-dioxane at 65 °C under nitrogen atmosphere. The structure and composition of terpolymers were investigated by ^{1}H (^{13}C) NMR spectroscopy. Interaction of terpolymers with human lung small cell carcinoma (SCLC) were investigated by using different methods such as cytotoxicity, statistical, apoptotic and necrotic cell indexes, double staining and caspase-3 immunostaining, light and fluorescence inverted microscopy analyses. Investigations into structure, composition, and antitumor activity relationships revealed that terpolymers containing a combination of ionisable amide-pyran linkages and H-bonded carboxylic groups exhibited higher cytotoxicity. It was observed that terpolymer with nearly alternating structure provides a maximum concentration of ionisable and H-bonded antitumor sites, and therefore, exhibits higher in vitro cytotoxicity, apoptotic and necrotic effects towards SCLC cancer cells.

1. Introduction

It is known that the water soluble anhydride-containing copolymers as polyanions and their functional derivatives have high biological and physiological activity, specially antimicrobial and antitumor properties.[1] Polyanions are also known for their potential to stimulate the immune system and invoke activities against tumors, viruses, and bacteria.[2–4] Copolymers of 3,4 -dihydro-2*H*-pyran (DHP) and its derivatives with acrylic acid, which contain tetrahydropyran rings and free carboxylic groups on the polymer backbone, as well as an alternating cyclocopolymer of divinyl ether (acyclic analog of DHP) with maleic anhydride (MA) have exhibited in vitro antitumor activities.[1–4] Antitumor activity of anion-active poly(DHP-*alt*-MA) and poly(DHP-*co*-MA-*co*-vinyl acetate) (co)terpolymers were studied by methyl-thiazol-tetrazolium testing method.[5] using calorimetric measurements of chemotherapic effect and quantitative evaluation of LD_{50} dose for the total of tumor cells (Raji cells-human Burkitt lymphoma cell line).[6] It was shown that hydrolyzed terpolymer has sufficiently high antitumor activity, which depends on the amount of hydrogen bonding carboxylic groups and their regular distribution in the side chain of functional macromolecules.[6–8] Han et al.[9,10] reported the synthesis, characterization and bioactivity of poly(DHP-*alt*-MA) and its derivatives, with different substituents (e.g., acetoxy, methoxy, ethoxy, methoxycarbonyl, formyl, acetoxymethyl, and tosyloxymethyl groups, as well as guanine derivatives) in the 2-position of pyran ring of the copolymer backbone. Authors found that these pyran-containing copolymers with a high density of carboxylic acid functionality as the polynucleotide analogues exhibit antitumor activities in vitro and in vivo,[9] which were found to have higher activities against the tumor cells (B16 and 3LL) than those of their acyclic analogues, especially alternating cyclocopolymer of divinyl ether with MA. This cyclocopolymer, known as pyran copolymer is one of the well known bioengineering polymers having a wide range of biological activity.[1,2] It processes antitumor, antiviral, antibacterial and antifungal activities, induces interferon formation, and acts as an anticoagulant and anti-inflammatory agent.[1,11–15] Hirano et al.[16,17] reported that the anhydride functionalized pyran copolymer 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.[17] It was demonstrated that the copolymer-SOD conjugate shows anti-inflammatory effect against rat re-expansion pulmonary edema at least 3 min of leukocyte

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adhesion.[16] Hydroscopic and water-soluble copolymers of DHP and its 2-carboxylate derivative with acrylic acid and acrylamide, which were synthesized by radical copolymerization into diethyl ether; antitumor activity were determined with *Lewis lond carcihom, carcihom, malignan and, melanoma*).[4]

Recently, Sasai et al.[18] reported the method to introduce a large amount of carboxyl groups onto polystyrene (PS) by the surface modification of PS Petri dish with poly(MA-*alt*-MVE) through plasma induced cross-linking reaction, followed by hydrolysis of copolymer to generate active sites for immobilization of the prostate cancer cell (LNCap). According to the authors, the microscopic images show a good attachment, adhesion and spreading behavior of cells on the PS/copolymer surface. 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 be included to a class of bioengineering polymer systems. Gam et al.[19] evaluated the in vitro cytotoxicities of glycinylmaleimide (GMI) copolymers using K-562 human leukemia cells and HeLa cells. According to the authors, the copolymers are less cytotoxic than monomeric glycinylmaleimide (GMI) at dosage of 0.02, 1.0 and 5.0 mg mL^{-1}. It was found that copolymers were very effective at any dosage tested. They 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. Cho et al.[20–22] opened up a new frontier for the pharmaceutical applications of maleimide copolymers. Authors also reported the synthesis and biological activity of polymeric antitumor compounds such as poly(glycinyl maleamic acid) derivatives,[20,21] methoxyitaconyl derivatives and homo- and copolymers of vinyl-(5-fluorouracil)-ethnoate (VFUE) with MA and acrylic acid,[22] and described their reduced toxicity and improved antitumor activity.

The pH and temperature sensitive copolymers of NIPAm were used in protein conjugation as cation-active polymers soluble in water and physiological medium,[23–27] as well as carrier system for DNA delivery,[28] affinity separation of genotoxins[29] and as reversible bioconjugates.[30] On the other hand, unlike homopolymer of NIPAm, specific behavior 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. Recently we have been reported that the functional copolymers of NIPAm and anion-active comonomers with controlled hydrophilic/hydrophobic balance have a wide range of medical, pharmacy and bioengineering applications.[31–34] Synthesis and characterization of bioengineering poly(NIPAm-*co*-DHP) copolymers were a subject of our recent publication.[35]

Jaracz et al.[36] described recent advances in tumor-targeting anticancer drug conjugates. According to the authors, traditional chemotherapy relies on the premise that rapidly proliferating cancer cells are more likely to be killed by a cytotoxic agent. In the last decade, various biosystems and antitumor agents have been developed so as to act as tumor-targeting drugs containing tumor recognition active sites and a cytotoxic warhead connected directly or with a suitable linker to form conjugates.[36–40] Lung cancer is the leading cause of cancer-related deaths, and its incidence continues to rise worldwide.[41] Generally this cancer classified in two major histological types: roughly 80% are non-small-cell lung cancers (NSCLCs), comprising adenocarcinoma, large-cell carcinoma, and squamous cell carcinoma. Most of the remaining cases are classified as small-cell lung cancer (SCLC).[42,43]

Zangemeister-Wittke et al.[44] reported that the most prominent advances in the treatment of SCLC, provided by preclinical and clinical studies during the last decade, that require further investigation are; (i) recent improvements in chemo- and radiation therapy; (ii) advances in targeting cell surface antigens, autocrine growth factors and their receptors, and modulators of drug resistance; (iii) advances in targeting the molecular basis of increased proliferation and apoptosis deficiency of tumor cells, such as the use of bcl-2antisense oligodeoxynucleotides or the replacement of inactivated or lost tumor suppressor genes. Miller et al.[45] showed that local therapy, either surgery or radiation alone, had no significant impact on the survival of SCLC patients. In the last three decades, several chemotherapeutic drugs, with activity against SCLC, were identified by many researchers,[45–47] including nitrogen mustard, cyclophosphamide, methotrexate, doxorubicin, etoposide, procarbacine, cisplatin, paclitaxel, docetaxel, vinorelbine, gemcitabine, ifosfamide, topotecan, teniposide, and vincristine. Another observation was that a combination of up to three drugs yield better results than individual drugs used alone or sequentially. According to the Ihde,[46] advances in chemotherapy reached a certain level in the eighties, despite many clinical trials testing further strategies to improve the results. On the other hand, phase studies of chemotherapy agents alone or regimens have shown toxic reactions as a leucopenia, neutropenia, anemia, erythema, diarrhea, and edema in patients with SCLC. However, a new method or drug has not been found to overcome SCLC cancer.

This work presents (a) the synthesis and characterization of novel antitumor active terpolymers with given stimuli-responsive properties and supramacromolecular structure prepared by complex-radical copolymerization of NIPAm with DHP···MA monomer complex, (b) interaction of synthesized functional copolymers with human lung small cell carcinoma (SCLC) as cancer cells, (c) investigation of structure–composition–antitumor activity relationship and (4) evaluation of antitumor activity by using a combination of different methods such as cytotoxicity, statistical, apoptotic, and necrotic cell indexes, double staining and caspase-3 immunostaining, and fluorescence inverted microscopy analyses.

2. Experimental

2.1. Materials

NIPAm monomer (Aldrich) was purified before use by distillation under moderate vacuum and re-crystallization from benzene solution with hexane. DHP monomer (Aldrich) was distillated before use. MA monomer (Fluka) was purified by re-crystallization from anhydrous benzene and sublimation in vacuum. 2,2′-Azoisobisbutyronitrile (AIBN) (Fluka) was twice re-crystallized from methanol/chloroform mixture before use. All other solvents and reagents were of analytical grade and used without purification. NCI-H82/An1 Human lung small cell carcinoma (SCLC) was obtained from the tissue culture collection of the SAP Institute (Turkey). Cell culture flasks and other plastic material were purchased from Corning (Israel). 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). The primary antibody, caspase-3 was purchased from Lab Vision.

2.2. Terpolymerization procedure

Terpolymerization of NIPAm, DHP and MA using various monomer feed ratios of NIPAm: [DHP···MA(1:1)] were carried out in 1,4-dioxane at 65 °C with AIBN radical initiator at a constant total concentration of monomers under nitrogen atmosphere. Reaction conditions: $[M]_{total} = 2.78$ mol/L, $[AIBN] = 6.5 \times 10^{-3}$ mol/L and monomer ratios of NIPAm/(DHP···MA) = 0.25–4.0. Appropriate quantities of monomers, 1,4-dioxane and AIBN initiator were placed in a standard pyrex-glass reactor, and was cooled by liquid nitrogen and flushed with dried nitrogen gas for at less 3 min, then

soldered and placed in a thermo stated heater with magnetic mixer at 65 ± 0.1 °C. Terpolymers were isolated from the reaction mixture by precipitation with diethyl ether, then washed with several portions of benzene and dried under vacuum at 40 °C. The terpolymer compositions were found by elemental (N content for NIPAm units) and chemical (acid number for MA units) analysis, and ^{1}H NMR spectroscopy using integral area of chemical shifts of monomer functional groups for quantitative analysis.

2.3. Characterization

FTIR spectra of the terpolymers (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) spectrometer with $CHCl_3$-d_1 or DMSO-d_6 as a solvent at 25 °C.

The CHNS-932 Model LECO Elemental Analyzer was used for the determination of nitrogen content in the terpolymers synthesized. Molar fraction of m_1 unit (mol % of NIPAm) in terpolymer was calculated according to the following equation:

$$m_1 = \frac{M_2}{\frac{A_N}{B} - \frac{\Delta M}{100}} \quad (1)$$

where M_2 is the molecular weight of alternating DHP-*alt*-MA (M_2) unit; A_N is the atomic weight of *N*; *B* is the content of *N* in the terpolymer (wt %); $\Delta M = M_1 - M_2$ (M_1 is molecular weight of NIPAm unit).

Acid number (AN, mgKOH/g) of copolymers containing anhydride and acid groups were determined by standard titration method. The copolymer compositions were calculated using chemical analysis data (acid number, Table 1) and the following equation:

$$m_2 = \frac{W_1 \cdot 100}{\frac{2M_{(KOH)}}{AN} - (W_2 - W_1)} \quad (2)$$

where, W_1 and W_2 are molecular weights of m_1 (NIPAm) and m_2 (DHP···MA) monomer units, AN is acid number and $M_{(KOH)}$ is the molecular weight of KOH.

Low critical solution temperature (LCST) values, related to coil-globule conformational transition at different pH media were obtained spectrophotometrically at 500 nm using acetic acid/sodium acetate (pH 4.0) and $Na_2HPO_4/Na_2PO_4{\cdot}H_2O$ (pH 7.4) buffers. The LCST measurements were performed in a UV spectrophotometer (UV 1602, Shimadzu, Japan) equipped with a heating system and temperature control unit. The temperature of the polymer solutions (1.0 wt %) at acidic and nearly neutral medium 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 of terpolymer solution was calculated from the absorbance versus temperature plot.

2.4. The analysis of cell culture

The number of apoptotic and necrotic cells were determined by Fluorescence Inverted Microscope (Leica, Germany). The above-mentioned microscopes also recorded the cell images. The independent sample t-test and p values of less than 0.05 were used for statistical analyses, where $p = 0.031$ for cytotoxicity values, $p = 0.029$ for apoptotic values, and $p = 0.033$ for necrotic values were considered significant. Every study was carried out three times. Data is presented in terms of ±SEM (standard errors of the mean).

2.5. Cytotoxicity

For cytotoxicity experiments, SCLC cells (20×10^3 cells per well) were placed in 24-well plates (DMEM). Different amounts of poly(NIPA-*co*-DHP) copolymer (C-1) and poly(NIPA-*co*-DHP-*alt*-MA) terpolymers (T-1, T-2 and T-3) (about 0–750 μg mL^{-1} in aqueous solutions as hydrolyzed copolymer and terpolymers, respectively) were placed in wells containing cells, respectively. The plates were kept in a CO_2 incubator (at 37 °C in 5% CO_2) for 24 h; the medium was replaced with fresh medium, and incubated under the same conditions for 24 h. Following this incubation, SCLC cells were harvested with trypsin–EDTA, and then dyed with trypan blue.[48] The number of living and dead cells were counted with a hemocytometer (C.A. Hausse & Son Phluila, USA), using light microscope at 200× magnification.[49]

2.6. Hematoxylin/eosin staining

SCLC cells (20×10^3 cells per well) were placed in 24-well plates. After treating with different amounts of functional (co)terpolymer (about 0–750 μg mL^{-1} in aqueous solutions) for a period of 24 h, the medium was removed, the cells were washed with distilled water and fixed in ethanol, and stained with hematoxylin/eosin. After staining, the cells were observed using light microscopy. Thus, showing the cellular and nuclear morphology in cultured cells stained with haematoxylin/eosin.

2.7. Analysis of apoptotic and necrotic cells

Double staining was performed to quantify the number of apoptotic cells in the culture, based on scoring apoptotic cell nuclei. SCLC cells (50×10^3 cells per well) were placed in 24-well plates. After treating with different amounts of functional (co)terpolymer (about 0–750 μg mL^{-1} in aqueous solutions) for a 24 h period, both attached and detached cells were collected, washed with PBS and then stained with Hoechst dye 33342 (2 μg mL^{-1}), propidium iodide (PI) (1 μg mL^{-1}) and DNAse free-RNAse (100 μg mL^{-1}) for 15 min at room temperature. Then, 10–50 μL of cell suspension was smeared on the slide and cover slip for examination using fluorescence microscopy.[50] While the nuclei of normal cells were stained light blue, apoptotic cells were stained dark blue by the Hoechst dye. The apoptotic cells, from their nuclear morphology, were identified as a nuclear fragmentation or chromatin condensation. Necrotic cells were stained red by PI. Necrotic cells lacking plasma membrane integrity and PI dye cross the cell membrane, providing that PI dye does not cross the non-necrotic cell membrane.

Table 1
Poly[NIPA-*co*-(DHP-*alt*-MA)] compositions determined by the different methods

Monomer feed (mol %)			^{1}H NMR analysis			Elemental and chemical analysis		Terpolymer composition (mol %)		
$[M_1]$	$[M_2]$	$[M_3]$	Am_1 (CH)	Am_2 (2CH)	Am_3 (CH_2)	*N* (%)	AN (mg KOH/g)	m_1	m_2	m_3
80	10	10	86	0.230	0.218	30	95	82.92*	8.30	8.78
								84.02	7.67	8.31
50	25	25	41	1.923	1.173	7.34	235	58.34*	20.30	21.36
								59.86	19.46	20.68
20	40	40	13	2.198	1.524	6.97	255	55.79*	21.09	23.12
								56.78	20.91	22.31

* These values for all monomer units were calculated by using ^{1}H NMR analysis data.

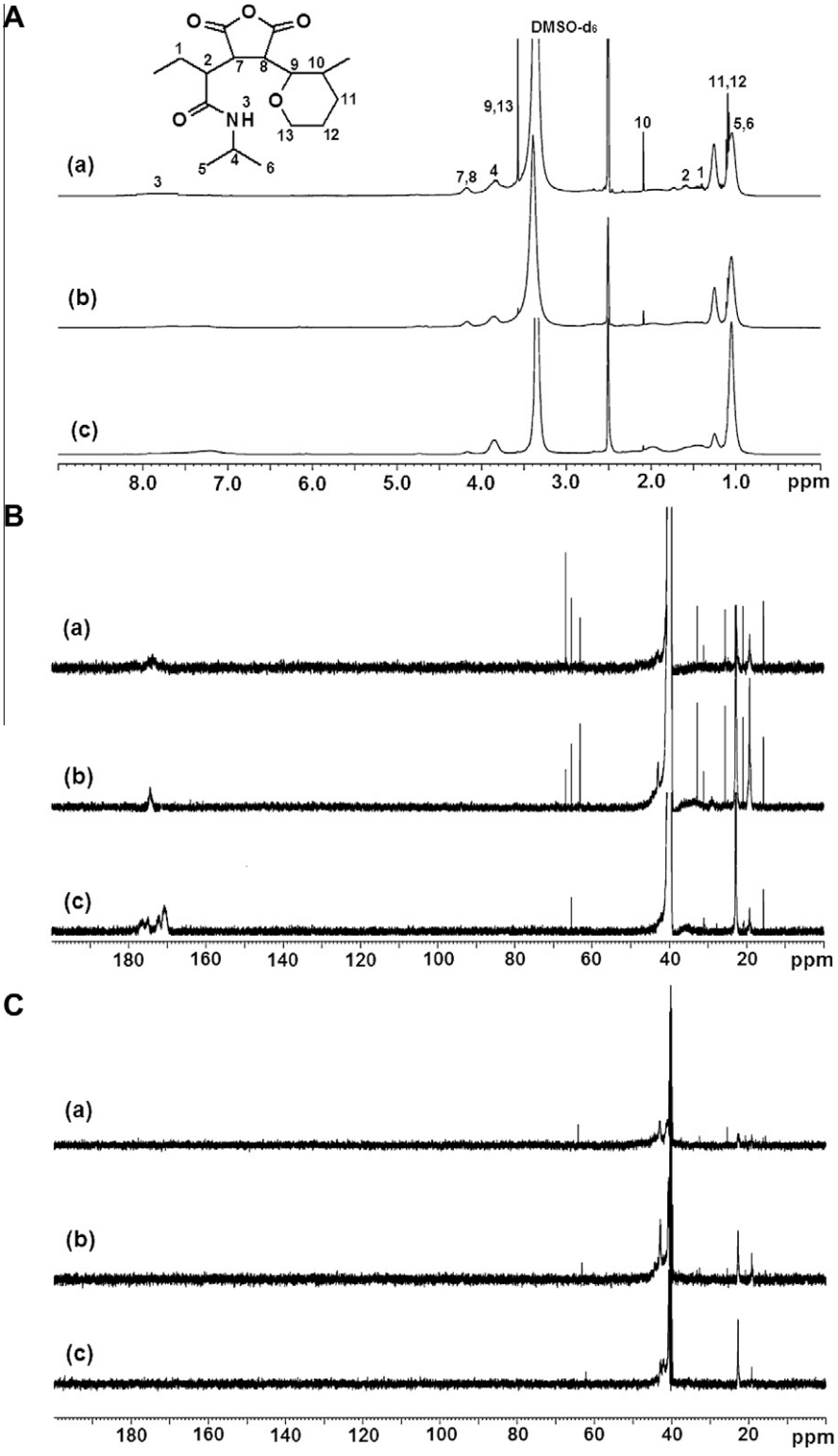


Figure 1. (A) ^{1}H NMR, (B) ^{13}C NMR and (C) ^{13}C NMR-DEPT-90 spectra of poly(NIPAm-*co*-MA-*alt*-DHP)s containing different amounts of NIPAm units (mol %) and carboxylic groups (acid number, mg KOH/g), respectively: (a) 84.0 and 95, (b) 59.9 and 235, (c) 56.8 and 255.

The results of the counted number of apoptotic and necrotic cells in 10 randomly chosen microscopic fields were expressed as a ratio of apoptotic and necrotic to normal cells. The number of apoptotic and necrotic cells were determined by Fluorescence Inverted Microscope (Leica, Germany) with DAPI filter, and FITC filter, respectively.

2.8. Immunocytochemistry stains

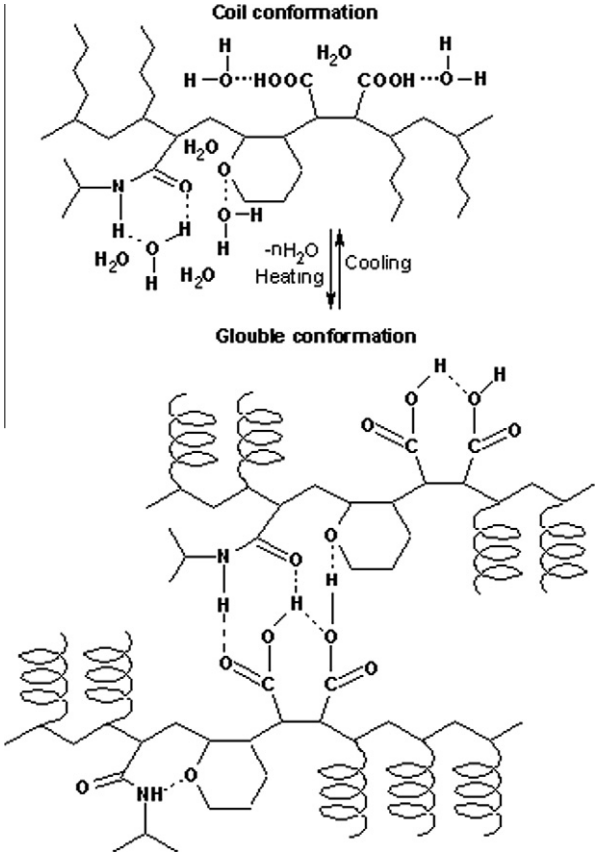
Approximately, 2 ml SCLC (Small cells lung carcinoma) cells ($20x10^3$ cells per well) were treated with (co)terpolymer (about 0–750 μg mL^{-1} in aqueous solutions). The acquired suspension was centrifuged for 5 min with 1670 g in a Hettich centrifuge.

Table 2
Characteristic FTIR absorbtion bands and their assignments for poly((NIPAm-*co*-DHP-*alt*-MA) terpolymers

Absorption band (cm^{-1})	Band assignment
NIPAm unit	
3430 (s), 3300 (s) and 3070 (s)	NH stretching broad bands for secondary amide group and its H-bonded complex
2970 (s) and 2875 (s)	CH stretching in CH_3
1650 (vs)	C═O amide I band
1545 (vs)	NH amide II band
1380 (s) and 1327 (m)	CH_3 deformation in isopropyl group
1268 (m)	NH–C═O *trans*-amide III band
1130 (m)	C–N stretching
1030 (m)	NH bending –NH···O< complex
800 (w) and 670 (m.-s)	NH deformation and CH bending (backbone)
DHP unit	
2930 (s) and 2770 (w)	CH_2 antisym. And sym. stretching
1455 (s) and 1230 (w)	CH_2 deformation (overlapping with CH_3)
975 (m) and 926 (m)	C–O–C band of cyclic ether
648 (w)	Pyran ring vibration
549 (w) and 576 (w)	CH bending (backbone) and C–O bending
MA unit	
1845 (m) and 1778 (s)	C═O stretching in anhydride group
625 (m.-w)	CH bending in –CH–CH– backbone

Table 3
Low critical solution temperature (LCST) of poly(NIPAm-*co*-DHP-*alt*-MA) terpolymers with different compositions

Terpolymers	NIPAm unit (mol %)	LCST (°C) pH 4.0	LCST (°C) pH 7.4
T-1	84.02	34.7	34.8
T-2	59.86	36.1	37.3
T-3	56.78	37.2	39.0
Poly(NIPAm)	100	28.5	26.2



Scheme 1. Schematic representation of a coil-globule transition in amphiphilic terpolymer macromolecules through conformational change of hydrophobic/hydrophilic linkages.

Cytospin preparations were fixed using 70% ethanol for immunocytochemistry. For an indirect immunocytochemistry procedure, cytology specimens were treated with 3% H_2O_2 for 10 min, diluted with water, and then rinsed in PBS (pH 7.4) for 5 min. Non-specific protein binding was blocked on the specimen by incubating with a blocking solution for 10 min. The primary antibody, caspase-3 (Lab Vision), was diluted at 1:300, and then incubated for 1 h at room temperature. Specimens were washed with a PBS buffer (pH 7.4) and incubated in the biotinylated secondary antibody solution for 10 min. Diaminobenzidine (Dako) served as the chromagen and Mayer's hematoxylin as the counter stain. For the negative control, the primary antibody was omitted in one of the slides.

The immunocytochemical staining results were reviewed independently and blindly by observers without the knowledge of treatment. The immune reactivity of the caspase-3 antibody is confined to the cytoplasm of apoptotic cells. The numbers of the caspase-3-positive cytoplasm stained cells in all fields using a x400 final magnification. For each image (at least 100 cells/field), three randomly selected microscopic fields were evaluated.

3. Results and discussion

3.1. Structure and composition of poly(NIPAm-*co*-MA-*alt*-DHP)s

Structure of synthesized copolymers was studied by ^1H (^{13}C) NMR spectroscopy. Obtained results are illustrated in Figure 1. In ^1H NMR spectra (A) of terpolymer (T-1), NH amide proton appears in the form of a broad peak at 7.82 ppm (NH proton appears at 6.44 ppm for NIPAm homopolymer[35]). This peak is shifted to 7.53 (for T-2) and 7.21 ppm (for T-3) region with increasing pyran/anhydride linkage content in terpolymers. This fact can be explained by the increasing effect of H-bonding through >O···NH– and –C═O···HN– complex-formation. Similar H-bonding effect in radical polymerization of NIPAm in the presence of hexamethylphosphoramide was observed by Hirano et al.[51] Results of comparative spectral analysis of terpolymers indicate that the chemical shifts of protons from NH (amide) and CH (isopropyl) groups of NIPAm unit and CH_2 groups (in CH_2–O pyran ring) of DHP unit show a significant sensitivity towards >O···HN– complexing. The formation of above-mentioned complexes is also confirmed by ^{13}C NMR (DEPT-90). The overlapping region for CH and CH_2 groups observed in ^{13}C NMR spectra (Fig. 1B) are resolved by employing DEPT-90 NMR (for the CH groups) measurement, the result of

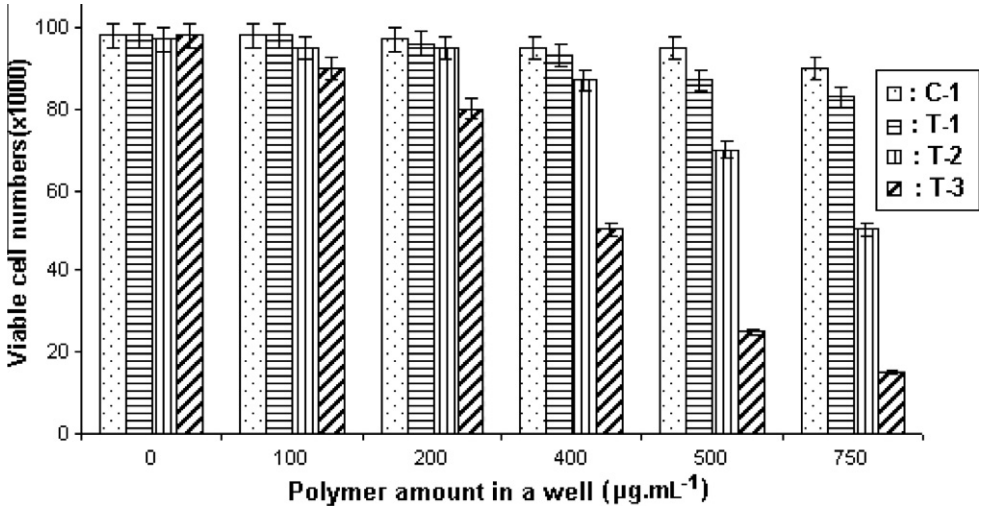


Figure 2. In vitro cytotoxicity of hydrolyzed poly(MA-*alt*-DHP) copolymer (C-1) and poly(NIPA-*co*-MA-*alt*-DHP) terpolymers in physiological conditions with different amount of NIPA units in terpolymers (mol %): 84.0 (T-1), 59.9 (T-2) and 56.8 (T-3) at 24 h incubation. Number of viable SCLC cells in wells. Results are presented as means ± SEM. * Significant difference from control ($p = 0.031$).

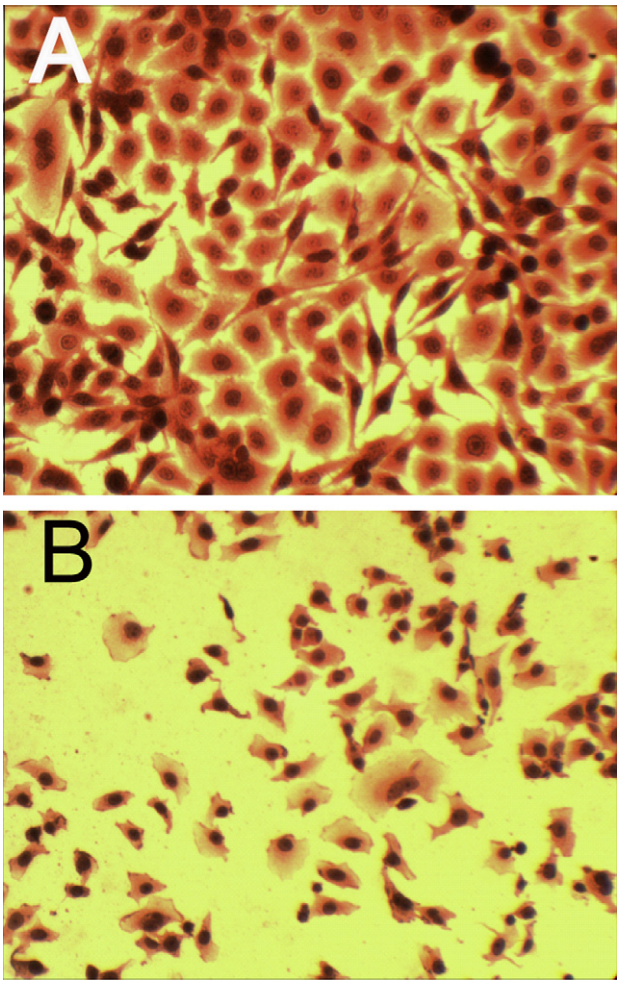


Figure 3. Light microscope image of (A) hematoxylin–eosin stained SCLC cells culture as a control, (B) T-3 terpolymer (500 μg mL^{-1} concentration)/SCLC cells conjugate (stained dye); dense spots were showed nucleus of cells, and distinct violet were indicated cytoplasm of cells at 24 h incubation. Images taken under 200× magnification, scale: 20 μm.

which is illustrated in Figure 1C. Both amide and anhydride carbonyl groups from NIPAm and MA units, respectively, were identified with characteristic peaks at 172.5–180 ppm in the ^{13}C NMR spectra of terpolymers (Fig. 1B).

Results of FTIR spectral analysis of synthesized terpolymers, including the characteristic bands and their assignments for NIPAm, DNP and MA units are summarized in Table 2. The formation of H-bonded linkage as a result of amide/carbonyl and amide/pyran interactions can be approximately confirmed by the presence of 3075 cm^{-1} (for the –NH···O=C– and for –NH···O< complexes) and 1026 cm^{-1} (NH bending in –NH···O< complex) bands. The compositions of the terpolymers, poly[NIPAm-*co*-(DHP-*alt*-MA)]s synthesized using various monomer feed ratios were determined by ^{1}H NMR method and were achieved by comparing the integral areas of the protons for CH of isopropyl group in NIPAm unit (Am_1), CH_2 of pyran ring in DHP unit (Am_2) and backbone CH–CH group in MA unit (Am_3) in the spectra of terpolymers. Molar fractions of the comonomer units (m_1, m_2, and m_3) were calculated according to the following equations:

$$m_1 = [1 + (n_1Am_2)\cdot(n_2Am_1)^{-1} + (n_1Am_3)\cdot(n_3Am_1)^{-1}]^{-1}\cdot 100 \quad (3)$$

$$m_2 = [1 + (n_2Am_1)\cdot(n_1Am_2)^{-1} + (n_2Am_3)\cdot(n_3Am_2)^{-1}]^{-1}\cdot 100 \quad (4)$$

$$m_3 = [1 + (n_3Am_1)\cdot(n_1Am_3)^{-1} + (n_3Am_2)\cdot(n_2Am_3)^{-1}]^{-1}\cdot 100 \quad (5)$$

Obtained results using ^{1}H NMR, elemental and chemical analysis data are summarized in Table 1. Obtained compositions (monomer unit ratios) of terpolymers indicated that H-bonding between amide and anhydride/carboxylic groups of NIPam and MA/MAc units, respectively, takes place a significant role in chain growing reactions providing an enrichment of the terpolymer macromolecules with NIPAm units. Similar effect previously was observed in radical copolymerization of NIPAm–MA monomer pair.[35]

Recently, we have demonstrated that aqueous solutions of the poly(NIPAm-*co*-MA) and poly(NIPAm-*co*-DHP) binary copolymers exhibit a reversible coil-globule transition that depends on the composition of the copolymers.[32,35] Similar effect was observed by Carter et al.[52] for the aqueous solutions of bioengineering branched NIPAm copolymers with end imidazole functionalities. It can be proposed that this reversible coil-globule transition is also realized in terpolymer solutions. In this conformational change of terpolymer macromolecules, H-bonded linkages play a significant role which provides an easy coil-globule transition under the employed testing conditions, and therefore in the interaction of functional macromolecules with SCLC cancer cells.

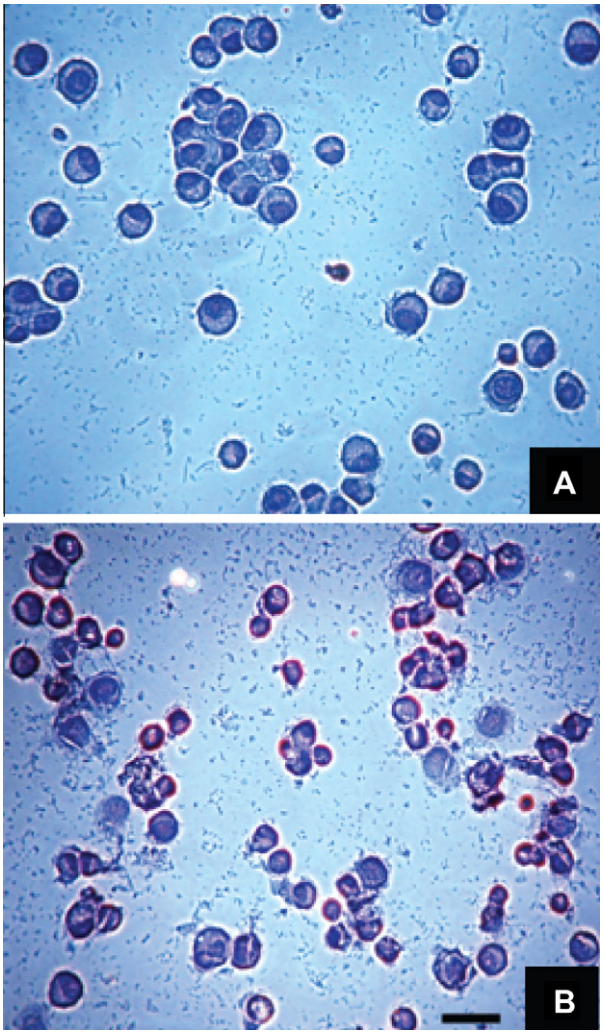


Figure 4. Light microscopy images of (A) non-apoptotic SCLC cells as a control group (stained with caspase-3 immunostaining kit); blue cytoplasm of cells were demonstrated non-apoptotic cells, and (B) 500 μg mL^{-1} T-3 terpolymer/SCLC cells conjugate (stained with caspase-3 immunostaining kit), where brown cytoplasm of cells image indicates the formation of apoptotic cells. Arrow shows apoptotic cell. Image were recorded with 200× magnification, scale bar shows 20 μm.

Physical structural changes, such as stimuli-responsive behavior (pH and temperature sensitive properties) and low critical solution temperature (LCST) of the terpolymers as a result of conformational changes of their macromolecules in aqueous solutions were performed by a temperature controlled UV-spectroscopy method at 500 nm. Obtained results are presented in Table 3. As seen from these data, poly(MA-*alt*-DHP) copolymer does not exhibit LCST behavior at pH 7.4 and 4.0 while poly(NIPAm-*co*-MA) copolymer with nearly the same content of NIPAm units exhibits coil-globule transition at pH 7.4 only.[32] It was shown that terpolymers exhibit pH-sensitive behavior and the coil-globule transition at a given LCST temperature which strongly depends on the content of NIPAm linkages and formation of hydrophilic/hydrophobic balance. The decrease of NIPAm units, and therefore the increase of acid number caused an increase in LCST temperature. The terpolymer solutions instantaneously transfer into equilibrium state as compared to those of poly(NIPAm) solutions in both neutral and acidic medium. This observed fact can be explained by H-bonding effect providing an easy conformational transition.

On the other hand, pyran-acid fragments prefer to interact with the solution rather than separating from the solution resulting in relatively higher LCST values. These results allow attributing the synthesized functional terpolymers to the class of pH and temperature sensitive bioengineering polymers with controllable hydrophilic/hydrophobic interactions.

Schematic representation of coil-globule transition can be illustrated as follows (Scheme 1):

3.2. Cytotoxicity of the (co)terpolymers

The effect of the (co)terpolymer as a natural polyanion analogue on SCLC cancer cells was investigated in this study. The cytotoxic behavior of C-1 copolymer and T-1, T-2, and T-3 terpolymers with different compositions {content of pH- and temperature responsive NIPAm linkage, mol %: (C-1), (T-1), (T-2), and (T-3)} were researched in regards to utility of antitumor drugs. Figures 2 and 3 (microscopy images) illustrate the number of viable cancer cells in each group after the incubation of the cancer cells with (co)terpolymer at their different concentrations for a 24 h incubating period in cell culture media, respectively. The wells containing cells without copolymers were also studied under the same conditions, and used as a controller. The following important results can be obtained from the graph illustrated in Figure 2. The C-1 copolymer does not display any observable toxicity in the chosen range of copolymer concentration. The toxicity of terpolymer is significant, probably because of the amphiphilic character of their structure containing a combination of ionisable amide-pyran fragments and H-bonded carboxylic groups (–COOH), which formed a supramacromolecular structure with antitumor active sites under the studied physiological conditions.

Observations showed that an increase of T-2 and T-3 concentrations in each well led to a higher degree of dying cells in comparison to C-1 copolymer (containing no carboxylic groups) and T-1 terpolymer, which was tested under the same conditions, T-1 terpolymer In particular showed relatively high amounts of NIPAm linkages (84.0 mol %), therefore a lower concentration of carboxylic groups and positively charged amide groups, displays significantly lower toxicity toward SCLC cells at 400 μg mL^{-1} for a 24-h incubation period. T-3 terpolymer containing a relatively higher content of DHP (20.9 mol %), maleic acid (22.3 mol %) units and 56.8 mol % of NIPAm linkages displays a higher in vitro cytotoxicity than other terpolymer. It is important to note that NIPAm containing smart linkages, rather than the individual copolymers, increases the cytotoxicity more profoundly; this is an important feature, which has a significant role in leading us to the present study. C-1 and T-1 polymers showed less toxicity against cultured cells at various quantities and different incubation times (Fig. 2). However, the toxicity of T-2 and especially T-3 terpolymers towards the SCLC cells increased in quantity, from 100 to 750 μg mL^{-1}. As seen in Figure 2, C-1 and T-1 did not show high toxicity although the copolymer amount was increased from 100 to 750 μg mL^{-1}, while a significant toxicity of T-2 and T-3 terpolymers (100 μg mL^{-1} and above) started to be observed when cancer cells were incubated for approximately 24 h. Light microscopy images are illustrated in Figure 3. As seen from these images, terpolymer T-3 showed higher toxicity at 400–750 μg mL^{-1}. This observed fact could be explained by simply alternating the structure of terpolymer, which provides a maximum concentration of ionisable and H-bonded antitumor sites in T-3 terpolymer. The results obtained indicate that C-1 binary alternating copolymer non-containing stimuli-responsive NİPAm units does not display any toxic effects on cultured SCLC cells, whereas, stimuli-responsive T-2 and T-3 terpolymers are definitely toxic to cancer cells.

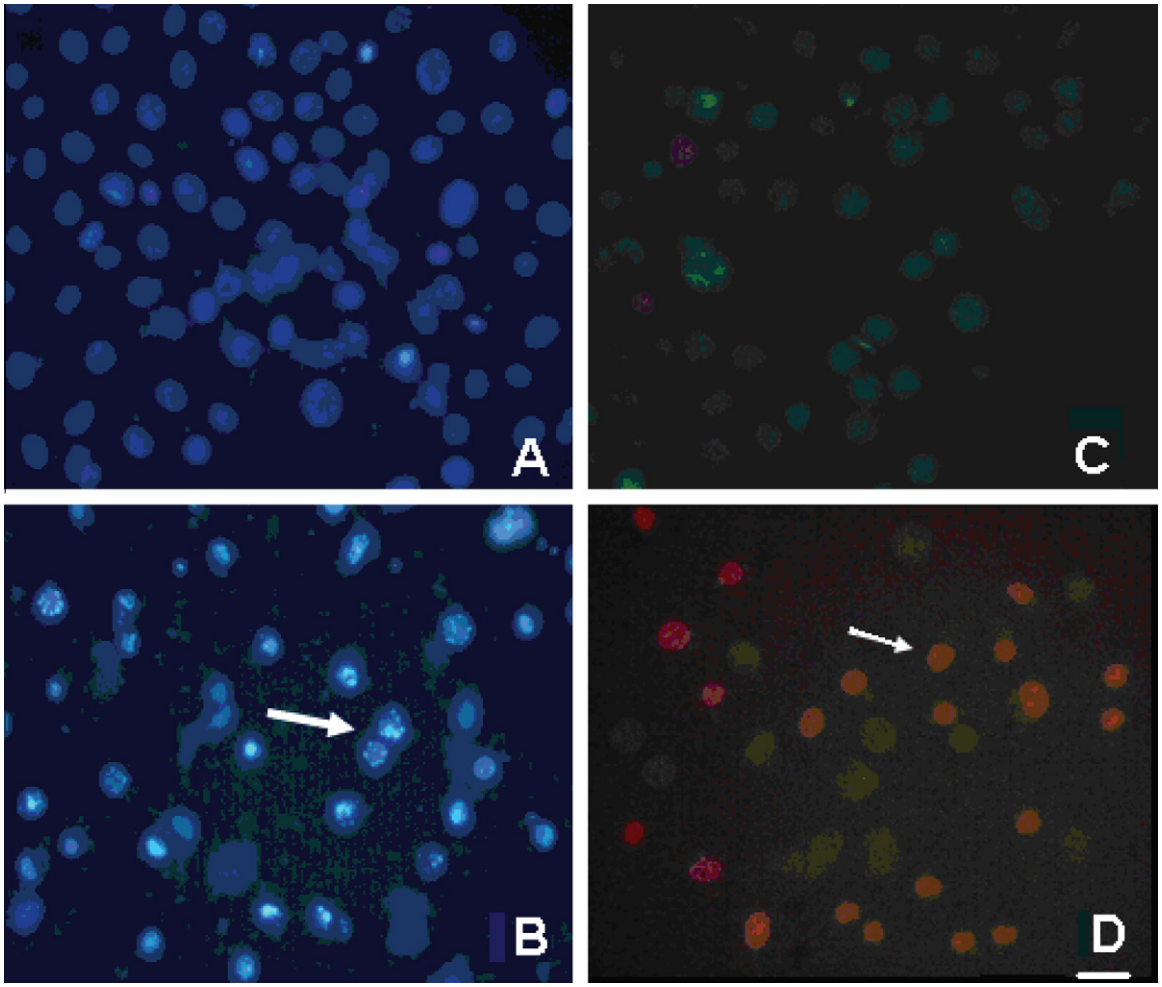


Figure 5. Fluorescence microscopy image of (A) nucleus of SCLC cells (stained with Hoescht 33342), where blue spots indicates nucleus of non-apoptotic cells as a control, and (B) shining and smashed nucleus(showed with arrow) of apoptotic cells in 750 μg mL^{-1} T-3 terpolymer containing medium, and (C) Fluorescence microscopy image of nucleus of SCLC cells (stained with Hoescht 33342), where formation of red spots demonstrates nucleus of necrotic cells and green spots demonstrates nucleus of non-necrotic cells as a control, and (D) nucleus of SCLC cells (stained with PI), where dense red spots indicates nucleus of necrotic cells in 750 μg mL^{-1} T-3 terpolymer containing medium. Images were recorded with 200× magnification. Photos A and B taken under DAPI filter and C and D taken under FITC filter, scale bar shows 20 μm.

3.3. Hematoxylin–eosin staining results

In this study, C-1 copolymer and T-1 terpolymer treated cancer cells have an intact nucleus with a concentration of about 100–750 μg mL^{-1} during the 24-h incubation. Cell morphology shows no change at the same concentration for 24 h while T-2 and T-3 terpolymers treated SCLC cells have no morphological change around 100–200 μg mL^{-1} concentration during this 24-h period. Membrane and cytoplasm structural changes of cancer cells were observed after their treatment with T-3 terpolymer around 12–24 h (Fig. 3). In addition, cell membranes were also lysed with T-3 copolymer around 12–24 h but no change was observed in the nuclei of cancer cells. Moreover, some of the cells (40% for SCLC) have been detached from the well at a concentration of 500–750 μg mL^{-1} T-3 terpolymer. Unaffected cells displayed similar morphological characteristics with untreated (control) cells.

3.4. Double staining and caspase-3 immunostaining results

The microscopic images of caspase-3 immunostaining and double staining and other images are illustrated in Figures 4 and 5, respectively. The obtained apoptotic indexes of SCLC cells for caspase-3 immunostaining were changed for the studied polymer systems as follows: C-1 (3%) > T-1 (12%) > T-2 (18%) > T-3 (30%) at 750 μg mL^{-1} and the 24-h incubation. If the SCLC cells are treated with C-1 copolymer and all terpolymers at low concentration for 24 h, number of apoptotic and necrotic cells does not yield high values (Fig. 6). However, if the polymer concentration is increased, the number of apoptotic and necrotic cells also increase, The number of apoptotic and necrotic cells especially increase when they are treated with T-3 terpolymer at 750 μg mL^{-1} concentration in cancer cell culture for 24 h. When treated with the other copolymer under similar conditions, the apoptotic index of cells drops below 20% (Fig. 6A).

The double staining and caspase-3 immunostaining results are similar to each other in cancer cells. In addition to these polymers, T-3 terpolymer has a toxic effect on cancer cells. After incubation around 100–750 μg mL^{-1} for a 24-h period, C-1 and T-1 resulted in less apoptosis, whereas incubation with T-2 and T-3 at the same concentration and for the same incubation time led to high apoptosis in SCLC cells. Both T-2 and T-3 may well constrain cell growth and viability in SCLC (Fig. 6A and B) cells. On the other hand, 100–750 μg mL^{-1} concentrated T-2 and T-3 terpolymer contents for 24 h generated an increase in necrosis stained with PI dye (Figs. 5B and 6B). It is important to note that incubation for 24 h at 750 μg mL^{-1}, T-3 produces the apoptosis supporting its high toxicity and necrotic effect. Furthermore, incubation without polymers as control cells resulted in a few PI-positive cells (Fig. 5C),

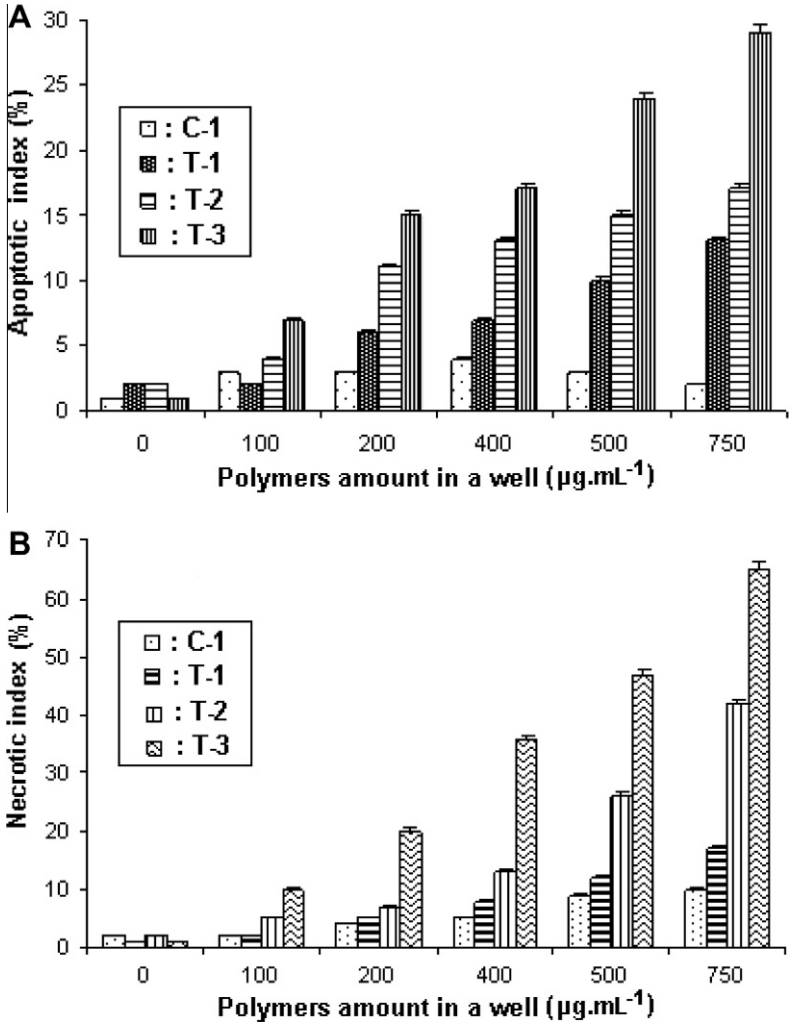


Figure 6. The analysis of (A) apoptotic and (B) necrotic SCLC cell indexes for 0–750 μg mL^{-1} concentration of copolymer and terpolymers at 24 h incubation. Results are presented as means ± SEM. * Significant difference from control (p = 0.029 and 0.033 for apoptotic and necrotic indexes, respectively).

whereas, cells exposed to T-2 and T-3 became highly PI-positive, suggesting that they are in necrosis. SCLC cells, incubated with a high dose of T-3 terpolymer, ruptured the cell membrane during the 12–24 h incubation period. Cancer cells released cell cytoplasm. Affected by T-3 terpolymer metabolic and morphologic changes may have occurred in the cells. T-3 terpolymer was more toxic than C-1 copolymer and T-1 and T-2 terpolymers. Thus, we have observed the most apoptotic and necrotic effects of T-3 terpolymer towards SCLC cells under the applied testing conditions.

4. Conclusions

This study presents the synthesis of novel bioengineering functional terpolymers and their hydrolyzed derivatives, containing a combination of ionisable amide, carboxylic and pyran functionalities, along with an ability to interact with small human lung cells carcinoma (SCLC). The copolymers were prepared by complex-radical terpolymerisation of NIPAm as a temperature and pH-sensitive monomer, DHP and MA monomers, which are responsible for the formation of H-bonding and supramacromolecular structure. The structure and compositions of all synthesized terpolymers were confirmed by ^{1}H (^{13}C) NMR analyses. Results obtained in relation to the terpolymer structure–composition–antitumor activity relationship investigations using various biochemical techniques such as cytotoxicity, hematoxylin–eosin, double staining and caspase-3 with immunocytochemical staining, apoptotic and necrotic indexes, as well as light and fluorescence microscopy methods indicated that poly(DHP-*alt*-MA) or terpolymers with a large number of NIPAm linkages (T-1) do not display cytotoxicity against SCLC, while terpolymers (T-2 and T-3) containing a given combination of ionizable amide and H-bonded pyran ring and carboxylic groups showed higher cytotoxicity and the most apoptotic and necrotic effects towards SCLC cancer cells. In general, the toxicity of terpolymers is significant, because of the amphiphilic character and stimuli-responsive behavior of their structure containing a combination of ionizable amide-pyran fragments and H-bonded carboxylic groups (–COOH), which formed a supramacromolecular structure with antitumor active sites under the studied physiological conditions.

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