Thermally-reversible gel for 3-D cell culture of chondrocytes

M. JASIONOWSKI[1,2,*], K. KRZYMINSKI[1,2], W. CHRISLER[2], L. M. MARKILLE[2], J. MORRIS[2], A. GUTOWSKA[2]
[1]*University of Gdansk, Department of Chemistry, 80-952 Gdansk, Poland*
[2]*Pacific Northwest National Laboratory, Richland, WA 99352, USA*
E-mail: marekj@chemik.chem.univ.gda.pl

Regeneration of destroyed articular cartilage can be induced by transplantation of cartilage cells into a defect. The best results are obtained by the use of autologus cells. However, obtaining large amounts of autologus cartilage cells causes a problem of creating a large cartilage defect in a donor site. Techniques are currently being developed to harvest a small number of cells and propagate them *in vitro*. It is a challenging task, however, due to the fact that ordinarily, in a cell culture on flat surfaces, chondrocytes do not maintain their *in vivo* phenotype and irreversibly diminish or cease the synthesis of the phenotypic markers for articular chondrocytes. Therefore, the research is continuing to develop culture conditions for chondrocytes with the preserved phenotype. We have investigated the use of thermoreversible gelling polymer based on *N*-isopropylacrylamide for the *in vitro* cell culture of chondrocytes.

1. Introduction

Articular cartilage is a highly specialized connective tissue that covers the ends of long bones within the synovial joint cavity and it is one of the few tissues in the body that does not heal spontaneously. However, some studies have demonstrated close to complete repair with hyaline cartilage in immature animals or in small defect [1, 2]. Intrinsic healing process may be impeded by that fact that articular cartilage is unique within the class of connective tissue since it contains no blood vessels, no nerve fibres, and no lymphatics [3]. Biochemically, articular cartilage is composed of a hydrated extensive extracellular matrix (ECM) (collagen, proteoglycans (PGs) and noncollagenous proteins) in which a small number of tissue-specific cells, named chondrocytes, are embedded [4, 5]. Chondrocytes occupy less than 10% of the tissue volume [4]. A particular feature of these cells is that they lack cell–cell contact, thus the connection between them has to occur via the ECM as well as the delivery of nutrients and removal of waste products from the cells [4].

The regeneration of damaged articular cartilage caused by disease, aging or trauma is still extremely limited and can be induced by transplantation of cartilage cells into a defect. Best results are obtained by the use of autologus cells, however, achieving large amounts of autologus cartilage cells causes a problem of creating a large cartilage defect at the donor site [6]. Vacanti *et al.* [7, 8] have described a new technology called tissue engineering that involves the morphogenesis of new tissues from constructs formed of isolated cells and specially designed polymer scaffolds. This technology requires the open implantation of the cell-polymer constructs. Extending these techniques to chondrocytes and formulating a system whereby the cell-polymer design could be delivered less invasively may greatly expand the application of tissue engineering to cartilage repair.

In this paper, we propose to use a reversible stimuli-sensitive hydrogel for the *in vitro* three-dimensional (3-D) cell culture of chondrocytes as well as for the injectable formulation that might be suitable for repair of cartilage defects. For the *in vitro* 3-D cell culture system we have developed a reversibly gelling copolymer of *N*-isopropylacrylamide (NiPAAm) and a hydrophilic component as acrylic acid (AcA). Reversible gelation of this hydrogel occurs in response to temperature change from ambient to body temperature. Recently, our group was investigating reversible sol–gel transitions of ionic copolymers of NiPAAm containing acidic (acrylic acid, acrylic acid dimer) as well as cationic (2-(*N*, *N*-dimethylamino)-ethyl acrylate) comonomers [9]. The key parameters affecting rheological behaviour (like elastic and viscous moduli, temperature range and kinetics of the sol–gel transition) as well as syneresis of the related gels were identified. These parameters were specified as molecular weight, concentration, comonomer content and ionisation degree of copolymers as well as ionic strength of the polymer's solution. Increase of network hydrophilicity, depending on the content of ionic comonomer, markedly decreased syneresis (desired feature), but on the other hand decreased the elastic

*Author to whom all correspondence should be addressed.

TABLE I Copolymers of *N*-isopropylacrylamide with AcA synthesised in dioxane. Numbers in parentheses represent standard deviations ($n = 3$)

Polymer code	Yield (%)	Total monomer concentration (M)	Initiator concentration (mM)	Ionic comonomer (mol%)	
				In feed	In copolymer
AAc-1	81	1.882	1.01	2.2	1.98 ($\pm$ 0.06)
AAc-2	84	1.878	1.01	2.0	1.85 ($\pm$ 0.04)
AAc-3	85	1.874	1.01	1.8	1.65 ($\pm$ 0.03)

modulus of gel at 37 °C (undesired feature). However, our gelling system exhibits advantage over other commercially available thermosensitive systems such as Pluronics or Poloxamers [10, 11] since the latter ones exhibit a limited stability of the gel state (they become liquids upon dilution) and they demonstrate a monotonic phase transition over a wide temperature gap [10, 11]. In contrast, reversible gels formed from the copolymers based on NiPAAm described in this paper exhibit stability upon dilution (e.g. when gelled, do not reverse to the liquid state) and relatively sharp phase transition falling around 35.5 °C [9, 12–14]. Further hardening of the gels occurs within next 3 °C.

For the most successful cartilage repair, chondrocytes have been isolated from articular cartilage, propagated in culture, transplanted into articular defects, and secured by a piece of periosteum [3, 6, 15]. In our study articular chondrocytes were harvested in a small amount and were propagated in the 3-D matrix of P(NiPAAm-co-AcA) to obtain a large amount of cells with a preserved phenotype. Another approach to prevent phenotypic change has been to embed cells in a scaffold such as agarose or collagen gel [16–19]. Encapsulation of chondrocytes in alginate beads has also helped in promoting chondrocyte morphology [20–22].

In our 3-D hydrogel the chondrocytes quantitatively sustain their morphology and continue to produce cartilage phenotypic marker (collagen type II). Such copolymer supports cell viability and metabolic activity, allowing this material to potentially be used as a base of thermally-reversible gel for 3-D cell culture of chondrocytes.

2. Materials and methods

2.1. Synthesis of poly(N-isopropylacrylamide-co-acrylic acid) copolymers and molecular weight determination

The polymers were synthesised by a free radical polymerisation in solution. The synthesis was carried out in 1,4-dioxane (HPLC grade, Aldrich, Inc.), using 2,2′-azobisisobutylonitrile (AIBN) (Eastman Kodak, Co.) as an initiator. *N*-isopropylacrylamide (Fisher, Co.) was crystallised from *n*-hexane and dried under vacuum. Acrylic acid was purchased from Aldrich, Inc. and distilled under reduced pressure prior to the use. The reaction was carried out under nitrogen at 70 °C for 18 h. The resulting solutions of copolymers were cooled to room temperature, diluted with acetone (1/1 v/v) and precipitated into excess of diethyl ether. Purification of crude copolymers involved multiple ultrafiltration of their aqueous solutions using membranes with molecular weight cut off (MWCO) of 30 000 kD (Millipore, Inc.). The purified products were then freeze dried (details of the synthesis are summarized in Table I).

The weight-average molecular weights (M_W) and the polydispersity indexes (M_W/M_N) were determined by a gel permeation chromatography (GPC) technique using polystyrene standards (Polysciences, Inc.). Chromatographic system was equipped with refractive index detector (Waters, Inc.) and light scattering detector (Wyatt, Inc.). The viscosity-average molecular weight (M_V) was determined using Cannon–Fenske viscometer (ASTM size equal to 200 and 350). Intrinsic viscosity [η] was calculated using established methods [23]. The Mark–Houwink equation was applied to obtain M_V values, using a and K constants equal to 0.51 and 14.5 e–04 dl/g, respectively [12, 24] (Data is shown in Table II).

2.2. Characterisation of the copolymers

2.2.1. Composition and NMR study

The content of AcA was determined by a reverse titration method. Typically, 100 mg sample of the polymer was dissolved in 5 ml of 0.15 M saline. 500 µl of 0.1 M NaOH standard solution and 20 µl of phenolphtalein solution was added to each sample and titrated with standardised 0.01 M HCl. Additionally, in order to evaluate the exact content of base in each sample, blank titration was performed in the same way with no polymer added.

The structure of the copolymers was proved using ^{1}H and ^{13}C NMR methods. The spectra were recorded using

TABLE II Weight-average and viscosity–average molecular weights for P(NiPAAm-co-AcA) ionic copolymers. Numbers in parentheses represent standard deviations ($n = 3$)

Polymer code	M_W (GPC) (kD)	(M_W/M_N) (GPC)	(η) (dl/g)	M_V (*C–F*) (kD)	η* (cP) +
AAc-1	315.4 (12.5)	1.14 (0.04)	0.874 (0.07)	315.1 (2.0)	113.2 (5.5)
AAc-2	313.5 (15.1)	1.17 (0.02)	0.701 (0.04)	233.7 (8.3)	127.3 (7.7)
AAc-3	300.1 (14.3)	1.18 (0.02)	0.776 (0.05)	265.4 (2.3)	144.9 (5.2)

+ 10 wt % solution in PBS at 20 °C. For experiment conditions see footnote under Table III.
Abbreviations: M_W: weight-average molecular weight; M_V: viscosity average molecular weight (Cannon-Fenske); M_N: number average molecular weight; GPC: Gel Permeation Chromatography; *C–F*: as estimated using Cannon-Fenske viscometers; [η]: intrinsic viscosity; η*: complex viscosity.

TABLE III ^{1}H NMR and ^{13}C NMR shifts (ppm) for AAc-2 copolymer recorded at room temperature in $CDCl_3$

^{1}H nuclei group	CS (ppm)/RPA	^{13}C nuclei	CS (ppm)
$-\underline{CH_3}$	1.07/6.0	$-\underline{CH_3}$	22.7
$-\underline{CH_2}-$	1.75/1.85	$-\underline{CH_2}-$	42.4
$=\underline{CH}$ (methine)	2.06/1.1	$=\underline{CH}$ (methine)	35.7 (vb)
$>\underline{CH}$ (isopropyl)	3.93/0.99	$=\underline{CH}$ (isopropyl)	41.4
$>N-\underline{H}$ (amide)	6.42/0.86	$>\underline{C}=O$ (amide carbonyl)	174.5
$-COO\underline{H}$	Not detected		

Abbreviations: CS: Chemical Shift as compared to tetramethylsilane (TMS); RPA: Relative Peak Area; vb: Very broad.

Varian VXR 300 spectrometer working at base frequency of 300 MHz. About 1.5 and 10 wt % solutions of the polymers in $CDCl_3$ were used for ^{1}H and ^{13}C NMR experiments, respectively. Additionally, $^{1}H-^{13}C$ HETCOR – type experiment was performed to obtain chemical shifts (CS) of ^{13}C nuclei in AAc-2 copolymer (Data is shown in Table III).

2.2.2. Phase transition and clouding behavior of copolymer solutions

The reversible sol–gel transition of the polymer solutions was analysed applying dynamic rheology methods. SR 2000 dynamic rheometer (Rheometric Scientific, Inc.) with 25 mm diameter parallel plates and the gap of 0.5 mm was used in all experiments. All solutions were prepared as a 10 wt % of copolymer in 0.1 M phosphate-buffered saline (PBS), pH = 7.4. Dynamic temperature ramp tests were performed under controlled stress of 2.0 Pa, frequency of 1.0 radian/s, ramp rate of 0.3 °C/min and heating–cooling cycle set at 20–40 °C (Data is shown in Table IV).

The lower critical solution temperature (LCST) was estimated by the cloud point determination method (CPT) using Hewlett-Packard HP 8453 UV-VIS spectrophotometer equipped with temperature-controlled cell. The CPT was regarded as the temperature at which the solution of copolymer maintains 10% of its initial transmittance at 500 nm [13]. 10 wt % polymer solutions in PBS (pH = 7.4) were heated from 20 to 40 °C with the ramp rate equal to 0.3 °C/min (Table IV).

2.2.3. Syneresis determination

Syneresis of the gels at elevated temperature was estimated as a mass of solvent excluded from the network divided by the total mass of a gel sample. Three samples (about 1.5 g each) of AAc-1, AAc-2 and AAc-3 copolymer solutions (10 wt % in PBS, pH = 7.4) were incubated at 37.0 °C up to 48 h. A Hamilton syringe was used to withdraw the PBS excluded from the gel samples at 12 h intervals. The solvent portions were then weighted and returned to the appropriate vials. Results become constant after 48 h of incubation and those values were used for calculations of synereis.

2.3. Cell isolation and culture conditions

All *in vitro* manipulations were performed in a laminar flow hood using aseptic technique. Fresh articular cartilage samples were harvested aseptically from the lower rim of the scapular (fibrocartilage) of 12-month old adult NZW rabbits. The articular cartilage specimens were transferred to the sterile 60 mm Petri dishes, diced into pieces of approximately 1 mm^3, cleaned and put into a trypsinizing flask containing 0.35% collagenase (Worthington Biochemical, Freehold, NJ), 0.05% hyaluronidase (Sigma, St Louis, MO) and 0.2% deoxyribonuclease (Sigma, St Louis, MO) in RPMI 1640 medium (Gibco, Grand Island, NY). The tissue was spun on a magnetic stirrer at 37 °C for 4 h to isolate chondrocytes. The cells were collected and centrifuged at 1200 rpm for 10 min. The cell pellet was resuspended in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum (Sigma, St Louis, MO) and antibiotics (penicillin and streptomycin) and seeded into 25 cm^2 polystyrene flask in an incubator at 37 °C in an atmosphere of 5% CO_2 in air. The culture was fed with fresh medium every other day, and examined daily for growth using an inverted phase-contrast microscope. Upon confluency, the cells were trypsinised (1X Trypsin-EDTA solution, 0.05% Trypsin, 0.53 mM EDTA × 4Na, Gibco, Grand Island, NY), washed and propagated in serial passages as a monolayer. Each passage was about one week and the cells were harvested at 80–100% confluence. Cells at passage 5 were harvested and then reseeded as passage 6 onto monolayer and in the polymer matrix.

TABLE IV Temperature-dependent properties of the P(NiPAAm-co-AcA) gels evaluated from dynamic rheological tests and transmittance measurements. Numbers in parentheses represent standard deviation ($n = 2$–3). All samples were prepared as a 10 wt % solutions in PBS, pH = 7.4

Polymer code	GFT (°C)	GMT (°C)	Elastic modulus (G′) at 37 °C (Pa)	CPT (°C)
AAc-1	36.68 (0.04)	32.62 (3.23)	12.63 (3.82)	35.56 (0.12)
AAc-2	35.46 (0.17)	29.92 (1.11)	77.80 (11.69)	34.48 (0.09)
AAc-3	33.87 (0.03)	26.78 (2.16)	248.17 (14.66)	33.54 (0.21)

Experiment conditions:
Dynamic Temperature Ramp Tests: heating range: 20–40 °C, heating rate: 0.3 °C/min, frequency: 1.0 rad/s, stress: 2.0 Pa,
Cloud Point determination: temperature ramp: 0.3 °C/min, wavelength = 500 nm.
Abbreviations: GFT: Gel formation temperature (GFT = G′/G″ cross-point on heating curve); GMT: Gel melting temperature (GMT = G′/G″ cross-point on cooling curve); CPT: Cloud point temperature, regarded as temperature at which the solution maintains 10% of the initial transmittance at 500 nm.

2.3.1. Monolayer culture

Chondocytes were propagated as monolayer cultures in 12-well culture plate (Falcon) with a cell density $2.5–3 \times 10^5$ cells/ml. Cells were counted in a hemocytometer and viability was assessed by the Trypan Blue exclusion test (Sigma, St Louis, MO).

Cell culture medium was changed every other day. Monolayer cultures were incubated at 37 °C in an atmosphere of 5% CO_2 and 95% humidity and cultured for one, two, three and four weeks.

2.3.2. Polymer gel culture

A 10 wt % solution of polymer in DIH_2O with pH adjusted to 7.4 was prepared and sterilised in an autoclave at 120 °C for 30 min. The polymer solution was combined with an equal amount of 2X RPMI 1640 medium supplemented with 20% (v/v) FBS and antibiotics (final concentration of polymer was 5 wt %). Chondrocytes with final concentration 5.5×10^5 cells/ml (passage # 5) were suspended in 5 wt % polymer solution and divided into 12-well culture plates pre-coated with sterile 2 wt % high-melting agarose (Biorad, Richmond, CA). 0.8 ml aliquots of polymer/medium/cells suspension was added per well. The culture plates were then placed into an incubator at 37 °C, 5% CO_2 until gelled completely and 2 ml of RPMI 1640 pre-warmed to 38 °C per well was added on the top of the gelled matrix in each well. The top medium was exchanged every other day and chondrocytes were cultured at 37 °C, 5% CO_2, 95% humidity for one, two, three and four weeks.

2.4. Histological analysis

The cells from polymer culture were collected by cooling down the system and were cytospun onto slides coated with poly L-lysine (Shandon, Pittsburgh, PA). One set of slides was fixed with 10% neutral buffered formalin (Baxter, Deerfield, IL) and stained with hematoxylin-eosin (H&E). A second set of slides was fixed with 60% citrate buffered acetone (Sigma, St Louis, MO) and stained with alkaline phosphatase (ALP). Monolayer cultures were fixed and also stained with H&E and ALP.

2.5. *In situ* fluorescent viability study

To assess the cell viability on a monolayer as well as in the 3-D polymer matrix, an *in situ* fluorescent viability study was performed using fluorescein diacetate (F-DA). F-DA which is not fluorescent, is converted to fluorescein (excitation/emission maxima 494/520 nm) through the cleavage of its acetate groups by intracellular esterases inside healthy cells.

After each consecutive week the assay of cell viability on cells cultured as a monolayer and in the polymer matrix was performed. A 60 mM solution of F-DA in DMSO was prepared and diluted to 60 μM in PBS. After removal of the top medium the polymer and monolayer samples were immersed in the solution pre-warmed to 38 °C F-DA for 10 min at 37 °C in the dark and then washed in the solution pre-warmed to 38 °C PBS for 5 min. The chondrocytes were viewed on an inverted microscope (Nikon Co., Japan) using epiilumination and standard Nikon filter sets for fluorescein (B-2A).

2.6. Western blotting analysis

The chondrocytes from confluent monolayer culture and from polymer disc culture were examined according to the procedures of Laemmli [25] by sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) in 10% (w/v) polyacrylamide gel. The polymer disc with total number of chondrocytes of ca. 6×10^5 was cooled down and melted, the monolayer culture with around the same number of cells was washed with PBS, trypsinised and spun down. Next, the cells from both cultures (monolayer and polymer) were lysed using lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1% Tween-20, DIH_2O), the cells were then loaded in the gel and the electrophoresis was performed. The gel was then stained with Coomassie Blue and de-stained overnight. Next, the proteins were electrophoretically transferred onto nitrocellulose membrane. The membrane was blocked with 5% non-fat milk in 30 mM Tris–HCl (pH = 7.4) containing 120 mM NaCl and 0.05% Tween-20 at room temperature for 1 h. It was then incubated with first antibody goat anti-type II collagen (Southern Biotechnology Associates, Inc.) for 1 h at room temperature, followed by rabbit anti-goat IgG HRP for 1 h at room temperature. Immunoreactivity was detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Inc., Rocford, IL) and visualised using LUMI-IMAGER F1 (Roche Diagnostics Co.).

2.7. Immunoprecipitation

The supernatants from the chondrocytes cultured in the monolayer and from the polymer matrix were harvested. Goat anti-type II collagen was added to the supernatant at 1 : 1000 dilution and incubated at 4 °C overnight. In order to immunoprecipitate the protein the Protein A/G Plus Agarose (Santa Cruz Biotechnology, Inc.) was used according to the methodology provided by the company. The protein bands were visualised using Coomassie staining.

3. Results and discussion

3.1. Synthesis and characterisation of the P(NIPAAm-co-AcA) copolymers

We have investigated thermoreversible gelation of copolymers of *N*-isopropylacrylamide containing small amount of AcA as a hydrophilic co-monomer. PNiPAAm is a well-known homopolymer that exhibits a lower critical solution temperature around 32 °C in water [12, 24]. Copolymerisation of NiPAAm with a hydrophilic monomers increases the LCST of resulting copolymer because their presence raise the overall hydrophilicity of the system [9, 24]. Moreover, adding the hydrophilic comonomer into the linear poly(NiPAAm) results in an adequate gel having lower syneresis than the homopolymer [9]. At physiological pH the carboxyl groups of AcA are almost entirely ionised and they hinder the dehydratation of the copolymer by

counteracting the aggregation of hydrophobic chains [24, 27]. Moreover, the repulsion between $-COO^-$ groups and *N*-isopropyl residues of NiPAAm as well as formation of hydrogen bonds between carboxylate and amide groups may resist the collapse of structure.

Details of the synthesis of linear P(NiPAAm-co-AAc) copolymers having about 1.6–2.0 mol % of AcA incorporated (AAc-1, AAc-2, AAc-3) are summarised in Table I. The yields of reactions and degree of AcA incorporation were relatively high in all cases, reaching the level of 80–85%.

Molecular weights (M_W) and molecular weights distribution (M_W/M_N) of copolymers was estimated by GPC and are summarised in Table II. In all cases the M_W values fall around 300 kDa with standard deviation among M_W values smaller than the actual experimental error. It suggests that the products are comparable in terms of their molecular weights. Molecular weight distributions, indicated as a M_W/M_N values, are narrow and fall in the range of 1.1–1.2 in all cases.

Viscosity-average molecular weights (M_V), determined from the solution viscosity, display, in all cases, close or slightly lower values than the adequate M_W ones (Table II). These findings support previously stated low polydispersity of the copolymers estimated by GPC.

Table II also presents the complex viscosities (η*) for 10 wt % polymer solutions at 20 °C in PBS. These values, falling in the range 110–150 cP, indicate relatively low solution viscosities, which make the systems easy transferable and facilitate their biological applications.

^1H and ^{13}C NMR data are presented in Table III. CS together with relative peak areas (RPA) are listed for AAc-2 copolymer, which was chosen for further application as a scaffold for chondrocyte seeding. Since the NMR technique provided inconclusive data in terms of quantitatitative analysis (due to low AcA content), this method was employed as a structure control tool. The ^1H and ^{13}C NMR spectra for AAc-1, AAc-2 and AAc-3 copolymers are similar and consist of five broad signals, resembling the pattern published elsewhere for PNiPAAm homo-polymer [26, 27]. Signals in the olefin range (around 6 ppm) were not detected by the ^1H NMR thus indicating the absence of the monomers in purified materials.

Sol–gel transitions of the P(NiPAAm-co-AcA) copolymer solutions were studied using dynamic rheology methods. The elastic modulus (G′) and the viscous modulus (G″) were recorded against the temperature. The data is summarised in Table IV and is illustrated in Figs. 1 and 2. The crossover point of the G′ and G″ curves has been suggested as an approximate criterion of the sol–gel transition temperature [28].

Change the temperature of copolymers' solutions from 20 to 37 °C manifests in about $0.8–16 \times 10^3$-fold increase of the elastic modulus. This phenomenon, resultant from the temperature-driven sol–gel transition, is illustrated for AAc-2 copolymer in Fig. 1. Hysteresis, observed for G′ and G″ values on the heating–cooling cycle, is a kinetic-controlled phenomena reflecting the resistance of the polymer chains to de-entanglement. Results presented in Fig. 2 indicate, that the gel formation temperature (GFT) and the gel melting temperature (GMT) reflect the content of AcA in copolymer.

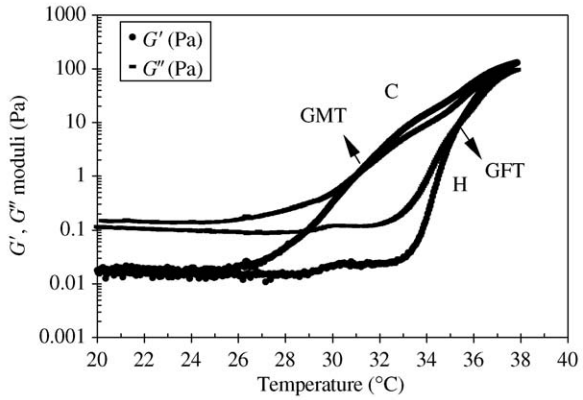

Figure 1 Changes in elastic (G′) and loss modulus (G″) for AAc-2 copolymer as a function of temperature. The sample was prepared as a 10 wt % solution in PBS, pH = 7.4. For experiment conditions see footnote under Table IV.

Interdependence of GFT *vs*. AcA content is practically linear for the copolymers studied. An increase in AcA content by 0.1 mol % manifests in shifting the GFT by about 1 °C towards higher values. Incorporation more than 2 mol % of AcA into the PNiPAAm shifts the GFT above 37 °C in PBS (pH = 7.4) thus making the material useless for the application proposed in this paper. Strong dependence of the elastic modulus (G′) at 37 °C on AcA content in copolymer is expressed in Table IV and is also illustrated in Fig. 2. Increase in an actual ionic comonomer content by about 0.1 mol % is reflected by the G′ value decrease for about 70–90 Pa.

The cloud point temperatures, estimating the LCST values for AAc-1, AAc-2 and AAc-3 copolymer solutions are listed in Table IV. In all cases the CPT values are 0.4–1.0 °C below the relative GFT values, but maintain the same order. It indicates the correlation between hydrophobic interactions among the copolymer chains and the phase transition of the system.

Syneresis is the feature of many hydrogels that characterises their shrinkage due to the hydrophobic interactions occurring in the system [29, 30]. The goal of the application proposed here is to minimise the syneresis since the network should stay within well-defined boundaries and maintain its mechanical properties (like G′ and G″ moduli values) being under

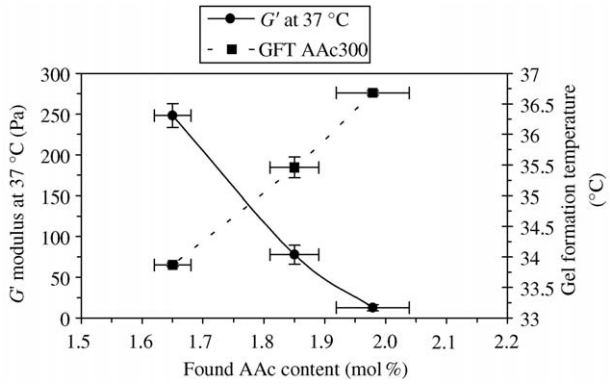

Figure 2 Correlation between elastic modulus (G′) at 37 °C and AcA content for P(NiPAAm-co-AcA) copolymers having averaged molecular weight around 300 kD. All solutions are prepared as 10 wt % in PBS, pH = 7.4. For dynamic temperature ramp test setups see footnote under Table IV.

experimental conditions. The syneresis of AAc-1, AAc-2 and AAc-3 gels at 37 °C is illustrated in Fig. 3. The results indicate, that syneresis increases fast with the decrease of AcA content. This plot resembles the interdependence of G′ versus AcA content, presented in Fig. 2. It suggests, that the two new features appear with the AcA incorporation to the PNiPAAm chain: (i) reduction of the syneresis (desired) and (ii) elastic modulus decrease (unfavourable). Rational designing of the polymer networks that might be used for cell culture should take into account these opposite tendencies. Among the materials presented here, the gel based on AAc-2 copolymer (10 wt % in PBS) is characterised by a moderate elastic modulus value (about 77.8 Pa) and relatively low syneresis (about 25%) at the physiological conditions (pH = 7.4, 37.0 °C) and thus it was selected as a matrix for chondrocyte seeding. Total reversibility of the gel–sol transition (Fig. 1) facilitates easy recovery and harvesting of cultured cells simply by lowering the temperature.

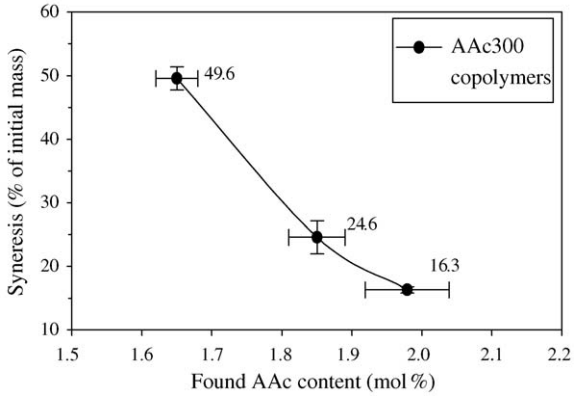

Figure 3 Correlation between syneresis phenomena and AcA content for P(NiPAAm-co-AcA) copolymers with M_W around 300 kD. All solutions are prepared as 10 wt % in PBS, pH = 7.4.

3.2. Histological analysis

The cells were harvested from adult rabbit scapular cartilage and propagated in a monolayer culture until confluency. Then the cells were harvested using trypsin solution and seeded into the copolymer gel. The monolayer cell culture was also continued as a negative control. After one, two, three and four weeks the monolayer and polymer culture was stained with H&E and ALP (Figs. 4 and 5). Our results indicate that the cells in monolayer culture appeared to be more fibroblast-like. The cells from AAc-2 copolymer gel culture display a typical chondrocytic morphology with densely stained nucleus (ALP activity in chondrocytes shown by ALP staining) and small round cell body (H&E staining). Thus, the cells recovered from polymer culture reexpressed the chondrocyte phenotype. To determine whether the polymer supports the survival of chondrocytes, cells from polymer gel culture (two week culture) were reseeded as a monolayer culture. Chondrocytes harvested from polymer gel culture were viable as indicated by exclusion of Trypan Blue dye and after a short period of time attached to the monolayer surface are started to look flattened and fibroblast-like (data not shown).

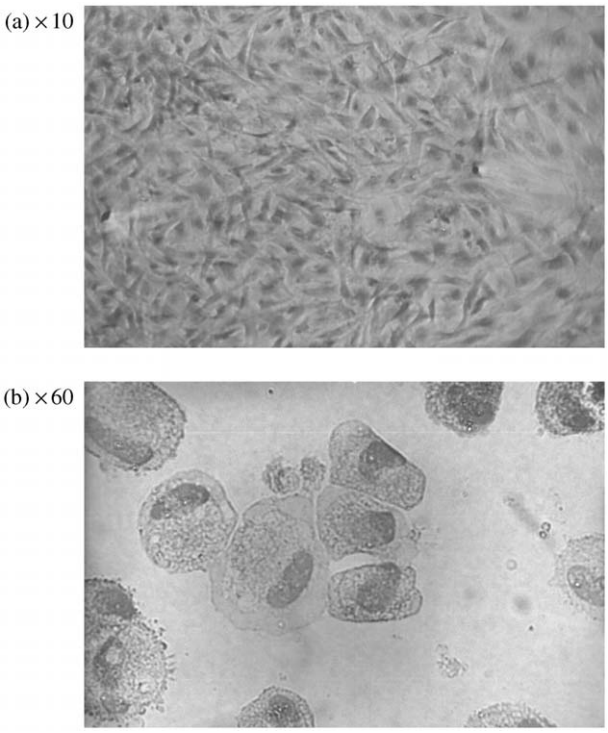

Figure 4 H&E staining of chondrocytes in (A) monolayer (B) hydrogel.

3.3. *In situ* viability study

In situ fluorescence images of rabbit articular chondrocytes cultured *in vitro* in 3-D AAc-2 hydrogel matrix are shown in Fig. 6. The cells maintained a round shape, characteristic of differentiated articular chondrocytes [31]. Chondrocytes cultured on a monolayer were used for comparison. These images are presented in Fig. 7.

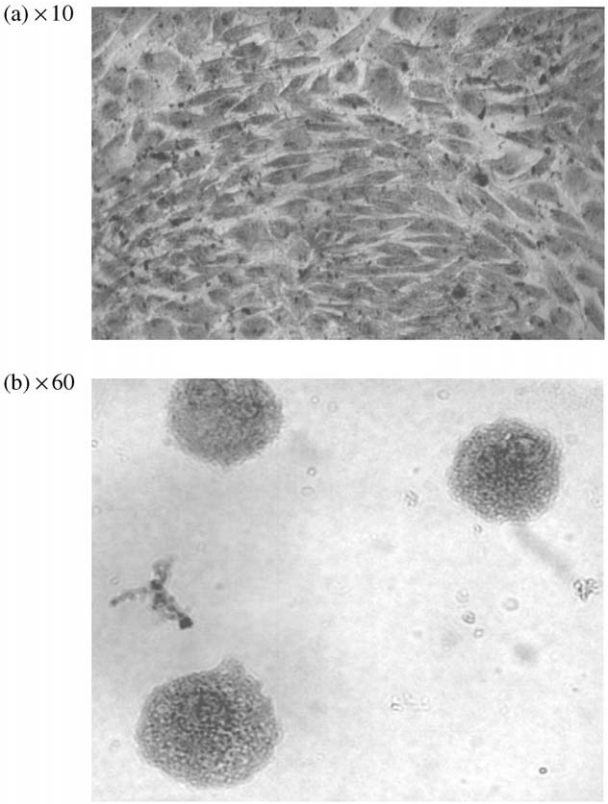

Figure 5 ALP staining of chondrocytes in (A) monolayer (B) hydrogel.

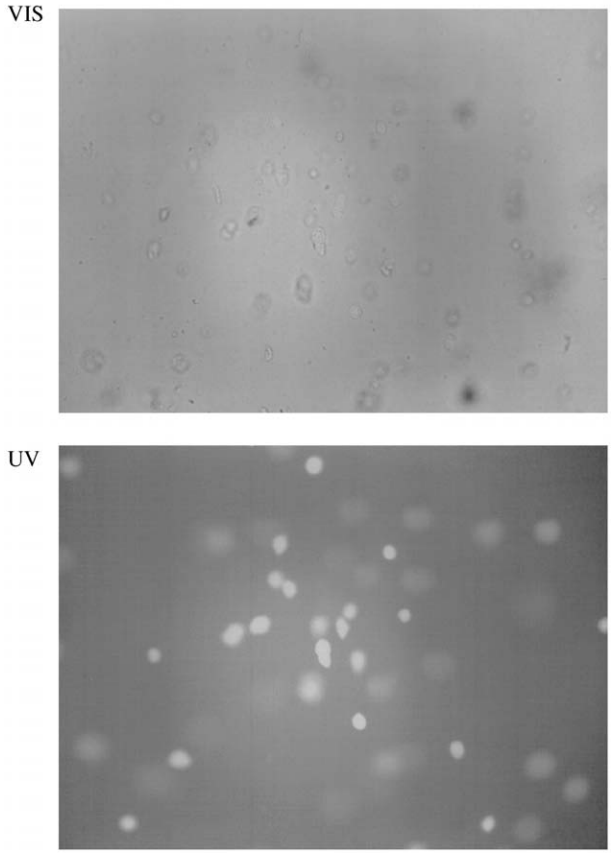

Figure 6 In situ fluorescent viability study of chondrocytes in hydrogel.

The cells cultured on the 2-D surface were viable but appeared to have a fibroblastic morphology, an indication of chondrocytes ''dedifferentation'' [19].

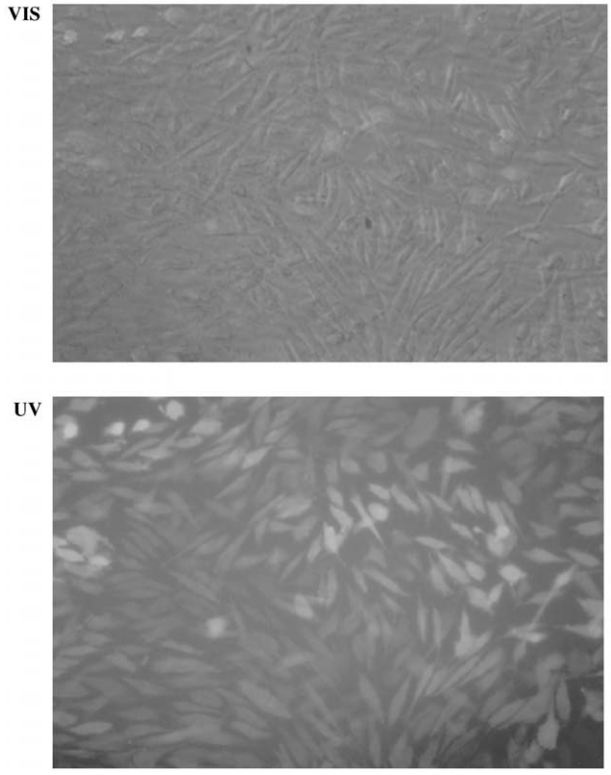

Figure 7 In situ fluorescent viability study of chondrocytes in monolayer.

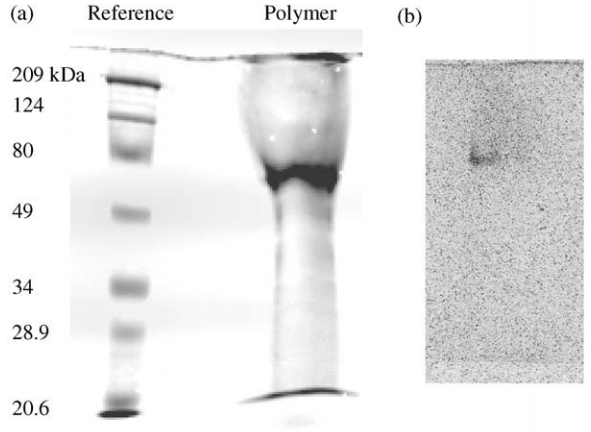

Figure 8 SDS-PAGE (A) and Western blot analysis (B) of chondrocytes.

3.4. Collagen type II synthesis in 3-D gel matrix

The ECM of all connective tissues is composed of a 3-D crosslinked network of insoluble collagen fibers. Currently, 14 genetically distinct collagens have been identified as structural components of the ECM of various connective tissues [32]. Articular cartilage is characterised by type II collagen [33, 34]. In adult tissue, approximately 90% of the collagens are of type II, which is found only in a few other tissues such as the vitreous body [32]. The type II collagen molecule is composed of three identical polypeptide chains α_1(II), which each consists of a single 300 nm-long triple helix with short telopeptides at each end [4]. The network of the collagen fibrils provides the tensile strength of cartilage and is essential for maintaining tissue volume and shape. In this work type II collagen has been detected in Acc2 matrix chondrocyte culture with the aid of SDS polyacrylamide gel electrophoresis and Western Blot analysis (Fig. 8) as described. Differentiated chondrocytes mainly express type II collagen ($[\alpha_1(II)]_3$), whereas dedifferentiation leads to suppression of type II and initiation of type I ($[\alpha_1(I)]_2$ $[\alpha_2(I)]$), type I trimer ($[\alpha_1(I)]_3$), and type III collagen ($[\alpha_1(III)]_3$) synthesis [35, 36]. Fig. 8 shows that on week four of culture, chondrocytes in AAc-2 matrix strongly expressed a phenotypic marker for articular cartilage – type II collagen. Flattened cells of chondrocytes in monolayer contributed most of type I collagen (data not shown) [37].

4. Conclusions

We have investigated the use of gel based on linear poly(*N*-isopropylacrylamide-co-acrylic acid) thermoreversible copolymer for the *in vitro* cell culture of chondrocytes. The solution of the copolymer having a molecular weight of about 300 kD containing 1.85 mol % of AcA demonstrates a sol–gel phase transition around 35.5 °C. The gel is characterised by a moderate elastic modulus value ($G' = 77.8$ Pa) and relatively low syneresis (about 25%) at physiological conditions (pH = 7.4, 37.0 °C). The system is injectable through a small-diameter aperture and does not exhibit acute toxicity towards living cells (ASTM protocol using L-929

fibroblast cell line). Therefore it was selected as a matrix for cell seeding.

On the basis of the results from the characterisation and cytotoxity studies, the rabbit articular chondrocyte viability studies, the histological studies and Western Blot analysis, the P(NiPAAm-co-AcA) hydrogel is suitable as a chondrocyte culture substrate. Preliminary data suggest, that it seems to be a proper material to be employed as a scaffold for the *in vitro* expansion of cells. Further studies have been undertaken to optimise the use of such a system in repairing of cartilage defects.

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