Thermoresponsive thin hydrogel-grafted surfaces for biomedical applications

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Abstract

Thin poly(N-isopropylacrylamide) (PIPAAm) hydrogels were introduced onto biomaterials surfaces for accelerating the kinetics of the swelling and shrinking for PIPAAm hydrogels in response to temperature changes. Thin PIPAAm hydrogels on the biomaterials surfaces exhibit rapid and reversible phase transitions and act as switching sequences to regulate the interaction between the surfaces and biological materials by external temperature-induced changes. By utilizing the temperature-dependent changes of thin PIPAAm hydrogels grafted onto surfaces, our laboratory has pursued unique approaches for developing useful biomedical materials as new types of chromatographic matrices and cell culture surfaces.

Aqueous thermoresponsive chromatography systems using PIPAAm-grafted stationary phases enable us to separate biomolecules with high biological activity. Additionally, thermoresponsive cell culture surfaces allow for the recovery of confluent cell monolayers, which have been clinically applied to ophthalmological treatments, dilated cardiomyopathy, esophageal ulcerations, periodontal disease, and cartilage injury. Furthermore, next-generation thermoresponsive cell culture surfaces for large-scale cell cultivation and the capture of specific cells have been considered a key technology for expanding small quantities of stem cells and isolating the resulting differentiated cells for therapeutic use.
1. Introduction

Stimuli-responsive polymeric hydrogels exhibit phase transitions in volume in response to external stimuli such as solvent components [1], pH [2], light [3], chemical species [4], and temperature [5]. In biomedical applications, much attention has been paid to thermoresponsive cross-linked poly(N-isopropylacrylamide) (PIPAAm) hydrogels, exhibiting temperature-dependent changes in volume in aqueous media. PIPAAm hydrogels have been investigated as drug delivery carriers for stimuli-responsive drug delivery systems [6] and as biomimetic actuators [7]. PIPAAm exhibits a reversible temperature-dependent phase transition in aqueous solutions at the transition temperature of approximately 32 °C, which is called the lower critical solution temperature (LCST) [5, 8]. In the case of macroscopic PIPAAm hydrogels, however, a sudden increase in temperature such as immersion in hot water above the LCST induced the formation of densely shrunken layer on the outermost of PIPAAm hydrogels, called skin layers (Figure 1A) [9]. The skin layer hampers the water penetration from inside the hydrogels to the outer environments, resulting in a very slow kinetics of the shrinking.

For accelerating the kinetics of the swelling and shrinking of PIPAAm hydrogels, our laboratory has conducted several strategies to avoid skin layer formation by tailoring molecular architectures at the molecular level such as the introduction of free mobile PIPAAm chains [10] or hydrophilic moieties [11] into the PIPAAm hydrogel networks. By contrast, the downscaling of PIPAAm hydrogels was also expected to exhibit the rapid response times. In general, the relaxation time of the swelling and shrinking of conventional hydrogels is proportional to the square of the characteristic hydrogel length [12]. Typically, the relaxation time of phase transition is $10^7$ s for a spherical hydrogel of 1-cm diameter, but only $10^{-1}$ s for that of
1-μm diameter, assuming that the collective diffusion coefficient of the hydrogels is on the order of 10⁻⁷ cm²/s [13].

When thin thermoresponsive hydrogel layers are grafted onto material surfaces, the grafted hydrogels show rapid and reversible temperature-dependent soluble/insoluble changes due to the hydration/dehydration of N-isopropylacrylamide side chains, resulting in hydrophilic/hydrophobic surface property changes [14]. Thus, the interaction between the thermoresponsive surfaces grafted with thin PIPAAm hydrogel and biological structures can be rapidly regulated by external temperature changes (Figure 1B). In addition, the thermoresponsive property of thin PIPAAm hydrogel layers is used to switch its functions as a temperature-dependent charge density (Figure 1C) and affinity interaction (Figure 1D).

Our laboratory has pursued unique approaches to utilizing thermoresponsive surfaces in useful biomedical applications. Thermoresponsive surfaces have been applied to new types of chromatographic matrices and cell culture surfaces. Thermoresponsive chromatographic matrices have been used to separate biologically active biomolecules in solely aqueous media [15]. Aqueous chromatographic systems using PIPAAm-grafted surfaces facilitate control over the retention time of biomolecules toward grafted PIPAAm as stationary phases by changing the external column temperature. Moreover, PIPAAm-grafted cell culture surfaces have exhibited temperature-dependent changes in cell detachment/attachment across the LCST [16, 17]. Confluently cultured cells on PIPAAm-grafted cell culture surfaces can be harvested as monolayers, called “cell sheets,” by lowering the culture temperature from 37°C to 20°C without the use of digestive enzymes or chelating agents. Our unique approach, referred to as “cell sheet engineering,” has been used to construct transplantable tissues composed exclusively of cells and three-dimensionally layered
tissues [18].

This study provides brief overviews of biomedical applications using thermoresponsive surfaces for bioseparation and cell-sheet-based tissue engineering. Moreover, the design of the next generation of thermoresponsive surfaces is also reviewed.

2. Thermoresponsive surfaces for bioseparation

2.1. Thermoresponsive chromatography

Chromatographic techniques are widely used for separation of biomolecules using columns, which are selected according to the size, hydrophobicity, ionicity, affinity of the target biomolecules [19]. Reversed phase chromatography (RPC), in which the interaction (partitioning) between the stationary phase and solutes is controlled by changing the polarity of the mobile phase, is commonly used as an effective separation tool, particularly in pharmaceutics and biochemistry [20]. RPC, however, has limited applications because solute bioactivity (particularly for proteins and peptides) is frequently compromised by the use of organic solvent mobile phases.

A novel aqueous chromatographic system that regulates hydrophobic and/or ionic interactions (partitioning) with solutes through external temperature changes was achieved by grafting thermoresponsive PIPAAm chains onto the surface of the device as the stationary phase (Figure 1B, C). Furthermore, temperature-induced conformational changes in the PIPAAm stationary phase will regulate the affinity of interactions between the functionalized surface and target biomolecules based on masking and forced-release effects (Figure 1D). This system has the advantage of preserving analyte bioactivity and has a low environmental impact (e.g., no organic-mobile disposal issue). It is important to note that the retention of
biomolecules is readily modulated simply by changing the external column temperature.

2.2. Thermoresponsive hydrophobic interaction chromatography

Our group collaborated with Kanazawa's group proposed the effectiveness and characteristics of thermoresponsive hydrophobic interaction chromatography in solely aqueous media [21]. The separation of hydrophobic steroids with different hydrophobicities was achieved on the surface of PIPAAm-grafted silica beads measuring 5 µm in diameter that were used as the high performance liquid chromatography (HPLC) packing material. While overlapping steroid chromatograms were observed at lower temperatures, the retention times of the steroids increased with temperatures above the LCST of PIPAAm, leading to highly resolved chromatograms. The force driving steroids toward PIPAAm-grafted stationary phases is mainly due to hydrophobic interactions (Figure 1B). The retention of hydrophobic solutes was enhanced by increasing the hydrophobicity of the thermoresponsive stationary phase, into which a hydrophobic co-monomer, n-butyl methacrylate (BMA), was introduced through copolymerization [22]. Additionally, the hydrophobic interaction between the grafted surfaces and solutes was amplified on the surface of thin PIPAAm hydrogel layers due to the enhanced partitioning of hydrophobic solutes toward the cross-linked PIPAAm layers (Figure 1B) [14]. Recently, PIPAAm brushes on the surface of column matrices were prepared by surface-initiated atom transfer radical polymerization (SI-ATRP), which facilitates the variation of the density and/or molecular weight of PIPAAm brushes by changing the density of the ATRP initiator and the ATRP duration [23, 24].

The use of polymeric beads as a base column matrix is useful because silica
bead matrices are unstable under alkaline conditions due to the hydrolysis of silica. Recently, porous cross-linked polystyrene [25, 26] and hydroxylated cross-linked polymethacrylate beads [27] were used as column matrices to improve stability under an all-aqueous mobile phase. A PIPAAm-grafted polymeric bead column helped enhance the retention of basic peptides, bradykinins, by increasing the pH level of the mobile phases due to the increasing deprotonation of basic amino acids in bradykinin; this led to an increase in the hydrophobicity of the peptides [26].

2.3. Thermoresponsive ionic interaction chromatography

Most bioactive molecules, such as drugs, peptides, and proteins, contain ionic moieties on their surfaces. Thus, the effective retention and separation of ionic biomolecules can be achieved by introducing the opposite ionic moiety into thermoresponsive stationary phases.

An anionic poly(IPAAm-co-acrylic acid-co-tert-butylacrylamide) (poly(IPAAm-co-AAc-co-tBAAm)) thin hydrogel on the surface of silica beads was used as the stationary phase to separate positively charged biomolecules in an aqueous mobile phase [28-30]. With increasing column temperature, the collapse and dehydration of thermoresponsive thin hydrogels on the surface prevented carboxyl groups within the polymer network from accessing the hydrophilic environment in the mobile phase, leading to an increase in the apparent pK\textsubscript{a} [28, 29] and eventually the reduction of surface charge densities (Figure 1C). The separation of catecholamines [28, 29] and basic bioactive peptides (angiotensin subtypes I, II, and III) [30] was achieved by using thermoresponsive aqueous chromatography on anionic poly(IPAAm-co-AAc-co-tBAAm) thin hydrogels as the stationary phases. The retention behavior of angiotensins can be dramatically modulated by applying
stepwise column temperature gradients, leading to a reduction in the duration of separating operations [30]. In addition, cationic poly(IPAAm-co-N,N-dimethylaminopropylacrylamide-co-BMA) (poly(IPAAm-co-DMAPAAm-co-BMA)) thin hydrogels were also applied to the column matrix to separate adenosine nucleotides (AMP, ADP, and ATP) [31] and oligonucleotides [32] in the aqueous mobile phase.

In contrast, with the aqueous mobile phase possessing the ionic strength of a buffer, the adsorption/desorption behavior of proteins on the ionic thermoresponsive surfaces was different from that of small biomolecules. Albumin was strongly adsorbed on the cationic thermoresponsive copolymer-grated stationary phases due to both enhanced electrostatic interaction with the cationic copolymer at low buffer salt concentrations at pH 7.0 and increased hydrophobic interaction with the dehydrated copolymer at high temperatures [33]. The enhancement in the electrostatic interaction was due to an increase in the Gibbs-Donnan effect at lower ionic strengths. Another plasma protein, γ-globulin, was not able to adhere to the surfaces at all the temperatures because γ-globulin is almost neutral at pH 7.0. With decreasing temperatures, captured albumin was eluted from the column because the expansion of the cationic copolymer chains reduced the extent of hydrophobic interaction and hampered albumin’s access to cationic moieties by steric hindrance. Additionally, on anionic thermoresponsive copolymer-grated stationary phases, lysozymes adhered strongly at higher column temperatures [34], exhibiting a retention behavior similar to that of the cationic thermoresponsive column. Purified lysozyme was isolated from whole egg white through the anionic thermoresponsive column by using stepwise temperature gradients.
2.4. Thermoresponsive affinity chromatography

By utilizing temperature-induced conformational changes in PIPAAm molecules, a novel concept of affinity regulation based on masking and forced-release effects was proposed (Figure 1D). PIPAAm chains and Cibacron Blue (CB) molecules, which have an affinity toward albumin, were independently immobilized onto column matrix surfaces [35]. Albumin may easily access immobilized CB ligands in the collapsed state of PIPAAm chains above the LCST, but it is hampered by the steric hindrance of the expanded PIPAAm chains below the LCST (i.e., masking effect). Through chromatographic analyses using PIPAA/CB coimmobilized beads as a column matrix, the binding capacity of albumin below the LCST of PIPAAm was shown to be significantly more reduced than that above the LCST. Furthermore, the captured albumin can be released from the matrix surface by lowering the temperature to below the LCST of PIPAAm (i.e., forced-release effect) while maintaining the properties of the mobile phase, e.g., pH and ionic strength.

By utilizing thermoresponsive affinity chromatography, the selective recovery of human albumin from human serum was achieved. By using the same concept, a thermoresponsive affinity chromatographic matrix, on which both *Ricinus communis* agglutinin (RCA120) lectin and lactose were co-immobilized within PIPAAm molecules, facilitated the temperature-regulated elution of the glycoprotein asialotransferrin [36].

Recently, the targets to be separated by using thermoresponsive surfaces have been extended to mammalian cells. Development of novel technologies for purifying specific cells without any labeling and modification is required for implanting the purified cells safely. Separation system using thermoresponsive surfaces would be useful because of the noninvasive recovery of the target cells. Currently, our laboratory has focused on the design of thermoresponsive polymer-grafted surfaces
to efficiently separate cells and the development of separation system such as chromatographic and microfluidic platforms.

3. Thermoresponsive surfaces for cell culture

3.1. Thermoresponsive cell culture dish

In the conventional cell culture method, cells proliferate and grow on the surface of a tissue culture polystyrene (TCPS) dish. Cultured cells are harvested by treating with trypsin to digest extracellular matrices (ECMs) and by chelating Ca$^{2+}$ ions to disrupt cell-cell junctions, followed by subculturing the cells onto another TCPS dish. These procedures degrade plasma membrane proteins and deposited ECMs, leading to a reduction in cell viability [17].

Over 20 years ago, our group proposed a new concept for cell culturing using thermoresponsive cell culture surfaces, on which cross-linked thin PIPAAm hydrogels are covalently grafted, allowing cultured cells to be recovered by lowering the temperature below the LCST [16, 17, 37]. In contrast with the conventional cell culture method, the detachment of cells from the surface of a PIPAAm-grafted thermoresponsive cell culture dish involves a unique process. At 37°C, various types of cells adhere to and proliferate on the thermoresponsive cell culture surface, which is hydrophobic due to the dehydration of the grafted PIPAAm above the LCST. When the culture temperature is decreased to 20°C, the PIPAAm-grafted surface becomes hydrophilic, resulting in the detachment of cultured cells. Cross-linked PIPAAm-grafted cell culture surfaces were prepared by irradiating a uniformly spread IPAAm monomer solution on TCPS dishes with an electron beam [16, 17]. Using this technology, a thermoresponsive TCPS dish called UpCell™ has been marketed globally by Thermo Fisher Scientific under the Nunc™ brand.
3.2. Cell-sheet-based tissue engineering

Tissue engineering, a concept proposed by Langer and Vacanti [38], has been widely accepted as a promising methodology for the \textit{in vitro} reconstruction of three-dimensional tissues and the \textit{in vivo} regeneration of damaged tissues and organs. In typical tissue engineering, the cells are seeded in or on biodegradable materials, which serve as temporary scaffolds and promote the reorganization of the cells to form a functional tissue. However, this approach has fundamental limitations, such as host inflammatory responses to the implanted scaffolds during their degradation [39].

By contrast to conventional approaches, our group has developed a novel method to prepare a cell sheet using a thermoresponsive cell culture surface and to build three-dimensional tissues \textit{in vitro} by stacking cell sheets [18]. A unique approach called “cell sheet engineering” has been used to construct ideal transplantable tissues composed exclusively of cells. Confluently cultured cells on the surface of hydrophobic PIPAAm-grafted TCPS at 37°C could be detached as a single cell sheet when the culture temperature was decreased to 20°C [40]. Detached cell sheets preserved the membrane proteins and ECMs beneath the cell sheet and cell-cell junctions because the cell sheets were recovered without the use of any enzymatic proteolysis treatment. Fluorescent labeling reveals that the recovered cell sheet retains typical ECMs such as fibronectin [40] and type IV collagen [41] beneath the cell sheets. ECM proteins such as fibronectin and type IV collagen are known to be major components of the basement membrane that underlies the epithelium, facilitating the transplantation of the cell sheets on the surface of the body. Furthermore, layering cell sheets facilitates the formation of
thick tissues that exhibit spontaneous pulsation of the layered cardiac cell sheets; such tissues are easily visible to the naked eye [42].

3.3. Cell-sheet-based clinical applications

The clinical parameters for the application of cell sheet engineering technology in regenerative treatments have been successfully created. The current status of clinical applications using cell-sheet-based regenerative treatment is summarized in Table 1.

Our first clinical research study involved the treatment of patients with burn injuries or scars using cultured epidermal keratinocytes [43]. A successful clinical study was conducted on the treatment of patients with unilateral or bilateral total corneal stem-cell deficiencies resulting from alkali burns or Stevens-Johnson syndrome [44]. An autologous oral mucosal epithelial cell sheet was transplanted on the ocular surface of patients with total corneal stem-cell deficiencies. In France, a clinical trial involving the treatment of a bilateral limbal stem-cell deficiency was conducted by the transplantation of autologous oral mucosal epithelial cell sheets [45]. Twenty-five cases with a one-year follow-up revealed that the treatment was safe and effective; only two patients experienced serious adverse events classified as not product-related. Based on these results, an application for marketing authorization has been submitted to the European Medicines Agency (EMA).

For the treatment of dilated cardiomyopathy (DCM) or ischemic heart disease, clinical research has begun, using autologous skeletal myoblast sheets. In the case of a 56-year-old male suffering from idiopathic DCM, autologous skeletal myoblast sheet transplantation on the surface of his dilated heart improved the patient’s clinical condition: the patient showed no arrhythmia, discontinued the use of a left ventricular
assist device (LVAD), and avoided cardiac transplantation [46]. Cytokines secreted from transplanted myoblast sheets were considered to induce the reduction of fibrosis, angiogenesis, and the recruitment of hematopoietic stem cells; however, myoblasts cannot differentiate into cardiomyocytes. Now, a Japanese medical device company, Terumo Corporation, is planning to begin a clinical trial to treat patients suffering from ischemic heart disease by transplanting autologous skeletal myoblast sheets.

Other clinical studies using cell sheet engineering have also been carried out for the treatment of esophageal ulcerations, periodontal disease, and cartilage injury. To treat esophageal ulcerations after the removal of a superficial neoplasm and to prevent stenosis, autologous mucosal epithelial cell sheets were transplanted [47]. Periodontal treatment using periodontal ligament-derived cell sheets has been applied to regenerate damaged periodontal support structures [48]. Chondrocyte sheet transplantation onto the knee joints has been applied in treatments to repair and regenerate articular cartilage [49]. Preclinical investigations using cell sheet engineering to create an air leak sealant for the lungs [50] and generate liver tissue [51] and pancreatic islets [52] have been conducted in animal models and summarized in other reviews [18, 53].

4. Next-generation thermoresponsive cell culture dishes

Successful cell-sheet-based therapies have been developed for the treatment of the superficial layer of tissues and organs due to the ease of transplantation. Our attention has recently shifted to address some challenging issues in tissue engineering. One of these issues concerns the expansion of a few cells (e.g., stem cells) to a large number of cells and their differentiation into desired cells. Furthermore, the expanded cells should be purified to clinically safe grades. To
overcome these challenges, our laboratory has designed the next generation of thermoresponsive cell culture surfaces.

4.1. Thermoresponsive microcarriers for large-scale cultivation

To produce a wide variety of recombinant proteins, including antibodies, vaccines, and enzymes, the large-scale cultivation of mammalian cells has been used in biotechnological and clinical applications. Recently, large-scale culture systems have been applied to the expansion of undifferentiated cells, such as human mesenchymal stem cells (hMSCs) and human embryonic stem cells (hESCs) on microcarriers (MCs) [54] and to embryoid body (EB) formation [54, 55]. Cell culture systems using MCs represent a promising way of expanding these cells to obtain sufficient quantities for the practical applications of tissue engineering and cell therapy. In the MC culture system, cells grow as a monolayer on the MC surface and are harvested by digesting the ECMs using trypsin during the subculturing process. However, repeated trypsin treatments may damage the cell membrane, resulting in a reduction in cell viability.

Recently, our laboratory developed a novel technology for large-scale cell cultivation in which the cultured cells can be harvested simply by lowering the temperature of the surface of PIPAAm-immobilized MCs [56, 57]. PIPAAm was grafted onto MCs by using SI-ATRP on the surface of chloromethylated polystyrene beads. By using the thermoresponsive MCs, Chinese hamster ovary (CHO-K1) cells were expanded by a factor of approximately 50 in a 7-day stirred suspension culture at 37°C. Furthermore, the cultured cells were harvested by lowering the temperature to 20°C; a harvest efficiency of 76.1±16.3% was attained by optimizing both the amount of grafted PIPAAm and the bead diameter.
The introduction of quaternary amino monomer (3-acrylamidopropyl trimethylammonium chloride; APTAC) into the grafted PIPAAm chains on the bead surfaces improved both the efficiency of cell detachment from the bead surfaces and cell proliferation [58]. Repulsive electrostatic interactions among positively charged PIPAAm copolymer-grafted bead surfaces facilitated the dispersion of MCs in the cell culture medium at 37°C, resulting in enhanced cell proliferation relative to a system featuring nonionic PIPAAm-grafted beads. In addition, the efficiency of cell detachment from the surface of the positively charged MCs after reducing the temperature to 20°C was also increased, presumably due to the enhanced hydration caused by introducing positively charged APTAC moieties. Thus, large-scale cell cultivation systems using thermoresponsive MCs have significant potential in expanding cell quantities for therapeutics without any proteolytic treatments.

4.2. Affinity regulation between cells and surface-immobilized ligands on thermoresponsive cell culture surfaces

Expanded human pluripotent stem cells such as hESCs and human-induced pluripotent stem cells (hiPSCs) in an undifferentiated state can differentiate into many types of cells, including cardiac cells. To obtain differentiated cells with high efficiency, expansion/differentiation methods, including the use of suspension cultures of EBs in bioreactors [55] and utilizing synthetic compounds to promote the differentiation of stem cells into desired cells [59], have been thoroughly investigated. Moreover, the obtained cells should be highly purified and contain no undifferentiated cells to satisfy clinical safety requirements. Currently, the most widely used methods for separating and purifying specific cells are flow cytometry and the magnetic-activated cell sorter system (MACS) technique. However, these methods
have several disadvantages, such as the requirement of a labeling procedure using a fluorescent dye-conjugated antibody. Thus, the development of labeling-free procedures for separating and purifying target cells with high efficiency is required to develop regenerative therapies that use stem cells as a source.

To regulate specific cell adhesion, our laboratory developed antibody-immobilized thermoresponsive cell culture surfaces. Anti-human CD90 antibody-immobilized thermoresponsive cell culture surfaces were prepared as a model to examine the temperature-dependent regulation of the affinity of interactions between CD90-positive cells and immobilized antibodies [60]. Thymic carcinoma cells (Ty-82) and neonatal normal human dermal fibroblasts (NHDFs), which express CD90 on their cellular membranes, were used as models. Both cells selectively adhered to anti-human CD90 antibody-immobilized thermoresponsive surfaces. Moreover, the cells were detached from the surfaces by lowering the temperature to 20°C and by applying additional forces, such as those induced by pipetting and contraction forces caused by shrinking the cell sheets. These results indicate that hydrated PIPAAm chains are able to diminish the affinity of interactions between immobilized CD90 antibodies and cell surfaces with a decrease in temperature.

A separation system using antibody-immobilized thermoresponsive surfaces would be useful for the noninvasive purification of specific cells because the recovered cells are free from any labeling agent such as antibodies. Although the system using a cell culture dish is unsuitable for large-scale cell separation, this problem will be overcome by incorporating, for example, a chromatographic system and microfluidic channels. Moreover, more precise separation and purification of cells can be achieved by controlling the flow of shear stress within the column or microfluidic channels.
5. Conclusions

This review introduced thermoresponsive thin hydrogel-grafted surfaces for chromatographic stationary phases, cell culture surfaces, and the application of these surfaces in cell-sheet-based tissue engineering. Aqueous thermoresponsive chromatography systems utilizing PIPAAm-grafted surfaces as stationary phases facilitate the separation of biomolecules with high biological activity. Furthermore, thermoresponsive cell culture surfaces allow for the recovery of confluent cell monolayers as contiguous living cell sheets. The biological activity of biomolecules and cells recovered from thermoresponsive PIPAAm-grafted surfaces can be preserved because the phase transition of grafted PIPAAm in aqueous media occurs at the LCST of 32°C, which is close to the natural temperature of the human body. We have already applied cell sheet engineering in clinical settings for the treatment of ophthalmological disease, dilated cardiomyopathy, esophageal ulcerations, periodontal disease, and cartilage injury. The development of next-generation thermoresponsive cell culture surfaces for large-scale cell cultivation and the capture of specific cells have the potential to expand small quantities of stem cells and to isolate the differentiated cells for therapeutic use.

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References


Cells, 23 (9), 1333-1342.


Table 1. Clinical applications using cell-sheet-based therapies.

<table>
<thead>
<tr>
<th>Tissue/organ</th>
<th>Target illness</th>
<th>Cell sheets</th>
<th>Implementation site (country)</th>
<th>Current status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corneal epithelium</td>
<td>Limbal stem-cell deficiency</td>
<td>Corneal limbal-derived cell sheet</td>
<td>Osaka University (Japan)</td>
<td>Started in 2003 [44]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Oral mucosal epithelial cell sheet</td>
<td>Les Hospices Civils de Lyon (France) Collaborator: Cellseed, Inc. (Japan)</td>
<td>Finished European clinical trial (25 cases with one-year follow-up [45]), and applied to the European Medicines Agency (EMA) for marketing authorization</td>
</tr>
<tr>
<td>Myocardium</td>
<td>Severe cardiac disease (e.g., ischemic heart disease, dilated cardiomyopathy)</td>
<td>Skeletal myoblast sheet</td>
<td>Osaka University (Japan)</td>
<td>Started in May 2007 [46]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Terumo Corporation (Japan) Collaborator: Cellseed, Inc. (Japan)</td>
<td>Japanese clinical trial in preparation</td>
</tr>
<tr>
<td>Esophagus</td>
<td>Prevention of stenosis after endoscopic submucosal dissection of esophageal cancer</td>
<td>Oral mucosal epithelial cell sheet</td>
<td>Tokyo Women’s Medical University (Japan)</td>
<td>Started in April 2008 [47]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Karolinska Institute (Sweden)</td>
<td>Approved by ethical committee, and in preparation</td>
</tr>
<tr>
<td>Periodontal ligament</td>
<td>Periodontal disease</td>
<td>Periodontal ligament-derived cell sheet</td>
<td>Tokyo Women’s Medical University (Japan)</td>
<td>Started in October 2011</td>
</tr>
<tr>
<td>Cartilage</td>
<td>Knee cartilage injury</td>
<td>Cartilage cell sheet</td>
<td>Tokai University (Japan)</td>
<td>Started in November 2011</td>
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</tbody>
</table>
Figure 1. Schematics showing the kinetics of the shrinking for (A) macroscopic PIPAAm hydrogel and (B-D) thin PIPAAm hydrogel-grafted surfaces when the temperature is increased from 20 to 37 °C.