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Thermoresponsive polymer surfaces and their application in tissue engineering

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Abstract: This short review addresses the synthesis of thermoresponsive polymer surfaces and their application for cell tissue engineering. Four classes of synthetic thermoresponsive polymers are discussed: poly(*N*-isopropylacrylamide)s, poly[oligo(ethylene glycol) methacrylate]s, polyoxazolines and polyethers, most notably, polyglycidol. Synthetic routes leading to thermoresponsive layers on solid support are described. Relationships between structures of the layers and their interactions with cells are analyzed. Chemical (copolymerization or the inclusion of biologically active species) and physical (patterning or the morphology of the surfaces) modifications of macromolecular surfaces are described and their relations to the growth and detachment of cell sheets are reviewed. The application of cell sheets grown and detached from thermoresponsive surfaces to treat diseases is also presented.

Keywords: thermoresponsive surfaces, intelligent polymers, cell culture, tissue engineering, poly(*N*-isopropylacrylamide), poly[oligo(ethylene glycol) methacrylate]s, poly(2-substituted-2-oxazoline)s, polyglycidol.

Termoczułe powierzchnie polimerowe i ich zastosowanie w inżynierii tkankowej

Streszczenie: Praca stanowi przegląd literatury dotyczącej syntezy termoczułych powierzchni polimerowych i ich zastosowania w inżynierii tkankowej. Omówiono cztery klasy termoczułych polimerów: poli(*N*-izopropyloakryloamidy), poli(metakrylany glikoli oligoetylenowych), polioksazoliny i polietery, w tym przede wszystkim poliglicydol. Opisano syntetyczne szlaki prowadzące do termoczułych warstw stabilnie i kowalencyjnie związanych ze stałym podłożem. Przeanalizowano zależności między strukturą warstw a ich oddziaływaniem z komórkami. Omówiono chemiczne (kopolimeryzacja, dołączenie do polimeru biologicznie aktywnego związku) i fizyczne (modelowanie, morfologia powierzchni) modyfikacje powierzchni polimerowych oraz ich związek ze wzrostem i odczepianiem komórek. Przedstawiono również zastosowanie wyhodowanych i odczepionych od termoczułych powierzchni komórek w postaci arkusza w leczeniu różnych chorób.

Słowa kluczowe: termoczułe powierzchnie, polimery inteligentne, hodowla komórek, inżynieria tkankowa, poli(*N*-izopropyloakryloamid), poli(metakrylany glikoli oligoetylenowych), polioksazoliny, poliglicydol.

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The paper addresses the application of thermoresponsive polymer layers for the purpose of cell sheet application in regenerative medicine.

Stimuli-responsive polymers are a class of macromolecular compounds that significantly and reversibly alter their properties under the influence of external stimuli: temperature, pH levels, and magnetic or electric fields. The temperature responsivity of polymers in contact with water has been frequently studied [1, 2].

In most cases, the solubility of polymers increases with increasing temperature. Polymer-solvent systems are however known where the polymer is soluble only below a certain temperature (under the so-called cloud point temperature T_{CP}). When this temperature is exceeded, the solubility is lost and the polymer precipitates in water. The mechanism of this phenomenon is understood. Below T_{CP}, macromolecules are kept in solution through hydrogen interactions involving chain elements and water molecules. When this temperature is exceeded (energy is pumped into the system), there is enough energy to overcome relatively weak hydrogen interactions responsible for the formation the hydration sphere and solubility. Once this hydration sphere is damaged (phenomenologically the order level is decreased while entropy levels increase), inter- and intrachain interactions begin to prevail and the polymer precipitates. This process is reversible.

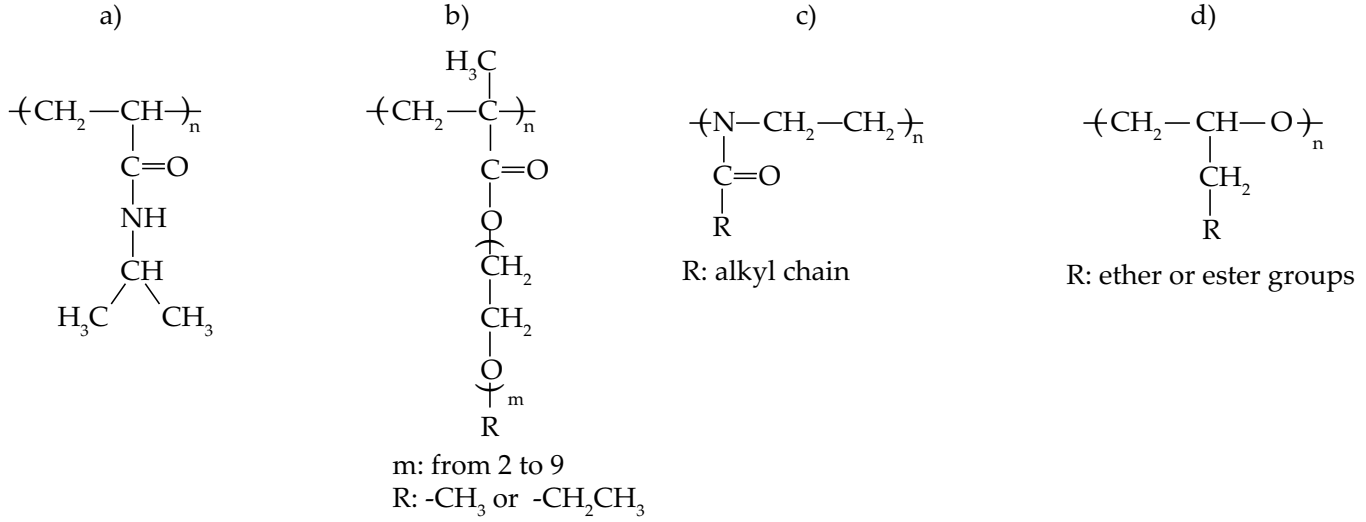
Thermoresponsive macromolecules may also be bound to a solid surface (polymer brushes) or crosslinked to form networks (gels). They still remain thermoresponsive. The observed change in hydrophilicity is in such cases evidenced by changes in the contact angle. At low temperature (below the transition point) the surface is hydrophilic. As the temperature is increased, the chains undergo a transition and the surface becomes hydrophobic.

The last effect offers interesting routes for the application of thermoresponsive polymer surfaces in regenerative medicine and especially in cell sheet engineering. There are many cells which need contact with a solid surface to grow. In most cases, cells attach to and grow on hydrophobic surfaces. Such cells detach and separate from hydrophilic surfaces. Therefore, it is intuitive to grow cells on hydrophobic surfaces – here at temperatures exceeding T_{CP} for the polymer on the surface – and to detach products obtained (cell sheet), rendering the culture substrate hydrophilic by simply lowering the temperature. So prepared cell cultures are suitable for tissue engineering. Of course, temperature changes must fall within a physiologically tolerable range. Most importantly, no enzymes or mechanical scratching tools are needed, thus creating opportunities to culture cell sheets or tissues.

In this paper, classes of thermoresponsive polymers used for cell sheet growth and detachment are discussed (Scheme A).

POLY(*N*-ISOPROPYLACRYLAMIDE)-BASED SURFACES FOR CELL CULTURING

Thermoresponsive surfaces based mainly on poly(*N*-isopropylacrylamide) (PNIPAM) and its copolymers dominate among thermoresponsive polymers for cell culturing. PNIPAM is the most intensively studied thermoresponsive polymer as its cloud point temperature is roughly 32 °C, what makes it attractive for use in medicine and pharmacy. Surfaces with grafted PNIPAM are thermoresponsive – their properties depend strongly on temperature. Pioneering studies on the application of PNIPAM-based support for cell culture and detachment were first published by Yamada *et al*. [3] and Okano *et al*. [4]. Procedures described in those works were called cell sheet engineering. They involved applying electron beam irradiation to graft PNIPAM onto commercially available tissue culture polystyrene (TCPS) dishes and to obtain material for hepatocytes sheet culturing and non-invasive detachment. As an unquestionable advantage of such a method is that the proteins associated with the



Scheme A. General scheme of (co)polymers described in the article: a) (co)poly(*N*-isopropylacrylamide)s, b) (co)poly[oligo(ethylene glycol) methacrylate]s, c) (co)poly(2-substituted-2-oxazoline), d) (co)polyethers based on polyglycidol

cell layer are not destroyed and cell-cell junctions and the extracellular matrix (ECM) are preserved. Thus the cell functioning is maintained [5, 6]. Different cells including corneal epithelia, renal epithelia, oral mucosal epithelia, myocardial, hepatocytes, keratinocytes, chondrocytes and skeletal myoblasts have been cultured on PNIPAM--based surfaces [7–13], and thus therapeutic treatments for animal or clinical models could be performed. Long--term and intensive investigations have led to the development of the commercially available (for research use only) PNIPAM-grafted TCPS named Nunc™ Dishes with UpCell™ Surface.

Below a more in-depth insight into the various techniques used to prepare PNIPAM responsive dishes, into factors influencing cell cultures and into the detachment and application of cell sheets in clinical studies is presented.

Methods used to obtain PNIPAM thermoresponsive substrates

Various approaches to grafting PNIPAM to different surfaces have been developed. The method most commonly used to coat the TCPS substrate with PNIPAM is that of electron beam irradiation [14–16]. Cell adhesion and detachment depend on the grafting density of the PNIPAM layer. Although electron beam facilitate the large-scale production of PNIPAM responsive dishes (UpCell™), this technique entails access to expensive equipment. Thus, several other approaches have been proposed as alternative methods.

Another method involving grafting PNIPAM onto silicon, glass or TCPS is that of vapor-phase plasma polymerization [17, 18]. Unlike those observed for e-beam PNIPAM surfaces, here thickness dependency on cell adhesion is barely observed. This method is advantageous in that the vapor-phase coating process is a one-step and solvent-free process. However, monomer fragmentation leading to the loss of chemical functional groups can appear and this method is also not quite appropriate for large-scale production.

The grafting of PNIPAM onto polystyrene [19], TCPS [20, 21] or polydimethylsiloxane [22] surfaces has also been performed by UV irradiation. Different reactions, *e.g.*, crosslinking through dimerization, photopolymerization or photografting, induced by UV in the presence of photosensitizers have led to the formation of stable PNIPAM layers. It has been shown that cell interactions with the surface are insensitive to polymer layer thickness [20, 21].

PNIPAM and its copolymer can also be grafted to a surface yielding thermoresponsive polymer brushes able for cell culturing. This technique involves surface-initiated living radical polymerization [23]: atom transfer radical polymerization (ATRP) and reversible addition-fragmentation chain transfer radical polymerization (RAFT). *Via* these techniques, well-defined and densely grafted PNIPAM-based (co)polymer brush layers of T_{CP} of 20 to 42 °C (depending on copolymer compositions) have been prepared [24–26]. It has been observed that for the layers obtained the layer thickness and density of polymer brushes affect cell adhesion and detachment.

Factors influencing cell adhesion and detachment

As noted above, many thermoresponsive culture surfaces with immobilized PNIPAM and its copolymers have been generated *via* different synthetic methods. It appears that only some of them are effective at initiating cell adhesion and detachment. The effects of several factors (*e.g.*, polymer layer thickness, grafting density levels, degrees of hydration and dehydration, and chain mobility levels) have been described (for details see [14, 15, 27–30]).

Cell adhesion and detachment processes observed for PNIPAM-based layers are reported to be based on changes in wettability (contact angle measurements) influenced by temperature alterations. However, the magnitude of the difference between $T > T_{CP}$ and $T < T_{CP}$ varies from $\Delta = 6–9°$ [31–33] to 50° [21]. Furthermore, reported angles at which cell adhesion and detachment occur show considerable differences. These findings suggest that while changes in wettability are integral to thermoresponsive layer behavior, other factors such as grafting density and polymer layer thickness influence cell adhesion/detachment.

The thickness of the grafted polymer plays also an important role in the control of cell adhesion/detachment in response to temperature. For PNIPAM layers on TCPS obtained *via* e-beam [32], cell adhesion and detachment are achieved for layers that are 15 nm to 20 nm thick. Though for all investigated PNIPAM surfaces the change in wettability with temperature is observed, for thin PNIPAM layers (of less than 15 nm) cells adhere and proliferate but do not detach whereas for thick coatings (of approximately 30 nm) no cell adhesion occurs. Thickness effects on cell adhesive characteristics have been attributed to interactions between grafted PNIPAM chains at the TCPS interface [32, 34]. Similarly, PNIPAM layers on glass (prepared by e-beam) exhibit polymer thickness dependency on cell adhesion and detachment although optimal polymer thickness is lowered to a range of 3.5–4.8 nm [34, 35]. Additionally, for PNIPAM brushes obtained *via* surface--initiated ATRP or RAFT, thickness dependence on cell culturing and detachment can be noticed. An optimum thickness range was from 10 up to 45 nm [25, 28, 31, 33, 36, 37]. It has been stated that the optimal combination of grafting density and chain length (which influence the final thickness) induces cell attachment and detachment. High polymer density and molar mass disturb cell attachment while excessively low density and chain length values hinder cell detachment. For such situation a mobility of the PNIPAM chains and their hydration is responsible.

Contrary to above discussed data, the thickness of the PNIPAM layer generated by plasma polymerization (ap-

T a b l e 1. Surfaces of PNIPAM copolymers with active species for cell culturing

Polymer	Active species	Cell type	Effect	Ref.
P(NIPAM-*co*-CIPAM)	RGDS	Human umbilical vein endothelial cells	Cell adhesion and proliferation in serum-free media. High peptide content prohibits cell detachment.	[42]
P(NIPAM-*co*-CIPAM)	RGDS	Bovine aortic endothelial cells	Cell adhesion and proliferation in serum-free media. Successful detachment of cell sheets.	[43]
P(NIPAM-*co*-CIPAM)	GnRGDS (n = 1, 6, 12, 16)	Human umbilical vein endothelial cells	Cell adhesion and proliferation in serum-free media depend on n.	[44]
P(NIPAM-*co*-CIPAM-*g*-PEG)	RGDS	Bovine aortic endothelial cells	Cell adhesion and proliferation in serum-free media. Cells did not detach at lower temperatures.	[45]
P(NIPAM-*co*-CIPAM)	RGD, GRGD, RGDS, GRGDS, PHSRN, PHSRN-RGDS, PHSRN-G_6-RGDS	Human umbilical vein endothelial cells	Cell adhesion and detachment depend on peptide sequences, and PHSRN peptides retard cell detachment.	[46]
P(NIPAM-*co*-CIPAM)	Insulin	Bovine carotid artery endothelial cells	Adhesion and proliferation of cells in serum-supplemented media. Successful detachment of cell sheets.	[47]
P(NIPAM-*co*-CIPAM)	Insulin, RGDS	Bovine carotid artery endothelial cells	Cell adhesion and proliferation in serum-free and serum-supplemented media. Successful detachment of cell sheets.	[48]
P(NIPAM-*co*-CIPAM)	Insulin, RGDS	NIH 3T3 fibroblasts	Cell adhesion and proliferation on patterned scaffolds. Successful detachment of cell sheets.	[49]
P(NIPAM-*co*-CIPAM) grafted with heparin	Basic fibroblast growth factor	NIH 3T3 fibroblasts	Thermoresponsive surfaces were able to hold the two to three times the number of cells than a PNIPAM surface with soluble biomolecules or wafers with only physisorbed fibroblast growth factors.	[50]
P(NIPAM-*co*-CIPAM) grafted with heparin	Heparin-binding epidermal growth-like factor (EGF)	Rat hepatocytes	Nearly double the amount of albumin was secreted from hepatocytes on a surface with bound EGF than from those on a surface placed in medium containing soluble EGF.	[51]

RGDS – arginine-glycine-aspartic acid-serine
GnRGDS – n × glycine-arginine-glycine-aspartic acid-serine
GRGD – glycine-arginine-glycine-aspartic acid
GRGDS – glycine-arginine-glycine-aspartic acid-serine
PHSRN – proline-histidine-serine-arginine-asparagine
PHSRN-RGDS – proline-histidine-serine-arginine-asparagine-arginine-glycine-aspartic acid-serine
PHSRN-G_6-RGDS – proline-histidine-serine-arginine-asparagine-6 × glycine-arginine-glycine-aspartic acid-serine

proximately 50–80 nm) does not impact cell attachment [27, 34, 38].

Detailed analyses [14, 15, 27–30, 38] show that only the combination of several factors (*e.g.*, chain mobility, grafting density, wettability, layer thickness, and the substrate on which PNIPAM has been grafted) can generate a material that efficiently promotes cell adhesion. Time needed to detach cells from thermoresponsive surfaces varies from 15 min to 24 hours depending on techniques applied and the thickness of the coating used. The temperature applied for detachment can be dependent on cell types [39, 40]. During detachment, cell metabolic changes occur and thus in addition to the above-mentioned parameters types of cells, culture media and additives used can also affect cell detachment.

T a b l e 2. Thermoresponsive surfaces produced by micropatterning for cell culturing

Thermoresponsive surface	Cell type	Details	Ref.
PNIPAM/P(NIPAM-*co*--butyl methacrylate)	Rat primary hepatocytes and bovine carotid artery endothelial cells	At 27 °C cells adhere only onto P(NIPAM-BMA) co-grafted domains. These domains adsorb serum proteins that facilitate cell adhesion.	[58]
PNIPAM/polyacrylamide	Human umbilical vein endothelial cells and neonatal normal human dermal fibroblasts	Multi-layer constructions of patterned endothelial cell sheets and confluent fibroblast sheets were prepared. Endothelial cell sheet fidelity was maintained within the multi-layered tissue. Microvascular-like networks formed in a multi-layered structure.	[59]
PNIPAM/P(NIPAM-*co*--butyl methacrylate)	Rat primary hepatocytes and bovine carotid artery endothelial cells	Double-layered cell co-culture exhibiting longer survival with the maintenance of cell functions then on TCPS was obtained. Physiological functions of HCs were enhanced by downsizing patterns.	[62]
PNIPAM/P(NIPAM-*b*-*N*--acryloylmorpholine)	Normal human dermal fibroblasts	Cells randomly adhered to the nonpatterned PNIPAM surfaces but adhered site-specifically to the patterned surfaces.	[63]
PNIPAM/polyacrylamide	Neonatal normal human dermal fibroblasts	Fibroblasts first adhered to the PNIPAM area and then invaded the PNIPAM/polyacrylamide domain. An oriented cell sheet was generated.	[64]
PNIPAM/polyacrylamide	NIH 3T3 cells	The temperature detachment of the adhered NIH 3T3 sheet was achieved more rapidly from the patterned surface than from a conventional PNIPAM surface.	[65]
PNIPAM/ P(NIPAM-*b*-*N*-acryloyl-morpholine)	Normal human dermal fibroblasts	A cell monolayer with a well-organized orientation was formed and maintained after thermally induced cell-sheet detachment.	[66]
PNIPAM/ P(NIPAM-*b*-*N*-acryloyl-morpholine)	Human skeletal muscle myoblasts	Cells were aligned on the surface and detached as a single anisotropic cell sheet with decreasing temperature.	[67]
PNIPAM/ P(NIPAM-*b*-*N*-acryloyl-morpholine)	Human skeletal muscle myoblasts, human-induced pluripotent stem cell-derived neurons and human umbilical vein endothelial cells	The seeding of human skeletal muscle myoblasts generated anisotropic cell sheets. Neurons and endothelial cells introduced in a following step allowed for the formation of complex structural networks.	[68]
PNIPAM patterned with fibronectin	Human aortic vascular smooth muscle cells	Fibronectin lanes patterned on PNIPAM promoted a parallel alignment of cells; on pure PNIPAM, area cells were randomly oriented. Cells maintained this configuration on respective substrates.	[60]
PNIPAM patterned with fibronectin	Rat primary hepatocytes and bovine carotid artery endothelial cells	Double-layered co-cultures maintained organization, allowing for cell constructs with tissue-like microarchitectures inside.	[69]
PNIPAM on molded PS support	Adult human aortic smooth muscle cells	Surface microtexturing allowed for the control of cell organization whereas PNIPAM coating allowed for intact cell sheet harvesting. Cell sheet organization was retained.	[61]

Thermoresponsive PNIPAM surface improvements

The introduction of bioactive molecules (peptides, growth factors or antibodies) to thermoresponsive polymer layers can significantly enhance a surface biocompatibility and can allow to control responses to stimulus influencing kinetics of cell adhesion and efficiency of detachment processes.

Works concerning surfaces of thermoresponsive PNIPAM copolymers enriched with active species culturing of chosen cells are shown in Table 1. The NIPAM copolymerization procedure involving 2-carboxyisopropylacrylamide (CIPAM) was developed by Aoyagi *et al*. [41] for this purpose. Reactive carboxylate groups of CIPAM allow for the conjugation of biomolecules to polymeric layers.

Rapid cell sheet recovery is essential to maintaining the biological functioning and viability of cells. To accelerate cell sheet harvesting, several approaches have been proposed [15, 52]. For one method, PNIPAM has been grafted onto a porous poly(ethylene terephthalate) membrane *via* e-beam radiation [53]. Cells detach more rapidly from PNIPAM-porous membranes than from a control surface as water penetrates the cell sheet not only from the periphery of the dish but also through the pores of membrane that is under the attached cell sheet.

To improve further the cell detachment, NIPAM has been polymerized using hydrophilic poly(ethylene glycol) macromonomers (PEG) [54]. Hydrophilic PEG chains form a number of pores for water diffusion and also increase the hydration of the PNIPAM layer, causing cells to detach rapidly. Another means of enhancing cell detach-

ment is based on the use of a polymer layer composed of comb-type grafted PNIPAM gel [55–57]. For this purpose, NIPAM and PNIPAM macromonomers have been grafted onto TCPS *via* e-beam radiation. The presence of more mobile PNIPAM grafts accelerates hydration and thus cell sheet detachment.

To construct co-cultures of cells and/or to achieve their selective attachment, various thermoresponsive surfaces have been prepared by micropatterning. Surface domains with varying responses to temperature were formed by using mask and electron beam irradiation [*e.g.*, 58], *via* photolithography [*e.g.*, 59] or by covering the thermoresponsive surface with a pattern of fibronectin [*e.g.*, 60]. An alternative approach involves grafting the thermoresponsive polymer onto a surface patterned by support molding [61]. Specifications of the micropatterned thermoresponsive surface used for cell culturing are given in Table 2.

Clinical application of cell sheets grown on thermoresponsive PNIPAM surfaces

As noted above various PNIPAM-based surfaces can be successfully applied for the culturing of different kinds of cells and for their detachment in a form of a sheet without enzyme treatment. Harvested cells preserve cell-cell junctions and their extracellular matrix (ECM). However, sheets of cells are rolled up during their detachment from surfaces. To prevent this and to transfer cell sheets grown on thermoresponsive surfaces to desired areas, the special manipulator has been developed [70]. For commercial and clinical applications, an automatic cell sheet apparatus with among others the cell sheet manipulator has also been developed [71]. All these caused that various therapeutic treatments involving animal or clinical models have been performed. Patients with corneal deficiencies have been treated using corneal epithelial cell sheets [7] or autologous oral mucosal cells [72]. It has also been demonstrated that layered myocardial cell sheets can be successfully transplanted into infarcted hearts [73]. Layered skeletal myoblast sheets have also been used to treat cardiomyopathy [74]. Combined therapeutic methods involving the use of autologous oral mucosal epithelial cell sheets have also been carried out on animal models and in clinical investigations to treat esophageal ulceration to prevent stenosis [75, 76]. The treatment of periodontal [77, 78], liver [79] or urinary bladder [80] diseases has also been reported.

THERMORESPONSIVE SURFACES FOR TISSUE ENGINEERING BASED ON POLY[OLIGO(ETHYLENE GLYCOL) METHACRYLATE]S

Poly[oligo(ethylene glycol) methacrylate]s (POEGMA) are an alternative class of thermoresponsive polymers that have attracted considerable interest for their use in biomedical applications [81–83]. This is due to several factors. Most monomers of oligo(ethylene glycol) methacrylates are commercially available and can be easily polymerized *via* controlled radical polymerization techniques (especially ATRP) [82]. The amphiphilic structure of these polymers [the side chain of oligo(ethylene glycol) is responsible for the solubility and formation of hydrogen bonds with water molecules whereas the main chain is responsible for competing hydrophobic interactions] causes many POEGMAs to exhibit thermoresponsive behavior [84–86]. POEGMAs present many advantages relative to commonly used PNIPAM. They are characterized by a narrow phase transition with slight hysteresis. The influence of external factors (pH, polymer or salt concentrations) on corresponding T_{CP} values is not significant. Moreover, POEGMAs are non-toxic, non-immunogenic and FDA (*Food and Drug Administration)* approved polymers [82, 83, 85].

Due to the many advantages of POEGMAs, they are promising in obtaining thermoresponsive surfaces [83]. They can be easily bonded to flat surfaces such as glass, gold titanium or polymers. The synthesis of thermoresponsive POEGMA surfaces can be performed by grafting to and grafting from the surface [87, 88]. Grafting to the surface involves a reaction between end-functionalized polymers and reactive groups present on the surface [89, 90]. For instance, Uhlig *et al*. [89] prepared a thermoresponsive polymer layer by covalent binding disulfide-functionalized poly[2-(2-methoxyethoxy)ethyl methacrylate-*co*-oligo(ethylene glycol) methacrylate] P(MEO_2MA-*co*-OEGMA) to gold. Grafting from the surface is most widely used to obtain POEGMA thermoresponsive surfaces. *Via* this method, the polymerization of monomers is initiated by an initiator that is attached to the surface [91, 92]. Dworak *et al*. [91] obtained poly[tri(ethylene glycol monoethyl ether) methacrylate] P(TEGMA-EE) surfaces *via* the grafting from technique where bromide groups immobilized on silica or glass substrate induced surface initiated atom transfer radical polymerization (SI-ATRP) of TEGMA-EE. It was also demonstrated that post-irradiation grafting using an electron beam can be applied for the preparation of polypropylene covered with P(TEGMA-EE) layers functionalized with short peptide ligands that promote cell adhesion [93]. All of the aforementioned methods generated stable layers.

The temperature responsive nature and biocompatibility of POEGMAs render these polymers suitable for preparing thermoresponsive surfaces for cell culturing and detachment. Good cell adhesion to these substrates is achieved through the appropriate control of their hydrophilic-hydrophobic balance. Cells can be cultured on POEGMA surfaces to form single cells and continuous cell sheets (which are of particular interest), which are detached without using enzymatic methods of cell separation. In Table 3 cases involving the preparation and application of POEGMAs to different surfaces are summarized.

T a b l e 3. POEGMA-based layers used as cell culture support

Polymer	Substrate	Synthesis method	Cell type	Assay*)	Single cell/ cell sheet	Ref.
P(MEO_2MA-*co*-OEGMA)	Gold	ATRP grafting to	L929 fibroblasts	CA (44 h) CD (30 min)	Single cell	[90]
P(MEO_2MA-*co*-OEGMA)	Glass/gold	ATRP grafting to	L929 mouse fibroblasts	CA (2–5 days) CD (30 min)	Single cell	[89]
P(MEO_2MA-*co*-OEGMA)	Si/SiO_2/PEI	SI-ATRP	L929 mouse fibroblasts	CA (2 days) CD (30 min)	Single cell	[94]
P(MEO_2MA-*co*-OEGMA)	Gold	SI-ATRP	L929 mouse fibroblasts, MCF-7 breast cancer cells	CA (4–5 days) CD (30 min)	Single cell	[95]
	Gold	SI-ATRP	L929 fibroblasts	CA (40 h) CD (30 min)	Single cell	[96]
P(MEO_2MA-*co*-OEGMA)	Glass	SI-ATRP	Murine L-929 fibroblasts	CA (2, 6, 24 h)	Single cell	[97]
	Glass/PEI	SI-ATRP	Murine L-929 fibroblasts	CA (24 h) (up to 60 min)	Single cell	[98]
PDEGMA	Gold	SI-ATRP	PaTu 8988t cells	CA (24 h) CD (83 % of cells detached)	Single cell	[99]
P(DEGMA-*co*-HEMA-*co*--$OEGMA_{360}$)	Glass	SI-ATRP	3T3 fibroblasts	CA (24 h) CD (single cell – 15 min; sheet – 180–240 min)	Cell sheet	[92]
P(TEGMA-EE)	Glass	SI-ATRP	Human fibroblasts	CA (2.5, 4, 8, 12, 24, 72 h) CD (40–60 min)	Cell sheet	[91]
P(TEGMA)	PP	Radiation grafting	Human fibroblasts	CA (24 h)	Cell sheet	[93]

*) CA – cell adhesion; CD – cell detachment.

POEGMA-based surfaces have mainly been used to culture different kinds of cells in a form of single cells. This phenomenon has been investigated, for example, in reference to gold surfaces coated with PDEGMA-*co*--POEGMA [89, 95, 96]. Mouse fibroblast and breast cancer cells were seeded onto this surface. Cells became adherent and were cultured from 2 to 4 days at 37 °C. The temperature of the cell culture medium was then decreased to 25 °C and single cell rounding was observed, confirming the occurrence of cell detachment.

However, the most interesting is the possibility to culture cells on POEGMA surface as a continuous sheet and their detachment in intact form by decreasing temperature below the T_{CP} of the polymer. Few publications have addressed this issue [92, 91, 93]. Dworak *et al*. presented a successful detachment of human fibroblasts grown on a glass/silica surface with an immobilized P(TEGMA--EE) layer [91]. Also the grafting of P(TEGMA-EE) onto polypropylene produced layers facilitating the attachment, growth and detachment of fibroblast sheets (within 10 minutes after temperature decreasing) [93]. To transfer such obtained fibroblast sheet to a new culture dish, a SUPRATHEL transfer membrane was applied [100].

Bioactive molecules have been introduced into thermoresponsive POEGMA surfaces by covalent binding [93, 101–104] or have been linked to wafers as separate units [105] (Table 4). The covalent conjugation of biomolecules with POEGMAs has been mainly performed through the reaction of hydroxyl groups present on the ends of side chains of OEGMAs units. Only IKVAVK has been introduced into a POEGMA chain by copolymerization [93].

THERMORESPONSIVE SURFACES FOR INTERACTION WITH CELLS BASED ON POLY(2-SUBSTITUTED OXAZOLINE)S

Another group of thermoresponsive polymers used for the investigation of their interactions with cells is that of poly(2-substituted-2-oxazoline)s (POx). POx, which are often referred to as pseudopeptides, are non-toxic and biocompatible [106] and do not accumulate in tissues [107]. It has been shown that it is possible to obtain POx copolymers with various side chains or end groups and different architectures [108]. Some of them are thermoresponsive, including poly(2-ethyl-2-oxazoline) PEOx (T_{CP} = 60 °C), poly(2-n-propyl-2-oxazoline) PnPOx (T_{CP} = 25 °C) and poly(2-isopropyl-2-oxazoline) PIPOx (T_{CP} = 36 °C) [109]. POx temperature transitions are easily adjustable through copolymerization within a broad temperature range [110]. These features render POx good candidates for the design of thermoresponsive support for cell sheet engineering.

Literature reports on (co)poly(2-substituted-2-oxazoline) surfaces suitable for interactions with cells are quite

T a b l e 4. Surfaces of POEGMA and its copolymers with active species for cell culturing

Polymer	Active species	Cell type	Effect	Ref.
P(OEGMA$_{526}$)	GRGDY	L929 fibroblasts	Enhanced adhesion and proliferation.	[101]
PHEMA P(OEGMA$_{360}$)	GGGRGDS	HUVEC	Differences in adhesion depending on polymer type.	[102]
P(OEGMA$_{360}$)	GRGDS	Mouse fibroblasts MC3TC	Differences in adhesion depending on grafting density.	[103]
P(DEGMA-*co*-HEMA-*co*-OEGMA$_{360}$)	GGGRGDS	3T3 fibroblasts	Cell adhesion and proliferation in serum free media. Successful temperature induced detachment.	[104]
P(TEGMA)	IKVAVK	Human fibroblasts	Adhesion and proliferation of cells.	[93]
P(DEGMA-*co*-OEGMA$_{475}$)	GRGDS	L929 mouse fibroblasts	Detachment of cells was successful and was dependent on temperature.	[105]

GRGDY – glycine-arginine-glycine-aspartic acid-tyrosine
GGGRGDS – glycine-glycine-glycine-arginine-glycine-aspartic acid-serine
GRGDS – glycine-arginine-glycine-aspartic acid-serine
IKVAVK – isoleucine-lysine-valine-alanine-valine-lysine

limited. Poly[2-methyl-2-oxazoline-*co*-(dec-9-enyl)-2-oxazoline] hydrogels enriched with adhesion mediating peptide RGD (arginine-glycine-aspartic acid) have been proposed as support [111–113] for cell culturing. The growth of fibroblasts on hydrogel discs is dependent on peptide levels, which also influence the cell morphology. For the development of 3D tissue culture systems, cells have been incorporated into the hydrogel structure either through their mixing with hydrogel precursors followed by UV-light curing [111, 112] or through the seeding of cells into porous structure of the scaffold [113]. More recently, the Vasilev group studied plasma polymerized poly(2--alkyl-2-oxazoline) thin films with controlled nanotopographical features, which impacted fibroblasts and stem cell adhesion, spreading and proliferation [114, 115]. In all of these works, however, thermoresponsivity was not used for cell culturing and their non-invasive detachment.

The idea of using nanolayers of thermoresponsive polyoxazolines for culturing and temperature-induced detachment has only been reported by Dworak group [116, 117]. Polyoxazoline layers were obtained *via* the grafting to technique through the termination of living cationic polymer chains of PIPOx [116, 117] and poly[(2--ethyl-2-oxazoline)-*co*-(2-nonyl-2-oxazoline)] (PENOx) [117] by active groups of the surface. All polymers exhibited T_{CP} values of 22–37 °C. PIPOx partially crystallized in acetonitrile, the solvent used for polymerization, formed fibrillary structured layers [117]. The thickness of POx layers were in the range of 4 to 11 nm and was dependent on the molar mass of the immobilized polymer and on the number of crystallites on the substrate. All PIPOx- and PENOx-based layers exhibited thermoresponsive behavior; the temperature-induced swelling (below T_{CP}) and shrinking (above T_{CP}) of POx layers with simultaneous changes in water affinity was observed. The surfaces were used for the culturing of fibroblasts. The degree of fibroblast adhesion to PIPOx surfaces was greater than that for PENOx surfaces [116]. For PENOx surfaces, neither the molar mass nor hydrophobic content of nonyloxazoline had significant effects on the adhesion of cells. Fibroblasts adhered to the same extent as that observed in the control sample. However, cell capacities for spontaneous detachment were found to depend on copolymer compositions (PENOx) and on the number of crystallites on the surface (PIPOx). For PENOx layers, full fibroblast detachment was only observed for the roughest surface and when the largest difference in contact angles between the hydrated and dehydrated layers was observed. For other PENOx layers, cells were removed from the substrate only in individual places. The PIPOx layers appeared to be effective at the detachment of a cell sheet. The capacity to detach depended however on the number of crystallites present on the surface – the fewer crystals present the more cells were detached. After lowering the temperature to 20 °C, a continuous fibroblast sheet was detached from the PIPOx with crystallite content up to 40 %, within 30 minutes [117].

POLYGLYCIDOL-BASED THERMORESPONSIVE SURFACES

Thermoresponsive polyglycidol (PGL) derivatives have been used as bioactive substrates in tissue engineering. Interactions with cells have been studied for thermoresponsive cryogels based on various hydrophobically modified high molar mass polyglycidol precursors of M_n = 1 250 000 g/mol with phase separation temperature equal to 25 °C [118]. Cryogels have been formed by photocrosslinking of a mixture of thermoresponsive poly(glycidol-*co*-ethyl glycidyl carbamate)s/photosensitizer after its freezing. Gels of high mechanical strength did not degrade during irradiation and importantly responded very quickly (within 20–25 seconds) to temperature changes. Obtained cryogels were hydrophobic un-

der cell culture conditions (37 °C) and could, therefore, promote fibroblast adhesion and proliferation.

Polymer layers based on poly(glycidol-*co*-ethyl glycidyl carbamate)s have also been applied to investigate their interactions with skin cells (fibroblasts and keratinocytes) [119]. Polymer layers of 20 nm to 60 nm thick were obtained using a grafting to technique. A change in their affinity to water with an increase in temperature was observed. The number of cells adhered to the modified polyglycidol surfaces depended on polymer layer thickness, same as for PNIPAM layers obtained *via* e-beam [32]. The fibroblasts were able to form a cell sheet monolayer on the studied surface only at low polymer layer thicknesses of up to 28 nm while the greatest thickness caused the formation of a discontinuous cell sheet. Both cell-free places and clusters of cells were observed likely due to the heterogeneity of the polymer layer. Keratinocytes were less likely to adhere than the fibroblasts. The addition of laminin (the main component of the intracellular matrix) to the culture medium enhanced the adhesion of keratinocyte and allowed for proliferation onto PGL-based surfaces.

Haag *et al*. [120–122] reported on sulfur-containing statistical copolymers composed of glycidyl methyl ether (GME) and glycidyl ethyl ether (EGE) with their thermoresponsiveness strongly affected by comonomer ratios. The polymers were synthesized by anionic ring opening polymerization with the use of protected sulfur-containing initiators or by postmodification reactions with thio reagents. Subsequently, sulfur anchoring groups of copolymers were used for the preparation of self-assembled monolayers on gold substrates. Fibroblast adhesion and detachment from these monolayers were compared with that of commercially available conventional TCPS and with PNIPAM dishes. Cell culture experiments showed that complete fibroblast sheets were formed on monolayers of thermoresponsive copolyethers with GME : EGE comonomer ratios of 1 : 3 and 1 : 5, confirming the effect of substrate hydrophobicity on this process. Conventional TCPS dishes show comparable cell culturing times as those of polyglycidol surfaces while the adhesion, proliferation and detachment of cells on commercial PNIPAM dishes occur significantly slower than on obtained layers.

CONCLUSIONS

Progress in regenerative medicine requires that implanted tissues become more readily available. The process of culturing tissues is demanding and not fully understood. Culture substrates exhibiting switchable affinity to cells are currently the tool of choice. Some synthetic polymers have the required properties. They are suitable for cell and tissue culturing at higher temperatures and permit easy detachment simply by lowering the temperature. Four classes of synthetic polymers have been so far studied: PNIPAM, a "gold standard" for thermoresponsive polymers, POEGMAs, new and biocompatible thermosensitive materials, polyoxazolines, the "pseudopeptides", and polyethers, among them polyglycidol. Studies reveal basic relations between the chemical nature of a polymer, its morphology, its modification, *e.g.*, through the addition of biologically active compounds, and the suitability of layers for cell sheet growth. The problem, very promising by itself, is however far from being perfectly understood and solved. Further studies must be conducted to address fundamental application requirements.

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