 DOI: 10.1002/masy.200751204

Synthesis and Characterization of a Biocompatible Copolymer to be Used as Cell Culture Support

Natalia Becerra,[1,2] *Luz Marina Restrepo,*[2] *Betty Lucy López**[1]

Summary: N-isopropylacrylamide (NIPAAm)/butylacrylate (BAc) copolymer was synthesized by emulsion polymerization in order to use it as a cell culture surface for corneal epithelium biosubstitutes. Results showed that the obtained polymers were thermosensitive hydrogels. The copolymer was characterized by Fourier transform infrared (FTIR), differential scanning calorimetry (DSC), thermogravimetric analysis (TGA) and swelling degree. 3T3 Swiss cells were used as feeder layers and they were used to test the cytotoxic effect of the hydrogels. The conditions to isolate, cultivate, expand and cryo-preserve human oral mucosal cells were established and analysis of several morphological and immuno-histochemical characteristics of the cultivated oral mucous were made.

Keywords: biocompatibility; biomaterials; hydrogels; stimuli-sensitive polymers; tissue engineering

Introduction

Polymer hydrogels are cross-linking polymer networks; their main property is their ability to absorb a large amount of water and their response to external stimuli such as temperature, pH and presence of solutes.

Polymer hydrogels are formed by hydrophobic units in addition to the hydrophilic ones and they can swell in aqueous media. But they can also swell in organic solvents and hydrophobic substances, if there is a greater amount of the hydrophobic units in the structure.

Hydrogels are useful for many applications such as immunoassays,[1] drug delivery systems,[2] separation process,[3] and immobilization of enzymes.[4] Hydrogels must be biocompatible polymers to be used in biomedical applications such as cell culture substrate or engineering tissue, providing that they do not induce cytotoxic responses and cells can grow and proliferate well on their surfaces. They must be temperature sensitive polymers, so that by reducing the temperature, the cells can be detached from the surface. Also, these polymers should have a proper hydrophilic/hydrophobic balance that allows appropriate diffusion of nutrients and metabolites[5] without change significantly its lower critical solution temperature (LCST), that must be near to the human body temperature (37 °C).

The poly (*N*-isopropylacrylamide) (PNIPAAm) is one of the most commonly studied thermo-reversible system since it is a biocompatible polymer and has a sharp phase-transition. PNIPAAm shows thermally reversible soluble-insoluble changes around its lower critical solution temperature (LCST) (32 °C) and cellular passive adhesion processes (interaction with cells but not alteration of cell morphology or metabolism). When the PNIPAAm is used as cell culture support, the hydrophobic hydrogels shrink above its LCST and the

[1] Grupo Ciencia de los Materiales, Universidad de Antioquia, Calle 62 N° 52 59 Medellín, Colombia
Fax: (+574) 210 6550,
E-mail: blopez@quimbaya.udea.edu.co
[2] Grupo de Ingeniería de Tejidos y Terapias Celulares, Universidad de Antioquia, Calle 62 52 59 Medellín, Colombia
Fax: (+574) 210 6550

cells spread and proliferate. Below its LCST, the hydrogels swell and spontaneously detach the cells.

PNIPAAm exhibits some disadvantages such as: In the first place it has an anomalous swelling behaviour, described in the literature as the overshooting effect.[6] At the beginning of the swelling process, the swelling curves exhibit a maximum in the water uptake, after which the swelling gradually decreases to an equilibrium value at longer times. In the second place, it has relatively weak hydrophobicity and mechanical properties, therefore it is necessary to introduce some chemical modifications as grafting of some functional groups or copolymerization with hydrophobic monomers, but the LCST may cause a decrease in the thermo-sensitivity of resulting material. Cell harvesting from cultures without loosing a significant amount of the freshly proliferating cells is still a challenge since often mechanical stress or chemical treatments have to be applied. Changing the surface properties of the cell culture support, from cell adhesive (not too polar) to cell repelling (very hydrophilic) by a small change in temperature within a suitable temperature window would allow cell harvesting under mild conditions in high yields.

The present study focuses on the synthesis of P(NIPA-co-BAc) hydrogels using a different N,N′-methylenebisacrylamide (NMBAAm) cross-linker concentrations, and the effect of the crosslink density on swelling degree is studied. The introduction of the BAc component may improve the mechanical strength of PNIPA hydrogels and diffusion of hydrophobic substances without modifying the LCST. The copolymers are characterized and their cytotoxic effect is evaluated.

Experimental Part

Materials

Reagent grade monomers N-isopropylacrylamide (NIPAAm) from Sigma, the cross-linker N,N′-methylenebisacrylamide (NMBAAm) and the surfactant sodium dodecyl sulphate (SDS) from Research organics, the accelerator N,N,N′,N′-tetramethylethylenediamine (TEMED) from Fluka, and the ammonium persulphate (APS) as the initiator from Aldrich. All chemicals were used as were commercially provided.

Hydrogel Synthesis

P(NIPAAm-co-BAc) hydrogels were synthesized by free-radical cross-linking copolymerization of NIPAAm and BAc in aqueous solutions. The amount of MBAAm in the monomer mixture was varied from 1.5 to 3.0% (m/m). The MBAAm, 97% w/w NIPAAm, 3% w/w BAc and 2% (w/w) SDS were dissolved in distilled water in a 30% monomers-70% water ratio; the solution was purged with nitrogen gas for 10 min and let to stabilize for 12 hours. Then, the initiator solution of 0,2% (w/w) APS and 0,2% (w/w) TEMED in water was added and the copolymerization was conducted for 3 h at 25 °C and at 60 °C.

The copolymerization was carried out into glass moulds. Upon completion of the reaction, the hydrogels were washed with water for 3 days to eliminate the unreacted monomers and the initiator. The hydrogel samples were then dried at 40 °C.[7]

Determination of Swelling Kinetics[8]

The swelling kinetics of the P(NIPAAm-co-BAc) hydrogels were measured gravimetrically at constant temperatures. The dried samples were hydrated in ultra-pure water and removed from water at regular time intervals. After, the water on the surfaces of the hydrogels was wiped off with moistened filter paper, and then the mass of the hydrogels was determined. The swelling ratio SR was defined as follows:

$$SR = \frac{M_s - M_d}{M_d} \quad (1)$$

Where M_s: the mass of the wet gel at is different times and M_d is the mass of the dry gel.

Determination of Equilibrium Swelling Ratio

To determine the equilibrium temperature, the hydrogels were equilibrated in distilled water at temperatures ranging from 25 to 37 °C following the same procedure used for the kinetic studies. The hydrogels were allowed to swell in an excess amount of ultra-pure water for at least 24 h at each predetermined temperature, in an oven at constant temperature. For each measure at predetermined temperature, the hydrogels were re-equilibrated in ultra pure water and their swollen mass was determined. The equilibrium swelling ratio was calculated with Equation (1).

Diffusion Model

To investigate the water diffusion model of the hydrogels, the initial swelling rates were fitted to the follow equation:

$$\frac{W_t}{W_\infty} = kt^n \tag{2}$$

$$D = 2\pi\left(r^2 + rL\right)\left(\frac{k}{4}\right)^{1/n} \tag{3}$$

This approximation is only valid for the first 80% of sorption/desorption ratio ($W_t/W_\infty < 0.8$). This equation was modified from the equation (6) of reference[9] because the geometrical shape of the samples was cylindrical. It was used to calculate the water diffusion coefficient (D).

For these equations, W_t and W_∞ are the amounts of water absorbed by the hydrogels at time t and at equilibrium, k is a characteristic constant, n characteristic exponent of the transport model, D is the diffusion coefficient of water trough the hydrogels, r and L are the radius and the initial length of the dried gels.

Fourier Transform Infrared Spectroscopy

The functional groups of the PNIPAAm-co-BAc hydrogels were determined in order to confirm the effective copolymerization reaction.

Differential Scanning Calorimetric

DSC modulated experiments were made (Q100 model, TA Instruments) in order to determine: glass transition temperatures (T_g) of dried samples and the LCST of the swollen hydrogel.

Thermogravimetric Analysis

The amount of water, residual monomers and decomposition polymer temperature were measured in a Q-500 model, TA instruments.

3T3 Swiss Feeder Layers Culture[10]

3T3 Swiss cells were cultured on Dulbecco's modified Eagle's medium (DMEM) (Gibco), supplemented with 10% fetal bovine serum (FBS) (Gibco). In order to establish the mitomycin C (MMC) (Sigma) concentration needed to inhibit cell proliferation, after 70% confluence, cells were incubated with 4, 8 and 10 μg/ml of MMC for two hours at 37 °C, then they were trypsinized and cultured at a density of 1×105 cells/cm^2.

For testing cytotoxic effect, direct contact assays were made. Hydrogels were washed and dried on culture plates and ethylene oxide sterilized. 3T3 Swiss cells were seeded at a density of 5×10^4 cells/cm^2 on the film and were incubated for 48 h. Cytotoxic effect was evaluated microscopically.

Isolation and Culture of Oral Mucous Epithelial Cells

Human epithelial cells were obtained from oral mucosal biopsies (surgical wastes) and washed with phosphate-buffered saline (PBS) (Gibco). After treatment with penicillin (100 μg/mL) and streptomycin (100 μg/mL) (Gibco) for 30 minutes, the biopsy was finely minced with surgical scissors, mixed with 25–50 ml of 0.025% Trypsin/EDTA solution, transferred into a 125 mL flask and stirred gently at 37 °C. Every 30 min, the suspended cells were harvested. The resulting cell suspension was washed with Dulbecco's modified Eagle's medium (DMEM) (Gibco), supplemented with 10% fetal bovine serum (FBS) (Gibco), resuspended, and counted.

The viability was determined by trypan blue dye exclusion. Fresh Trypsin–EDTA was added to the flask repeating this treatment three or four times.

Isolated cells were plated over 3T3 Swiss feeder layers (25 cm^2 cell culture Corning flasks). After 70% confluence (14–15 days) cells were sub cultured, seeded on chamber slide system (Nunc-Lab tek) at 1×105 cells/well and used for morphological analysis (hematoxilin-eosine, cytokeratines pool)

Results and Discussion

Swelling Kinetics and Diffusion Coefficient

Table 1 shows the equilibrium swelling ratio of PNIPAAm-co-BAc hydrogels with different amounts of cross-linking agent. Increasing the degree of cross-linking, the swelling values decrease; because the polymer cross-linking leads to a decrease in the free volume and chain flexibility.

Figure 1 shows the swelling ratio vs. time at 25 and 37 °C. of the PNIPAAm-co-BAc hydrogels. The copolymer presents a normal swelling behaviour at 37 °C and a little overshooting effect at 25 °C. The equilibrium swelling ratio at 37 °C is lower than at 25 °C. The results for the assays at 27 °C, 29 °C, 30 °C, 32 °C, 35 °C (data no shown) confirmed this behavior. The temperature had an inverse effect on equilibrium swelling ratio for the hydrogels, this is, the swelling ratio decreased with increasing temperature. This evidence shows that the synthesized copolymers are temperature-sensitive.

Diffusion Model

The (apparent) diffusion coefficient (D) and transport characteristic constants (n, k) were calculated with the data obtained from the swelling kinetics. The values founded at 25 °C and 37 °C are presented in Table 2.

Table 1.
Equilibrium swelling ratio vs, % mol of cross-linking agent of the PNIPAAm-co-BAc hydrogels.

% mol	Equilibrium swelling ratio	
Cross-linking	25 °C	37 °C
1.5	5,69	1,27
2.5	2,40	0,69
3.0	4,01	0,78

The diffusion exponent (n) values for the hydrogels with 2.5 and 3% cross-linker behaves according to a regular Fickian diffusion process at 37 °C ($n \sim 0{,}5$),[11] while non-Fickian behaviors was observed at 25 °C ($n > 0{,}5$).

The data for 1.5% cross-linker composition hydrogel, could not be fitted to theoretical model, because all the time W_t/W_∞ was higher than 0,8.

According to this model, the hydrogels with 2,5 y 3,0% cross-linker concentrations at 37 °C should have better properties for the diffusion of nutrients and metabolites, in aqueous media cell culture.

As a consequence of increasing cross-linking degree, the diffusion into the gel is less, and lower values of the diffusion coefficient were obtained. Because a high crosslink density decreases the space between the copolymer chains and consequently, results in a rigid structure with a smaller free volume that can not be expanded and absorb large quantities of water.

Infrared Analysis of the PNIPAAm-co-BAc Hydrogels

In Figure 2, the IR spectrum for 2.5% cross-linking content hydrogel is showed. For the other samples, the IR spectra were similar (not showed), with the absorption bands of the functional groups having very close values of frequency.

In the copolymer spectra it is seen the NH, C–H, C=O bands. The low intensity of CH olefinic- and the C=C stretching bands did not appear in the spectra, indicating a small amount of residual monomer in the synthetized hydrogels.

DSC Analysis

Glass Transition Temperatures

Figure 3 shows the thermograms of dried PNIPAAm-co-BAc synthesized with different amount of cross linking agent. The glass transition temperatures (T_g) of the

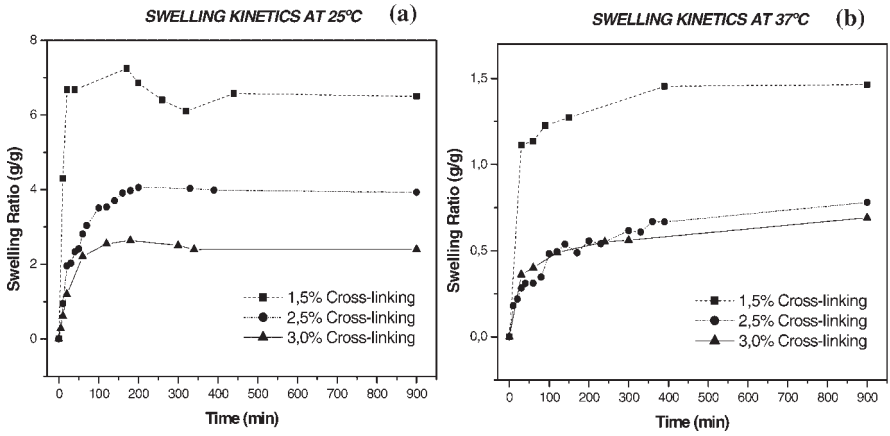

Figure 1.
Swelling ratio of the PNIPAAm-co-BAc hydrogels, (a) at 25° and (b) 37 °C, n = 3.

Table 2.
Equilibrium swelling ratio of PNIPAAm-co-BAc hydrogels vs, % mol of cross-linking agent.

$T = 25\ ^{\circ}C$				$T = 37\ ^{\circ}C$			
% *A.E**	$k \times 10^2$	*n*	$D \times 10^4$ (cm^2/s)	% *A.E*	$k \times 10^3$	*n*	$D \times 10^4$ (cm^2/s)
1,5	–	–	–	1,5	–	–	–
2,5	0,49	0,95	2,3	2,5	1,13	0,49	1,8
3,0	5,30	0,60	0,48	3,0	1,42	0,41	0,31

synthesized hydrogels increase with the cross-linking agent concentration indicating that the chain flexibility decreases, since the cross-linking involves the formation intermolecular connections through chemical bonds, then this process necessarily results in a reduction of the chain mobility and a reduction of the free volume.

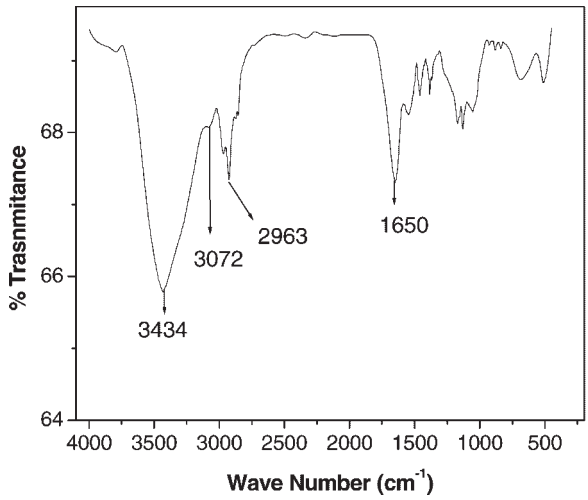

Figure 2.
IR spectrum hydrogel with 2.5% cross-linking.

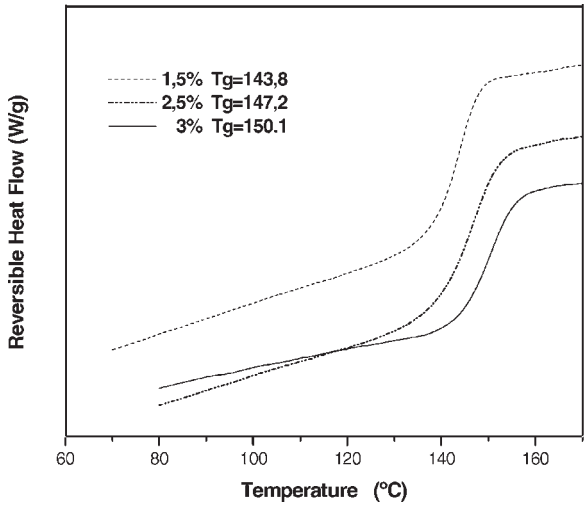

Figure 3.
Thermograms of dried PNIPAAm-co-BAc.

LCST of the Swollen Hydrogels

Figure 4 shows the LCST of the swollen hydrogels. The LCST of the PNIPAAm-co-BAc with 2.5 and 3% cross-linker did not change comparing with the PNIPAAm, since the objective is to synthesize a thermosensible hydrogel with the transition around physiological temperature (37 °C) for been used as a cell culture support, these results indicate that the hydrogels synthesized can be considered as potential material for this application.

Thermogravimetric Analysis (TGA)

Figure 5 shows the TGA thermograms for the hydrogels prepared with different cross-linking percent, each thermogram presents three weight losses. The first, at 100 °C, corresponds to water loss. The second corresponds to residual monomer, this indicates that it is necessary to eliminate this residual amount of monomer before using it as a cell support, because it could be toxic to the cells. The third lost corresponds to the decomposition temperatures. The decomposition

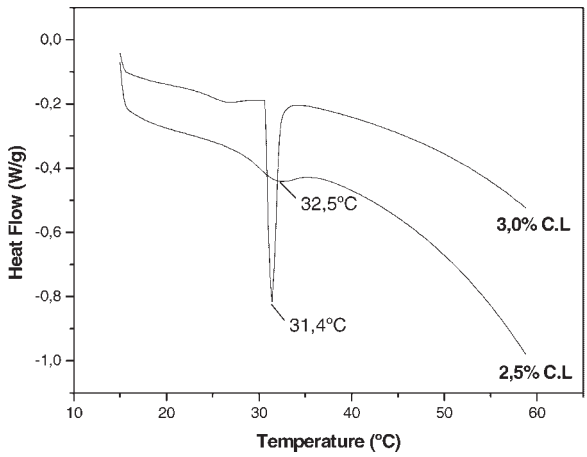

Figure 4.
LCST of the swollen hydrogels (C.L: Cross-Linker).

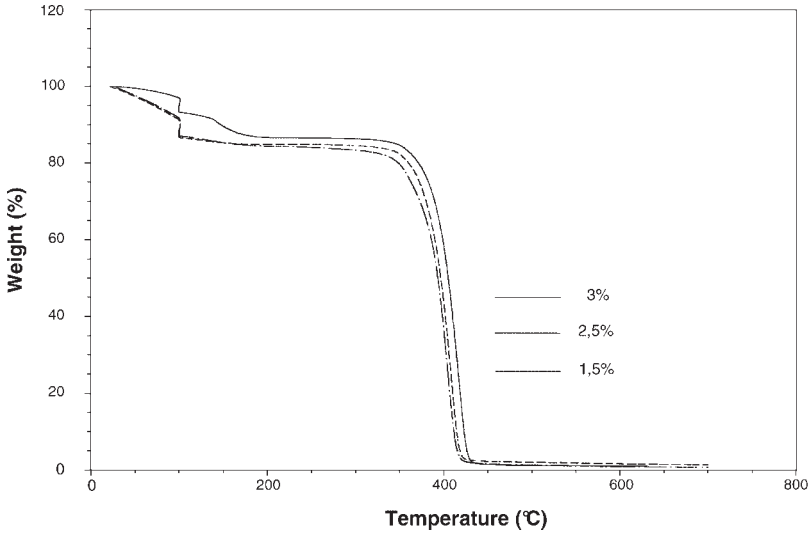

Figure 5.
TGA hydrogels thermograms.

temperatures were 404 °C, 408 °C and 416 °C for the copolymers with 1.5, 2.5 and 3% cross-linking agent respectively, indicating that thermal stability increases with the cross-linking degree.

3T3 Swiss Feeder Layer Culture

Table 3 shows that the the MMC concentration needed to inhibit 3T3 Swiss cells proliferation was 10 μg/ml.

3T3 Swiss Cytotoxicity

The cells behaviour on the synthesized copolymers hidrogels were monitored during 48 hours (n = 5), then photographic images of cells were obtained using an inverted microscope. The image shows elongated cell shapes, which are characteristic of the morphology of 3T3 Swiss cells (Figure 6). Theses preliminary experiments did not show a toxic effect on 3T3 Swiss cells.

Table 3.
Direct contact assay.

MMC [=] μg/ml	3T3 Swiss culture behavior
4 (n = 1)	At this concentration cells did not respond. They continued growing
8 (n = 1)	At this concentration cells did not respond. They continued growing
10 (n = 3)	Cell proliferation was inhibited at this concentration

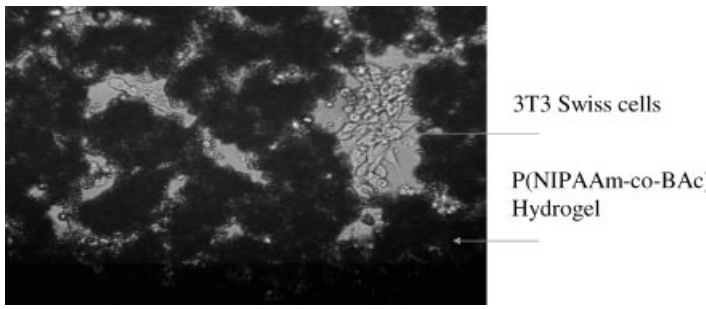

Figure 6.
Image of the direct contact between the 3T3 Swiss cells on the hydrogels.

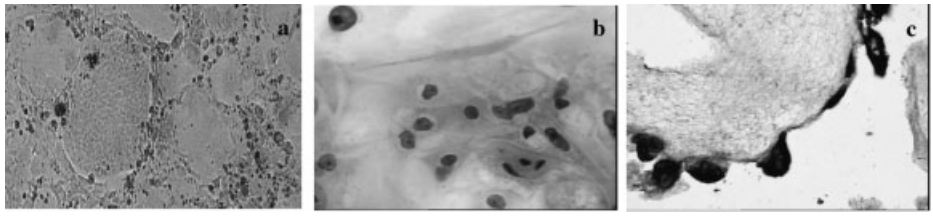

Figure 7.
Image oral mucous cell culture.

Culture of Oral Mucous Epithelial Cells
Epithelial cells were seed on MMC treated 3T3 Swiss cells. After 14-15 days approximately epithelial mucosal cells form colonies on the feeder layer. (Figure 7a).

Hematoxilin-eosine stain showed that cell morphology was compatible with epithelial cells (Figure 6b). Cytokeratin expression by immunohistochemical analysis suggests the epithelial origin of the cells obtained from oral mucosal cultures (Figure 6c).

Conclusion

NIPAAm-co-BAc copolymeric hydrogels had similar thermosensitive characteristics to PNIPAAm gels. Therefore, they can be considered as a cell culture supports, where cells would spread, proliferate at 37 °C and form well hydrated expanded structures that spontaneously detach cells at 25 °C.

Water sorption for the higher cross-link concentration hydrogels at 37 °C was a Fickian process, which indicates that the diffusion of metabolites and nutrients in an aqueous media would improve on hydrogels supports at incubation temperature (37 °C).

The preliminary results for the hydrogels cytotoxicity showed that the growing and cellular adhesion were normal after 24h. However other cytotoxicity and genotoxicity assays will be further investigated in next studies.

For hydrogels at 37 °C, water sorption for the higher cross-link concentration was a Fickian process, which indicates that the diffusion of metabolites and nutrients in an aqueous media would improve on hydrogels supports incubated at this temperature.

The preliminary results on the cytotoxic effect of the hydrogels showed that growing, proliferation and cellular adhesion were normal after 24hours. However other cytotoxicity and genotoxicity assays will be further investigated.

Acknowledgements: This study was supported by grants from the CODI (Comité para el Desarrollo de la Investigación-Sostenibilidad) University of Antioquia and COLCIENCIAS young researches program. The authors gratefully acknowledge Dr Alvaro Meana for supplying the 3T3 Swiss cell line, Dr Luis Correa for his help with immuno-hystochemical analysis and the Polychar 15 committee for economical support.

[1] H. A. Monji N, *Applied Biochemistry and Biotechnology* **1987**, *14*, 107.
[2] A. S. Huffman, A. Afrassiabi, *et al.*, *Journal of Controlled Release* **1986**, *4*, 213.
[3] S. Champ, W. Xue, *et al.*, *Polymer* **2001**, *42*, 6439.
[4] L. C. Dong, A. S. Hoffman, *Journal of Controlled Release* **1986**, *4*, 223.
[5] C. S. Patrickios, T. K. Georgiou, *Current Opinion in Colloid & Interface Science* **2003**, *8*, 76.
[6] E. Diez-Pena, I. Quijada-Garrido, *et al.*, *Macromolecules* **2003**, *36*, 2475.
[7] G. Odian, in: "*Principles of Polimerization*", W. Interscience, **2004**, p. 4.
[8] W.-F. Lee, Y.-C. Yeh, *European Polymer Journal* **2005**, *41*, 2488.
[9] T. Caykara, S. Kiper, *et al.*, *European Polymer Journal* **2006**, *42*, 348.
[10] K. Nishida, M. Yamato, *et al.*, *N Engl J Med.* **2004**, *351*, 1187.
[11] M. P. Mullarney, T. A. P. Seery, *et al.*, *Polymer* **2006**, *47*, 3845.

