

Substrates for cell cultivation based on thermosensitive imidazole copolymers

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ABSTRACT. Cell cultures are needed in various fields: the study of cell structure and function, models in drug screening, and other biomedical applications. Most tissue-derived cells can only grow on solid substrates. Thus, cell culturing involves three steps: cell adhesion on the surface, cell growth and division on the surface, and cell detachment from the surface for further use. Thermo- and pH-sensitive polymers are promising substances for cell culture coatings. Changes in temperature and/or pH can drastically change surface properties, resulting in gentle cell detachment. We synthesized copolymers with pendant imidazole and hydrophobic groups that exhibit the properties of weak polymeric bases capable of thermosensitivity due to hydrophobic interactions. Plastic surface can easily be coated with copolymers by pouring over the copolymer solution. The modified plastic surface is a good substrate for culturing adenocarcinomic human alveolar epithelial cells. The cells show strong adhesion to the copolymer film and high viability after detachment under the influence of temperature and/or pH changes.

Keywords: thermosensitive polymer, imidazole, polyelectrolyte, surface modification, cell culture, cell adhesion and detachment

1. Introduction

Adhesive cell culture on various substrates such as glass, polystyrene, natural and synthetic polymers has become a popular method for supporting cells *ex vivo* in laboratories. They are used to gain knowledge about biological processes inside cells, to study the toxicity and efficacy of drugs in a cell model before animal and human studies, to develop new biomaterials for medical applications, and to create 3D models of cell cultures closely resembling specific tissues and organs for a more accurate physiological response to the research object. (Verma et al., 2020). Although some cells grow in suspension and do not depend on attachment to the substrate (e.g., blood cell lines), most tissue-derived cells die from anoikis without proper cell adhesion or aggregation (Paoli et al., 2013).

One of the main challenges of modern 3D cell culture is that most methods result in cell aggregation into spheroids 20-500 μm in diameter, since such aggregation is limited due to the lack of a vascular system for oxygen and nutrient delivery (Langhans, 2018). However, detaching a cell sheet from the surface

without breaking intercellular interactions during cell detachment with only cell-substrate connections broken can create sheets with adjustable widths by adding layers on top of each other, and the sheet area will be limited only by the selected culture dishes. Cell sheet production has a future in transplant medicine by creating cell sheets from different cell types (Yamato et al., 2001; Sekine et al., 2013). Moreover, in ordinary cell culture there is a need to maintain a certain number of cells in a Petri dish by manually detaching the cells with chemical agents (Na_2EDTA and trypsin) after the cells have grown a monolayer on the substrate. In the monolayer the cells cannot continue their reproduction and release a huge number of metabolites that can trigger the processes of cell aging and programmed cell death - apoptosis. In cell biology, smart materials with properties of cell detachment under certain stimuli are needed to simplify manipulations, improve cell detachment and create new models for drug screening and transplantation medicine.

Natural cell adhesion happens through the interaction of cell membrane receptors (integrins) with extracellular matrix proteins, such as collagen,

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fibronectin, laminin, which have special amino acid sequences with adhesive properties (RGD: Ruoslahti, 1996; GFOGER: Knight et al., 2000; PHSN: Dillow et al., 2001). Nevertheless, the easiest way to achieve cell adhesion in a daily cell culture is to use plasma-treated polystyrene laboratory tissue culture dishes, where the surface charge of the dishes will depend on the gases used to treat the plasma (Lerman et al., 2018). Mostly, it is a net negative charge or both negative and positive charges (Barker and LaRocca, 1994). Interestingly, the cell membrane has a negative charge (Fairhurst et al., 2007; Gallardo et al., 2017) and for passive adhesion to matrix positive charge on the surface of biomaterial is essential. After passive adhesion, the cell begins to spread over the surface and establish strong focal adhesions using integrins and the actin cytoskeleton, which require an energy source, adenosine triphosphate (ATP), and are called active adhesion. (Okano et al., 1995). Studies of functional groups similar to those in amino acids (-COOH, -CH₂OH, -CONH₂ and -CH₂NH₂) have shown that polar and positively charged surfaces have better conditions for cell growth, adhesion and proliferation than negatively charged surfaces (Lee et al., 1994). Cell detachment techniques such as trypsin, Na₂EDTA, and scraping reduce cell viability, disrupt the integrity of membrane receptors, disrupt intercellular connections, and the ECM molecules secreted by cells, which reduces the quality of experiments and makes it impossible to collect cell sheets for regenerative medicine.

Stimulus-sensitive polymers have become a valuable tool in cell biology due to their unique properties of harmless cell detachment with increased cell viability and preservation of cell membrane receptors (Chen et al., 2015; Kurashina et al., 2017), cell sheet recovering by keeping intact cell-cell junctions and extracellular matrix (ECM) (Akiyama and Okano, 2015) in comparison with more conventional detachment methods: chelating agents (Na₂EDTA) and enzymes (trypsin). Smart polymers are sometimes used in cell sorting methods (Matsuda et al., 2007; Zhao et al., 2016). Poly-(N-isopropylacrylamide) (PNIPAM) is the gold standard and widely investigated polymer in this field because the hydrophobic character of the polymer changes to a highly hydrophilic one when the temperature changes from 37°C to 20°C, which impairs cell adhesion to the polymer substrate, but electron beam (EB) irradiation, which is used to graft PNIPAM onto laboratory dishes, is an expensive procedure. Although some modified PNIPAM coatings can overcome this disadvantage, they still face another problem: making a surface with sufficient polymer grafting density. (Akiyama and Okano, 2015).

Although temperature is the most abundant stimulus in cell culture, there are pH (Chen et al., 2012), mechanical stress (Akiyama et al., 2018) and electricity (Gao et al., 2016). Moreover, we believe that only one stimulus cannot provide sufficient quality for cell sorting, and that a larger number of modifiable parameters improves the procedure. In addition, the combination of stimuli allows for finer regulation of cell detachment. That is why new polymers with

temperature and pH stimulus were synthesized to enhance cell adhesion and detachment parameters, cell sheet collection, and cell sorting quality. One of the main features of these polymers would be a simple modification of most substrates connected with cell culture (cover glass, polystyrene, PDMS) that have a great potential in creation of smart materials in cell culture like microfluidics.

The work is aimed at synthesizing thermo- and pH-sensitive polymers, coating plastic surfaces, and studying cell growth on the modified surfaces. The pH sensitivity of the polymers was achieved by introducing imidazole groups, which are not strongly protonated at neutral pH and therefore should be less toxic than amine groups.

2. Materials and methods

2.1. Chemical reagents and cell culture

Acryloyl chloride, 3-(1H-imidazol-1-yl)propan-1-amine, dipropylamine, triethylamine (Sigma-Aldrich, Acros Organics, USA) were purified by distillation before use. Dibenzylfluorescein was used without purification (Sigma-Aldrich, USA). DMF was dried with CuSO₄ (30 minutes) and distilled at 5 mm Hg. Asobis(isobutyronitrile) (Sigma-Aldrich, USA) was recrystallized from ethanol. NaOH was purified from carbonate impurities by filtering its 50% solutions. Ethanol were purified according to (Keil et al., 1966) Dichloromethane and 3-methylbutan-1-ol were purified by distillation. Solutions were prepared using deionized water (resistance 18.2 MΩ·cm, Millipore Simplicity UV, USA). A cellophane membrane (3.5 K) was used for dialysis.

CalceinAM (Invitrogen), Propidium Iodide (95%, J&K Scientific), Fetal Bovine Serum (FBS, Biowest), NaOH (98%, Ekspit, Russia), Dulbecco's modified eagle medium (DMEM), gentamicin (10 mg/mL), phosphate-buffered saline (PBS) (pH = 7.4) were obtained from Biolot, Russia. Sterile 24-well microbiological polystyrene plates were used (non-pyrogenic, non-cytotoxic, DNase/RNase/DNA – free, SPL Life Science Co., Ltd). A549 adenocarcinomic human alveolar epithelial cell line (from American Type Culture Collection ATCC) were obtained by Biolot, Russia.

2.3. Modification of plastic microbiological assay plates

24-well microbiological polystyrene plates were modified with a polymer solution in EtOH:CH₂Cl₂ 85:15 v/v mixture. Poly(vinyl butyral) (PVB) or 3-methylbutan-1-ol were added to the polymer solution with the aim to obtain non-fragile films (Table 1). Solutions were evaporated under a closed cap for 48 h on an orbital shaker (90 rpm). The plates were stored at room temperature for 24 h and heated for 2 h at 80°C. The fluorescent dye dibenzylfluorescein was added to the polymer solutions in order to visualize the coatings and check their stability. The coatings were stable for 7 days at 37°C in phosphate buffer (pH 7.4, 50 mM).

Table 1. Compositions for the plates modification

Coating	Polymer	Polymer conc., %	Additive	Additive conc., %	Solution volume, μL	*Coating thickness, μm
PV5-1-A	ZS-682-5	0.1	PVB	0.01	300	1.307
PV5-2-A	ZS-682-5	0.1	3-methylbutan-1-ol	0.005	300	1.307
PV6-1-A	ZS-682-6	0.1	PVB	0.01	300	1.307
PV5-1-B	ZS-682-5	0.02	PVB	0.01	300	0.261
PV5-2-B	ZS-682-5	0.02	3-methylbutan-1-ol	0.005	300	0.261
PV6-1-B	ZS-682-6	0.02	PVB	0.01	300	0.261

* Theoretically, taking the polymer density as 1.

2.3. Study of the cell growth on the modified surfaces

24-well assay plates modified with different polymers were sterilized under UV light (250 nm) for 30 minutes. A549 cells were seeded at concentration 50,000 cells *per well* and incubated in DMEM medium supplemented with 10% fetal bovine serum, gentamicin (50 $\mu\text{g/ml}$) at 37 $^{\circ}\text{C}$, 5% CO_2 in a humidified atmosphere for 72 h until a monolayer cell formation for most samples. Then cells were imaged on an inverted Microscope Leica DMi8 (cells unaffected by CalceinAM chelating properties), washed with 1x phosphate-buffered saline (PBS), stained by CalceinAM for 30 minutes, washed with PBS again, stained by propidium iodide (1 $\mu\text{g/ml}$) for 10 minutes, and washed with PBS. Fluorescent images were obtained via Microscope Leica DMi8 in a Rhodamine and FITC filters. All images were edited via LAS X software.

2.3. Instrumentation

^1H NMR spectra were recorded using a Bruker DPX 400 (Bruker Biospin Corporation, Billerica, MA, USA) at 400.13 MHz, in DMSO-d_6 for ZS-682-5 and in D_2O for ZS-682-6. IR spectra were recorded on an Infracum FT-801 instrument (SIMEX company, Novosibirsk) using KBr pellets.

The molecular weight (MW) of ZS-682-5 in chloroform was determined by the light scattering method (Photocor, Russia) using plots of inverse scattering intensity versus concentration. MW was calculated from the segment cut off by a straight line on the y-axis, according to the Debye equation.

Potentiometric titration was carried out on a Multitest ionometer (NPP Semiko, Novosibirsk, Russia) in water with the addition of 0.1 M NaCl, the polymer concentration was 7.5 mM. Before measurement, HCl was added to the solution to pH 2.5 and the solution was titrated with 0.1M NaOH.

Fluorescence microscopy was carried out using a MOTIC AE-31T and Leica DMi8 inverted microscopes. Ultraviolet analytical cabinet "UFK-HD" (LLC "PETROLASER", St. Petersburg) was applied to detect the stability of coatings in an aqueous environment using dibenzylfluorescein.

3. Results and discussion

3.1. Synthesis and study of copolymers

The copolymers were obtained by reacting poly(acryloyl chloride) with 1-(3-aminopropyl)imidazole (API) and dipropylamine in the presence of triethylamine (Scheme 1), similar to (Danilovtseva et al., 2017). Structure of the copolymers was confirmed by ^1H NMR (Fig. 1) and their composition was analyzed

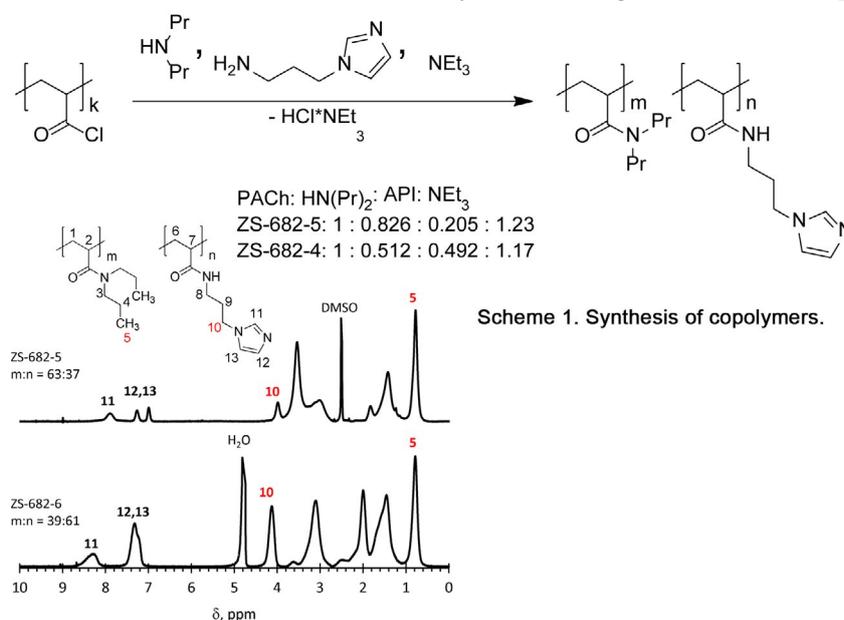


Fig.1. ^1H NMR of copolymer ZS-682-5 in DMSO-d_6 and ZS-682-6 in D_2O .

by signal intensity ratio at 0.5-1.1 ppm (CH_3 groups) and 3.9-4.4 ppm (CH_2 group attached to the imidazole cycle).

ZS-682-5 copolymer is insoluble in water, but soluble in organic solvents (ethanol, chloroform, dichloromethane). It exists in mono-macromolecular form in chloroform (hydrodynamic radius of 4.3 nm), and its molecular weight has been measured as 29.8 kDa. Comparing this value with the degree of polymerization of the original PACH (220 (Zakharova et al., 2018)), we can conclude that there is no noticeable change in macromolecule length during the modification reaction. ZS-682-6 copolymer contains more hydrophilic groups and it is soluble in water and ethanol but insoluble in chloroform. Increasing the ionic strength (0.15 M NaCl, pH 4 and 7 buffers) prevents solubility in aqueous medium. This copolymer gives associates in aqueous solutions (hydrodynamic radius 56 nm), which do not allow a correct measurement of the molecular weight. Estimation of the molecular weight by minor monomolecular peak (3.3-3.6 nm) gives 30 kDa which agree with ZS-682-5 data.

The ZS-682-6 copolymer precipitates out of water at pH greater than 6.5 at 20°C. Light scattering (90°) and transmittance studies (Fig. 2) show the formation of turbidity at 46°C. The data of dynamic light scattering (Table 2) show the presence of macromolecules in aggregates of two sizes: 30 and 200-400 nm in radius. Large aggregates decrease in size and contribution to the scattering intensity when heated and disappear at 40°C. This behavior explains decrease in scattering from 10 to 30°C in Fig. 2. Heating ZS-682-6 should enhance the compactization of macromolecules and their aggregates due to hydrophobic interactions, resulting in redistribution between large and small aggregates. Polymer precipitation at about 50°C does not show large particles in DLS due to the effect of multiple scattering (Danilovtseva et al., 2015).

ZS-682-6 was studied by potentiometric titration at pH below 6.5, in the region of solubility (Fig. 3). pK of conjugated acid ionization of the imidazole units increases with increasing degree of ionization (α), because a decrease in the positive charge on the polymer chain hinders further elimination of protons from it. Increasing the temperature reduces the basicity of the imidazole units due to compactization of the macromolecules, which increases the electrostatic effect on the acid-base properties.

3.2. Observation of cell adhesion, growth, and proliferation on polymer coatings of different thicknesses

Using the polystyrene surface for control and synthesized polymers, cells were cultured on different substrates. Endothelial cells A549 were used as a well-characterized cell line with medium adhesive properties to substrates. In addition, A549 cells are a popular model for 3D cell culture due to their epithelial origin of lung adenocarcinoma and strong cell-cell interactions. Being too hydrophobic, untreated polystyrene showed a lack of cell attachment to its

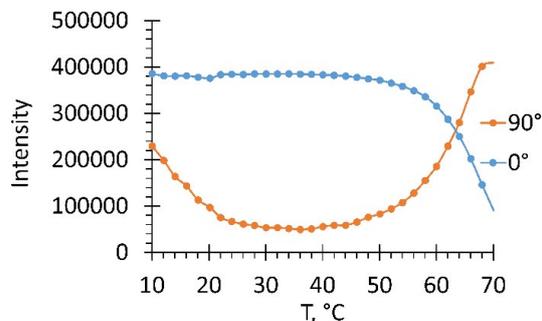


Fig. 2. Light scattering (90°) and transmittance data for ZS-682-6 copolymer at pH 6 in sodium acetate buffer (20 mM). The polymer concentration was 7.5 mM (in polymer units) and the heating rate was 0.6 deg/min.

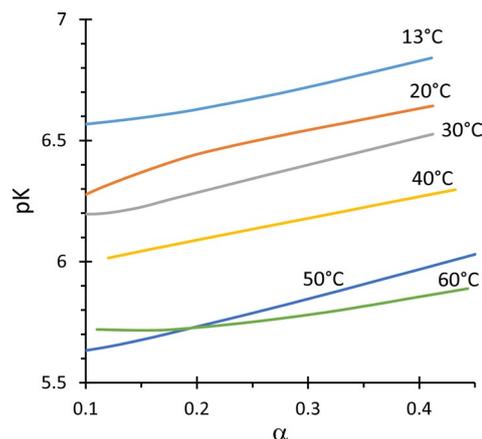


Fig. 3. Dependence of conjugated acid pK vs. α for ZS-682-6 copolymer.

Table 2. Particle size in aqueous solution of ZS-682-6 at pH 6

T, °C	R ₁ , nm	I ₁ , %	R ₂ , nm	I ₂ , %
10	30	60	281	40
20	30	71	396	30
30	28	76	190	24
40	39	100	-	-
50	42	100	-	-
60	53	100	-	-

surface. Treatment with PV6-1-B polymer significantly improved cell adhesion to the substrate, although some cells remained rounded, i.e., poorly attached to the surface (Fig. 4). Meanwhile, a thicker version of this PV6-1-A polymer coating had no cell attachment and only irregularities in the polymer coating were visible. The cell morphology on PV5-1-B and PV5-2-B surfaces corresponds to a typical monolayer epithelial cell line with close cell-cell interaction. PV5-1-B and PV5-2-B are completely suitable for conventional cell culture. On thicker polymer layers PV5-1-A and PV5-2-A cell attachment was reduced to a small number that showed weak proliferation rate due to dependency on cell adhesion quality. Here, we recognized the importance of thickness in developing stimuli responsive polymer coatings. Thus, further experiments are needed to find the most appropriate polymer thickness to regulate cell adhesion.

3.3. Cell detachment under temperature and pH stimuli from different polymers

Response to the stimuli was examined by changing temperature by adding DPBS (DPBS temperature was similar to room temperature and corresponded to 25°C), or by simultaneously changing temperature by adding DPBS and increasing pH to 8.2 by adding 0.1 M NaOH solution. The PV6-1-B surface responded sufficiently to pH and temperature stimuli, and cells easily detached from the surface (Fig. 5). Moreover, using both stimuli, a change in pH and temperature, compared to a change in temperature, showed a significantly higher level of cell detachment, as can be seen by morphological changes from slight to complete rounding of the cells. The cells were then successfully detached from the surface. Although the PV5-1-B and PV5-2-B surfaces also showed some delamination in response to pH and temperature stimuli, it was limited to the center of the polymer coating, which may be due to the uneven thickness of the polymer layer, and cells are sensitive to the thickness of the cell layer.

3.4. Life/Dead cell staining after detachment

Cells recovered after detachment from various polymers were stained with two dyes: CalceinAM (green) for live cells and Propidium iodide (red) for dead cells (Fig. 6). It is worth noting that staining and imaging procedures take time and occur at room temperature, which led to rounding of cells on stimulus-sensitive polymers. Cells that were separated from the PV6-1-B polymer and then seeded onto the same new polymer layer showed a high concentration of separated cells. Moreover, the detachment procedure and prolonged cell culture on the PV6-1-B layer showed no significant signs of cytotoxicity even when compared to the mild chemical detachment method using Na₂EDTA. PV5-1-B and PV5-2-B layers showed poor cell retrieval after detachment even when both stimuli were used. Nevertheless, cells showed good viability in these layers.

Conclusions

New copolymers containing weakly basic imidazole groups and hydrophobic fragments were obtained. Water-soluble samples show thermo- and pH-sensitivity. The plastic surface can easily be coated with copolymers by pouring over the copolymer solution. The modified plastic surface is a good substrate for culturing A549 cells. The

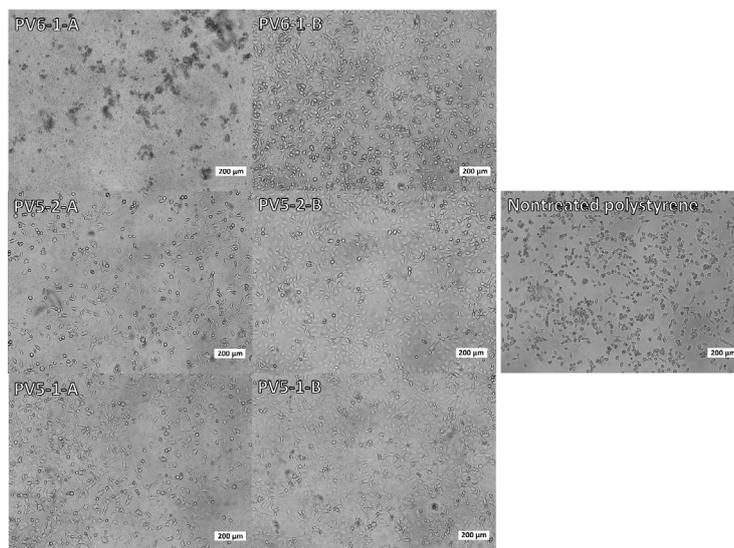


Fig. 4. Adhesion of A549 cells to various substrates. Nontreated polystyrene showed poor adhesion, most cells are rounded. PV6-1-A didn't show any cell adhesion at all while cells on a thinner PV6-1-B layer have a moderate adhesion and only some cells are unattached. Although cells attached and proliferated restrictively on the PV5-1-A and PV5-2-A thick polymer coatings, the PV5-1-B and PV5-2-B polymer substrates showed perfect cell adhesion and resulted in a cell monolayer, proving the suitability of their composition for cell culture.

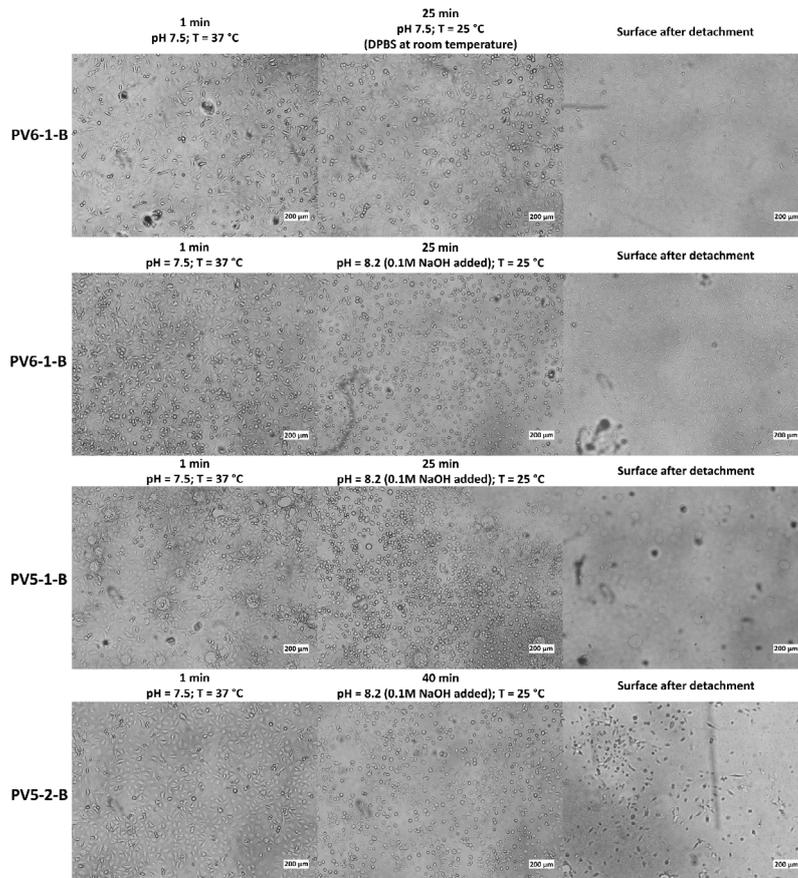


Fig. 5. Characteristics of A549 cell detachment in response to changes in temperature and pH from polymer substrates. PV6-1-B demonstrated significant cell detachment in response to changes in temperature and pH across the surface. PV5-1-B and PV5-2-B delamination was limited to the center of the well surface and was more sensitive to changes in pH than to changes in temperature (not shown).

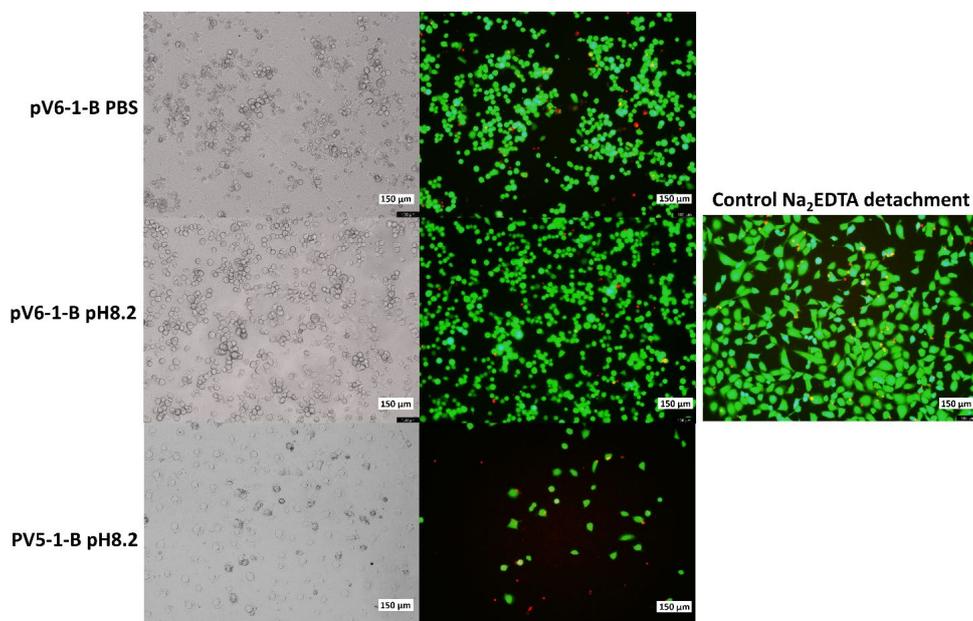


Fig.6. Life/Dead cell analysis after detachment and comparison with the control detachment via Na_2EDTA . A549 were detached at high concentrations from the PV6-1-B polymer under temperature and pH change. Moreover, cell viability after detachment from the PV6-1-B polymer is high and similar to the Na_2EDTA detachment. The number of cells detached from the PV5-1-B and PV5-2-B surfaces via stimuli were low although cells remained with metabolic activity (PV5-2-B has no significant difference from PV5-1-B).

cells show strong adhesion to the copolymer film and high viability after detachment under the influence of temperature and/or pH changes.

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Conflict of interest

The authors declare no conflict of interest.

References

- Akiyama Y., Matsuyama M., Yamato M. et al. 2018. Poly (N-isopropylacrylamide)-Grafted Polydimethylsiloxane substrate for controlling cell adhesion and detachment by dual stimulation of temperature and mechanical stress. *Biomacromolecules* 19(10): 4014–4022. DOI: [10.1021/acs.biomac.8b00992](https://doi.org/10.1021/acs.biomac.8b00992)
- Akiyama Y., Okano T. 2015. Temperature-responsive polymers for cell culture and tissue engineering applications. *Switchable and Responsive Surfaces and Materials for Biomedical Applications*: 203–233. DOI: [10.1016/b978-0-85709-713-2.00009-2](https://doi.org/10.1016/b978-0-85709-713-2.00009-2)
- Barker S.L., LaRocca P.J. 1994. Method of production and control of a commercial tissue culture surface. *Journal of Tissue Culture Methods* 16(3–4): 151–153. DOI: [10.1007/bf01540642](https://doi.org/10.1007/bf01540642)
- Chen S., So E.C., Strome S.E. et al. 2015. Impact of detachment methods on M2 macrophage phenotype and function. *Journal of Immunological Method* 426: 56–61. DOI: [10.1016/j.jim.2015.08.001](https://doi.org/10.1016/j.jim.2015.08.001)
- Chen Y.-H., Chung Y.-C., Wang I.-J. et al. 2012. Control of cell attachment on pH-responsive chitosan surface by precise adjustment of medium pH. *Biomaterials* 33(5): 1336–1342. DOI: [10.1016/j.biomaterials.2011.10.048](https://doi.org/10.1016/j.biomaterials.2011.10.048)
- Danilovtseva E.N., Aseyev V., Belozeroва O.Yu. et al. 2015. Bioinspired thermo- and pH-responsive Polymeric Amines: multimolecular aggregates in aqueous media and matrices for Silica/Polymer nanocomposites. *Journal of Colloid and Interface Science* 446: 1–10. DOI: [10.1016/j.jcis.2015.01.02](https://doi.org/10.1016/j.jcis.2015.01.02)
- Danilovtseva E., Maheswari Krishnan U., Pal'shin V. et al. 2017. Polymeric Amines and Ampholytes derived from Poly(Acryloyl Chloride): synthesis, influence on silicic acid condensation and interaction with nucleic acid. *Polymers* 9(11): 624. DOI: [10.3390/polym9110624](https://doi.org/10.3390/polym9110624)
- Dilloo A.K., Ochsenhirt S.E., McCarthy J.B. et al. 2001. Adhesion of $\alpha 5\beta 1$ receptors to biomimetic substrates constructed from peptide amphiphiles. *Biomaterials* 22(12): 1493–1505. DOI: [10.1016/s0142-9612\(00\)00304-5](https://doi.org/10.1016/s0142-9612(00)00304-5)
- Fairhurst D., Rowell R.L., Monahan I.M. et al. 2007. Microbicides for HIV/AIDS. 2. Electrophoretic fingerprinting of CD4 + T-Cell model systems. *Langmuir* 23(5): 2680–2687. DOI: [10.1021/la063043n](https://doi.org/10.1021/la063043n)
- Gallardo A., Martinez-Campos E., Garcia C. et al. 2017. Hydrogels with modulated ionic load for mammalian cell harvesting with reduced bacterial adhesion. *Biomacromolecules* 18(5): 1521–1531. DOI: [10.1021/acs.biomac.7b00073](https://doi.org/10.1021/acs.biomac.7b00073)
- Gao T., Li L., Wang B. et al. 2016. Dynamic electrochemical control of cell capture-and-release based on redox-controlled host-guest interactions. *Analytical Chemistry* 88(20): 9996–10001. DOI: [10.1021/acs.analchem.6b02156](https://doi.org/10.1021/acs.analchem.6b02156)
- Keil B., Herout V., Hudlicky M. et al. 1996. *Laboratorní technika organické chemie [Laboratory technique of organic chemistry]*. Praha: Mir. (in Czech)
- Knight C.G., Morton L.F., Peachey A.R. et al. 2000. The Collagen-binding A-domains of integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ recognize the same specific amino acid sequence, GFOGER, in native (Triple-helical) collagens. *Journal of Biological Chemistry* 275(1): 35–40. DOI: [10.1074/jbc.275.1.35](https://doi.org/10.1074/jbc.275.1.35)
- Kurashina Y., Hirano M., Imashiro Ch. et al. 2017. Enzyme-free cell detachment mediated by resonance vibration with temperature modulation. *Biotechnology and Bioengineering* 114(10): 2279–2288. DOI: [10.1002/bit.26361](https://doi.org/10.1002/bit.26361)

Langhans S.A. 2018. Three-dimensional in vitro cell culture models in drug discovery and drug repositioning. *Frontiers in Pharmacology* 9. DOI: [10.3389/fphar.2018.00006](https://doi.org/10.3389/fphar.2018.00006)

Lee J.H., Jung H.W., Kang I.K. et al. 1994. Cell behaviour on polymer surfaces with different functional groups. *Biomaterials* 15(9): 705–711. DOI: [10.1016/0142-9612\(94\)90169-4](https://doi.org/10.1016/0142-9612(94)90169-4)

Lerman M.J., Lembong J., Muramoto S. et al. 2018. The evolution of polystyrene as a cell culture material. *Tissue Engineering Part B: Reviews* 24(5): 359–372. DOI: [10.1089/ten.teb.2018.0056](https://doi.org/10.1089/ten.teb.2018.0056)

Matsuda T., Saito Y., Shoda K. 2007. Cell sorting technique based on thermoresponsive differential cell adhesiveness. *Biomacromolecules* 8: 2345–2349. DOI: [10.1021/bm070314f](https://doi.org/10.1021/bm070314f)

Okano T., Yamada N., Okuhara M. et al. 1995. Mechanism of cell detachment from temperature-modulated, hydrophilic-hydrophobic polymer surfaces. *Biomaterials* 16(4): 297–303. DOI: [10.1016/0142-9612\(95\)93257-e](https://doi.org/10.1016/0142-9612(95)93257-e)

Paoli P., Giannoni E., Chiarugi P. 2013. Anoikis molecular pathways and its role in cancer progression. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* 1833(12): 3481–3498. DOI: [10.1016/j.bbamcr.2013.06.026](https://doi.org/10.1016/j.bbamcr.2013.06.026)

Ruoslahti E. 1996. RGD and other recognition sequences for integrins. *Annual Review of Cell and Developmental Biology* 12(1): 697–715. DOI: [10.1146/annurev.cellbio.12.1.697](https://doi.org/10.1146/annurev.cellbio.12.1.697)

Sekine H., Shimizu T., Sakaguchi K. et al. 2013. In vitro fabrication of functional three-dimensional tissues with perfusable blood vessels. *Nature Communications* 4(1): 1399. DOI: [10.1038/ncomms2406](https://doi.org/10.1038/ncomms2406)

Verma A., Verma M., Singh A. 2020. Animal tissue culture principles and applications. *Animal Biotechnology*: 269–293. DOI: [10.1016/B978-0-12-811710-1.00012-4](https://doi.org/10.1016/B978-0-12-811710-1.00012-4)

Yamato M., Utsumi M., Kushida A. et al. 2001. Thermo-responsive culture dishes allow the intact harvest of multilayered keratinocyte sheets without disperse by reducing temperature. *Tissue Engineering* 7(4): 473–480. DOI: [10.1089/10763270152436517](https://doi.org/10.1089/10763270152436517)

Zakharova N.V., Simonova M.A., Khairullin A.R. et al. 2018. Effect of pH on the behavior of a random copolymer of acrylamide. *Polymer Science A* 60(2): 127–133. DOI: [10.1134/S0965545X18020153](https://doi.org/10.1134/S0965545X18020153)

Zhao X., Wang L., Wang P. et al. 2016. Fabrication of thermoresponsive nanofibers for cell sorting and aligned cell sheet engineering. *Journal of Nanoscience and Nanotechnology* 16(6): 5520–5527. DOI: [10.1166/jnn.2016.11738](https://doi.org/10.1166/jnn.2016.11738)