Use of Thermosensitive Polymer Material on the Basis of N-Isopropylacrylamide and N-Tert-Butylacrylamide Copolymer in Cell Technologies

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We developed thermosensitive polymer substrates on the basis of N-isopropylacrylamide and N-tert-butylacrylamide co-polymer and studied their interaction with cultured substrate-dependent mammalian cells. It was shown that these polymers promote cell adhesion and proliferation at a level comparable to polystyrene treated for cell culturing and provide effective cell detachment after lowering culturing temperature below a critical level determined by phase transition temperature in aqueous solutions of polymers. A dependence of phase transition temperature on the ratio between N-isopropylacrylamide and N-tert-butylacrylamide was demonstrated. Differences in the dynamics of cell detachment from the surface of polymer substrates with various proportions between the components were shown.

Key Words: *therm sensitive polymers; cells; cell layers; non-enzymatic detachment*

Maintenance of differentiated state and biological activity of specialized cells during their *in vitro* culturing and subsequent transplantation is an important problem of modern medicine. A promising approach is the use of thermosensitive substrates on the basis of poly-N-isopropylacrylamide (PNIPAAm), a thermosensitive polymer carrying C=O and N-H groups typical of protein molecules and a polyleucine analog by its chemical composition. The transition of PNIPAAm from insoluble to water-soluble state occurs at a phase transition temperature of 33°C (lower critical solvation temperature, LCST). At 37°C the polymer is in insoluble state and can be used as the substrate for cell culturing. Lowering the culturing temperature below LCST leads to polymer hydratation and cell detachment from its surface without treatment with proteolytic enzymes and dissociating agents.

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The PNIPAAm-based thermosensitive substrates have several advantages over traditionally used culture plastic. Culturing on the thermosensitive surface makes it possible to obtain intact cell cultures and maintain their specific activity through many passages [5]. There are immunocytochemical and biochemical data that lowering of the incubation temperature leads to detachment of cultured cells and synthesized matrix bound to the basement membrane from PNIPAAm surface [3]. Characteristics of PNIPAAm substrate opens wide prospects for the use of this polymer for culturing of specialized cells, creation of tissue equivalents, and cell biological studies.

Polymerization of PNIPAAm polymer is usually carried out using high-energy electron beam [6] and technique of photoinduced polymerization on polystyrene surface [1]. Drawbacks of these methods are that they require multiple washouts from unreacted monomers toxic for cells, create non-uniform surface, and that the apparatus generating high-energy electron beam

is unavailable for the majority of investigators dealing with cell cultures.

Here we propose a new approach to the formation of thermosensitive substrates consisting in the use of alcohol solutions of linear copolymers PNIPAAm and N-tert-butylacrylamide (NtBAAm),

MATERIALS AND METHODS

High-molecular-weight polymers PNIPAAm, poly-NtBAAm (PNtBAAm), and PNIPAAm/NtBAAm copolymers (molar ratios 85/15, 65/35, and 50/50) were synthesized by the method of free radical polymerization [4].

LCST of these polymers was determined by changes in light transmission in 0.02% aqueous solutions of polymers preheated to 37°C during their cooling. The measurements were carried out on a Hitachi U3410 spectrophotometer equipped with thermocontrolled cuvettes (optical path 1 cm) at λ=500 nm. The cuvettes were cooled at a rate of 0.2°C/min, accuracy of temperature measurements was 0.1°C. LCST was determined as the temperature corresponding to a sharp increase of light transmission in polymer solutions.

The polymer substrates were formed on a surface by slow drying of 5% polymer solutions in absolute alcohol. For evaluation of swelling, the polymer films were formed on coverslips and kept in distilled water at prescribed temperature for 15 min. The degree of swelling of polymer substrates was determined as the ratio of water weight in swollen polymer to the weight of dry polymer measured after drying of the swollen sample. The samples were weighed on Mettler HL52 balances, accuracy 0.05 mg.

We used NCTC fibroblasts, strain L929. The cells were cultured in DMEM supplemented with 10% FCS, 50 μg/ml penicillin, 50 μg/ml streptomycin, and 1% L-glutamine at 37°C in a humid atmosphere containing 5% CO_2. Seeding density was 30-50 thousands cells/cm^2. Living cells were counted in a Goryaev chamber, viability was determined by trypan blue exclusion. Seeding and examination of cells on the surface of thermosensitive substrates was carried out on a thermocontrolled surface at 37°C.

Non-enzymatic detachment of cells from the polymer surface was carried out at 5°C. The detachment was observed under a Biolam-MPZ microscope (the time needed for detachment of 90% cells from the substrate was recorded).

RESULTS

The study of phase transitions of the synthesized polymers in aqueous solutions showed that all materials are thermosensitive and have LCST in the physiological range. The critical temperature corresponding to polymer transition from collapsed into solvated state linearly decreased with increasing the percent of NtBAAm monomer moieties in the PNIPAAm/NtBAAm copolymer (Fig. 1).

All studied substrates except PNIPAAm promoted cell adhesion and proliferative activity at a level comparable to that observed in cultures grown on culture plastic (Fig. 2). On the surface of PNIPAAm/NtBAAm polymers the cells had typical spindle shape, while on the surface of PNIPAAm looked weakly flattened or form cell aggregates (Fig. 3).

We hypothesized that weak flattening and low proliferative activity of cells on PNIPAAm surface are determined by high hydratation of linear PNIPAAm polymers at culturing temperature of 37°C, *i.e.* close to the phase transition temperature. Introduction of hydrophobic monomers NtBAAm into copolymer composition decreased the phase transition temperature and made the surface moderately hydrophobic, which determined typical flattening and growth of cells.

The study of swelling of PNIPAAm/NtBAAm polymer films in water at different temperatures revealed rapid decrease of substrate swelling rate with increasing the percent of NtBAAm (Fig. 4). Thus, the increase in the content of hydrophobic monomers NtBAAm not only decreased the phase transition temperature of the test copolymers, but also decreased the rate of system response to temperature shifts.

Evaluation of non-enzymatic detachment of cells from the substrate upon lowering the culturing temperature below the critical level showed sharp deceleration of cell detachment with increasing the percent of NtBAAm. The time of detachment of

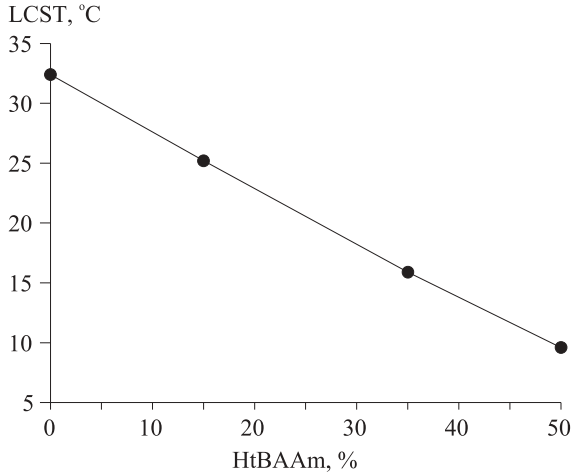

Fig. 1. Solvation temperature as a function of NtBAAm percent in PNIPAAm/NtBAAm copolymer.

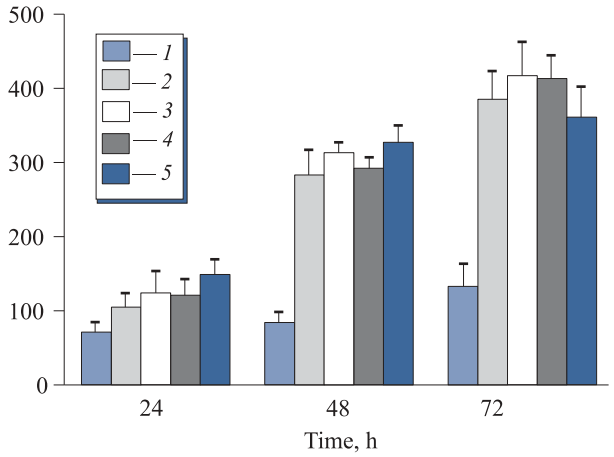

Fig. 2. Growth of NCTC L929 fibroblasts on substrates. Ordinate: number of cell (% of seeded). *1*) PNIPAAm, *2*) PNIPAAm/NtBAAm (85/15), *3*) PNIPAAm/NtBAAm (65/35), *4*) PNIPAAm/NtBAAm (50/50), *5*) specially treated polystyrene.

NCTC L929 fibroblasts at 5°C from the substrate was 0.5±0.5 min for PNIPAAm, 2.0±0.5 min for PNIPAAm/NtBAAm (85/15), 30±5 min for PNIPAAm/NtBAAm (65/35), and 180±15 min for PNIPAAm/NtBAAm (50/50).

Since physiological activity of cells at 5°C is very low, decreased thermosensitivity of the polymer substrates is the main cause of decelerated detachment of cells from the surface with increasing the percent of NtBAAm in the polymer.

Cell binding to matrix is retained even at 4°C [2], and therefore the extracellular matrix is detached with cells. Cell detachment from the surface of thermosensitive substrates without treatment with enzymes and dissociating agents preserved cell contacts, membrane proteins and extracellular matrix synthesized by cells and makes it possible to obtain cell layers for transplantation, which opens wide prospects for the use of these polymers in cell technologies. In our experiments, less abrupt detachment of cell from the surface of PNIPAAm/NtBAAm (65/35) and PNIPAAm/NtBAAm (50/50) substrates preserved the integrity of cell layers, while rapid detachment from PNIPAAm and PNIPAAm/NtBAAm (85/15) led to the formation of cell spheroids of different size. Thus, the proposed substrates provide new possibility for regulating cell detachment rate and varying the size of cell aggregates.

The synthesized PNIPAAm/NtBAAm substrates are characterized by a number of advantages over PNIPAAm. High purity of synthesized linear polymers allowed omitting multiple washouts, while the proposed method of polymer film formation from alcohol solutions ensured sterility of substrates and provides the possibility of applying the films on complex surfaces. Copolymerization of PNIPAAm and NtBAAm not only improved adhesion and growth characteristics of the substrates

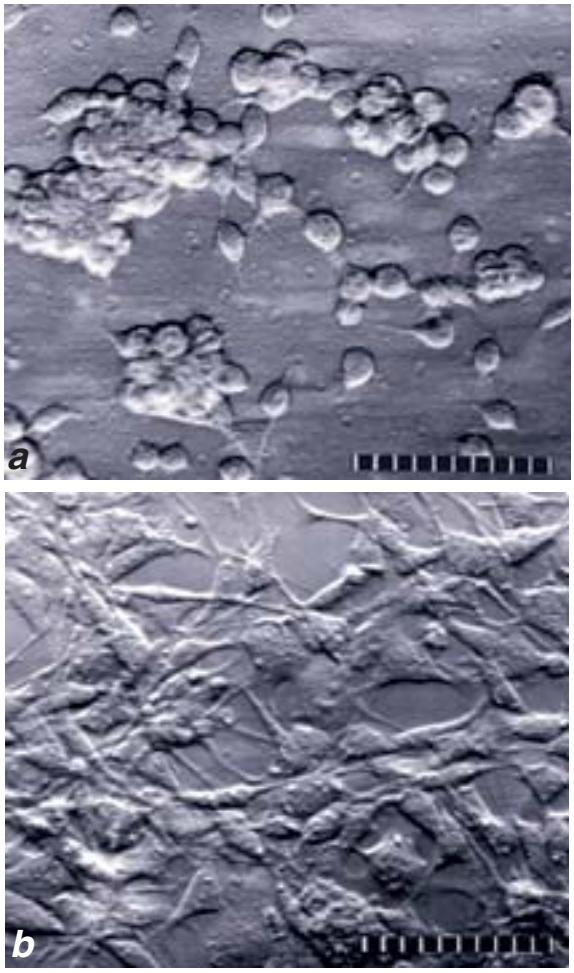

Fig. 3. NCTC L929 cells 48 h after seeding on PNIPAAm (*a*), PNIPAAm/NtBAAm (85/15) (*b*) surfaces. Scale: 100 μ.

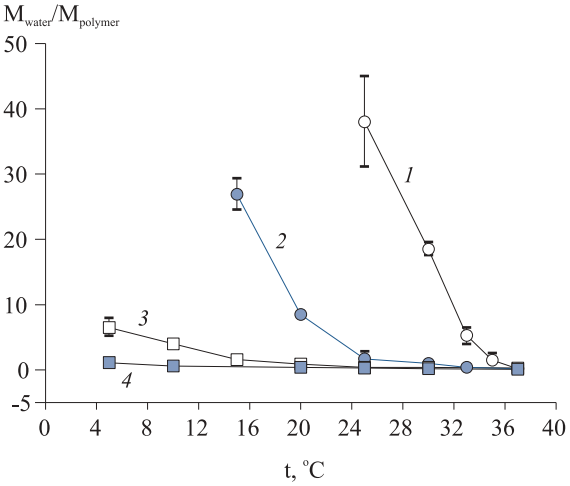

Fig. 4. Swelling of polymer films in water at different temperatures (t). *1*) PNIPAAm, *2*) PNIPAAm/NtBAAm (85/15), *3*) PNIPAAm/NtBAAm (65/35), *4*) PNIPAAm/NtBAAm (50/50). M: weight.

compared to PNIPAAm alone, but also allowed regulation of temperature and rate of cell detachment.

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