19 Design of Temperature-Responsive Cell Culture Surfaces for Cell Sheet-Based Regenerative Therapy and 3D Tissue Fabrication

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Abstract

This chapter describes the concept of "cell sheet engineering" for the creation of transplantable cellular tissues and organs. In contrast to scaffold-based tissue engineering, cell sheet engineering facilitates the reconstruction of scaffold-free, cell-dense tissues. Cell sheets were harvested by changing the temperature of thermoresponsive cell culture surfaces modified with poly(*N*-isopropylacrylamide) (PIPAAm) with a thickness on the nanometer scale. The transplantation of 2D cell sheet tissues has been used in clinical settings. Although 3D tissues were formed simply by layering 2D cell sheets, issues related to vascularization within 3D tissues and the large-scale production of cells must be addressed to create thick and large 3D tissues and organs.

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Keywords

Temperature-responsive polymer · Poly(*N*-isopropylacrylamide) · Tissue engineering · Cell sheet · Regenerative medicine

19.1 Cell Sheet Engineering Using Temperature-Responsive Cell Culture Surfaces

Biological tissues and organs form their own shapes and exhibit specific functions by assembling multicellular cells with extracellular matrices (ECMs). The concept of "tissue engineering" [42] is emerging as a means to generate three-dimensional (3D) cellular tissues *in vitro* and to utilize them for replacement of damaged tissues. In typical tissue engineering, natural or artificial scaffolds composed of biodegradable materials are temporally used to form the shape of desired tissues. After implantation, sacrificial scaffolds in the engineered tissues should be ideally replaced with host ECMs and cells. To date, several biodegradable scaffolds, including porous synthetic polymers [43] and hydrogels [11], have been proposed for matching physical properties (e.g., the elastic modulus), exhibiting mass transport properties (e.g., the diffusion of oxygen and nutrients), cellular adhesiveness to scaffolds, and cellular signaling ligands.

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By contrast, artificially assembling cells into 3D tissues without scaffolds offers fundamental advantages in forming tight connection among cells (e.g., the formation of gap junctions in myocardium) [21] and in preventing unfavorable host responses to biomaterials (e.g., inflammatory reactions accompanied by the degradation of scaffolds) [95]. Two-dimensional cellular tissues referred to as "cell sheets" resemble native tissue structures that maintain cell-cell binding as well as adhesive proteins on the basal side. For example, epithelial tissues, including epidermal, esophageal, stomach and intestinal tissues, form sheet-like structures via cell junctions and adhere to connective tissues through a basement membrane. Therefore, cell sheets are highly effective at artificially constructing epithelial tissues.

"Cell sheet engineering" involves creating and manipulating transplantable 2D cell sheets and/or building 3D tissues by layering cell sheets [92]. Cell sheets are fabricated using a temperature-responsive cell culture dish where the surface is grafted with a temperature-responsive polymer. This chapter focuses on the design of temperature-responsive cell culture dishes for clinical applications of 2D cell sheet-based regenerative therapies. Issues related to the fabrication of 3D cell sheet-based tissues and organs are also described.

19.1.1 Temperature-Responsive Cell Culture Surfaces

The temperature-responsive polymer poly(*N*-isopropylacrylamide) (PIPAAm) has been used in several biomaterial applications because PIPAAm exhibits reversible temperature-dependent hydration and dehydration across the lower critical solution temperature (LCST) of 32 °C, which is close to body temperature, 37 °C (Fig. 19.1a) [23]. When temperatures fall below 32 °C, PIPAAm is dissolved and exhibits an expanded chain conformation in water because of hydration of PIPAAm, whereas above the LCST, PIPAAm is aggregated and insoluble in water due to the dehydration of isopropyl groups of PIPAAm. Owing to the reversible changes of hydration and dehydration, PIPAAm-grafted surfaces undergo temperature-dependent hydrophilic and hydrophobic changes across the LCST [73]. In addition, the conformation of the grafted PIPAAm chain and its chain mobility affect wettability changes occurring across the LCST on a PIPAAm-grafted surface [87, 88].

A pioneering study on temperature-responsive cell culture surfaces was conducted by Yamada and Okano et al. in 1990 [89]. A temperature-responsive cell culture surface, cross-linked PIPAAm-grafted tissue culture polystyrene (TCPS) (PIPAAm-TCPS), was prepared via electron beam (EB) irradiation. In this report, the authors showed that almost all hepatocytes adhered onto PIPAAm-TCPS at 37 °C were only detached by lowering the temperature. To our knowledge, PIPAAm-TCPS is the first temperature-responsive surface to achieve cell adhesion/deadhesion switching. On the other hand, Takezawa et al. found that human fibroblasts can be cultured on a TCPS surface coated with a mixture of PIPAAm and collagen [74]. Although the cells did not adhere onto PIPAAm-coated TCPS, cell adhesion and proliferation occurred on TCPS coated with a mixture of PIPAAm/collagen. For the PIPAAm coating, the addition of collagen proved essential for cell adhesion. When temperatures were decreased to 15 °C, the cells became detached and floated in the medium. The re-adhesion of the floating cells onto another plastic dish resulted in the formation of multicellular spheroids and in the outgrowth of fibroblasts from the spheroids.

Grafting of a nanoscale PIPAAm layer is a key factor for PIPAAm-TCPS to exhibit temperature-dependent alteration of cell attachment and detachment. To reveal the effects of grafted PIPAAm thickness, the temperature-dependent adhesiveness of cells was investigated for two types of PIPAAm-TCPS, for which the graft PIPAAm layers were thick (30 nm) and thin (20 nm) [3]. Bovine carotid artery endothelial cells did not adhere to the PIPAAm-TCPS surface with a 30 nm-thick PIPAAm layer even at 37 °C, where PIPAAm chains were dehydrated (Fig. 19.2a)). By contrast, cells adhered to the PIPAAm-TCPS surface with a thinner PIPAAm

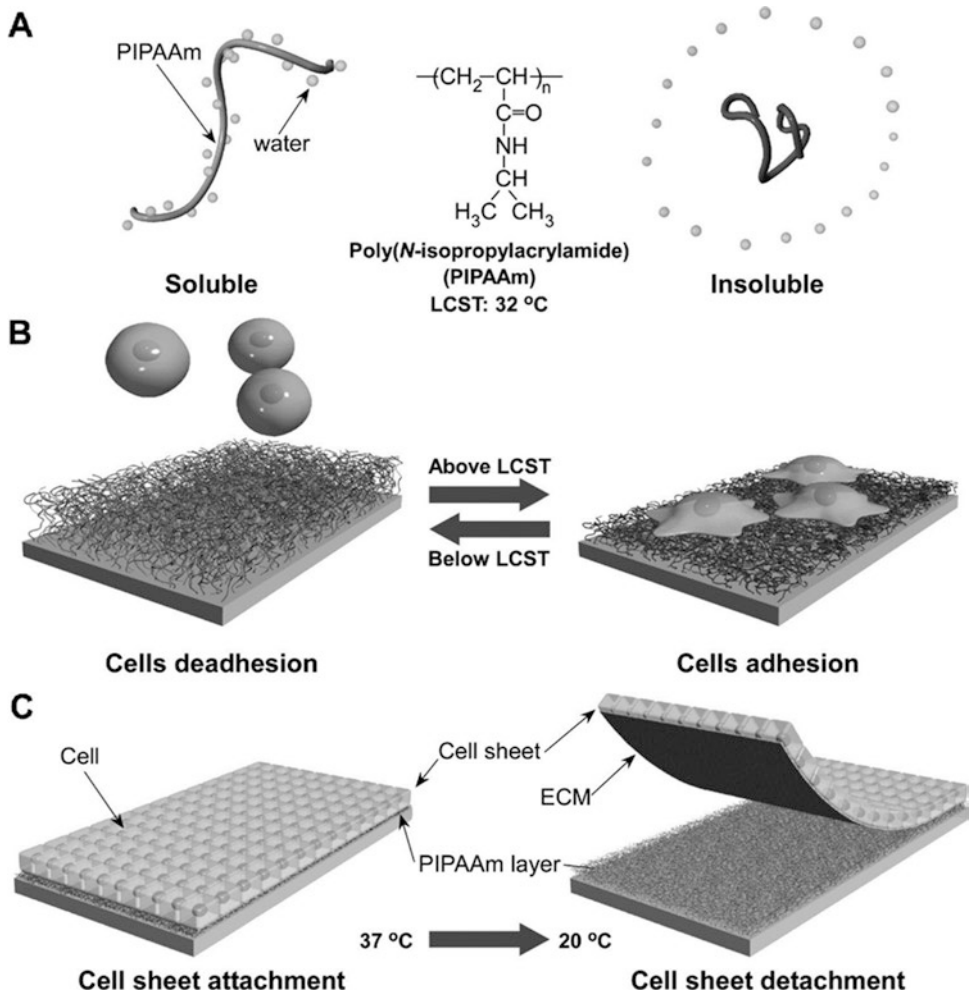

Fig. 19.1 Schematic illustration of the temperature-responsive character of (**a**) PIPAAm in aqueous solution and (**b**) PIPAAm grafted on a substrate and of (**c**) cell sheets recovered from a PIPAAm grafted substrate. (**c**) Attached cells proliferate to confluence 37 °C (right) and detach as a contiguous sheet with ECM from the PIPAAm-grafted substrate when the temperature is decreased to 20 °C (right). (Reprinted permission from [78]) with permission from Oxford University Press)

layer (20 nm) at 37 °C. By decreasing the temperature to 20 °C, the adhered cells detached from the thinner PIPAAm grafted surface. The non-cell-adherent character of layers thicker than 30 nm PIPAAm was supported by the fact that no detectable fibronectin was found on the surface.

We considered the hypothesis that PIPAAm chains at the interface of basal TCPS were aggregated and strongly dehydrated due to the hydrophobic TCPS. In contrast to flexible PIPAAm chains, such as soluble polymers and hydrogels, the mobility of covalently immobilized PIPAAm chains on TCPS was restricted. This restriction and dehydration of PIPAAm chains at the interface of the TCPS surface gradually extended toward the outermost PIPAAm-TCPS region. For the thin PIPAAm layer (15–20 nm), this gradual restriction and dehydration promoted the dehydration of PIPAAm chains in the outermost surface region, generating sufficient hydrophobicity for cell adhesion to PIPAAm-TCPS surfaces at 37 °C. On the other hand, for the thick PIPAAm layer (above 30 nm), the dehydration of PIPAAm chains at the outermost surface did not occur as

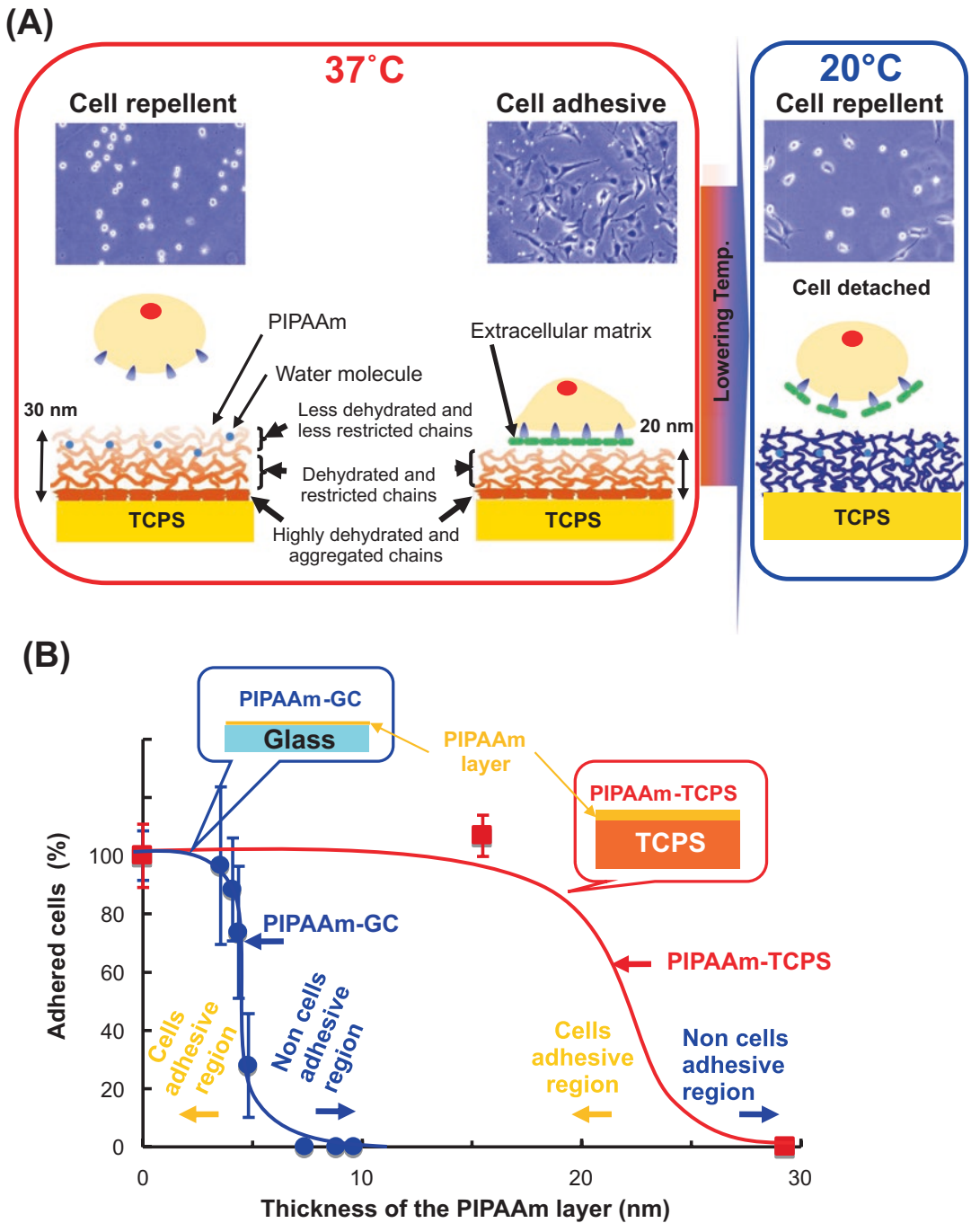

Fig. 19.2 The dependence on the graft polymer thickness of the subsequent cell adhesion behavior of temperature-responsive cell culture surfaces. (**a**) The influence of the mobility of grafted PIPAAm chains with different thickness on the hydration of polymer chains at 37 °C (left red-colored box) and 20 °C (right blue-colored box). Hydrophobicity of TCPS promoting aggregation and dehydration are shown as the black TCPS region. The molecular mobility of the grafted polymer chains increases according to the distance from TCPS interfaces. (**b**) Correlation between the spread cell density and thickness of the grafted PIPAAm. Red-colored circles and blue-colored diamonds denote PIPAAm-TCPS and PIPAAm-GC values, respectively. (Reprinted from [2, 17]) with permissions from John Wiley and Sons and The Society of Polymer Science, Japan, respectively)

efficiently. Therefore, cells were unable to adhere to the thicker PIPAAm-TCPS even at 37 °C. Based on this technology regarding the controlled thickness of grafted PIPAAm, a temperature-responsive cell culture dish, UpCell®, has been made commercially available.

Phenomena wherein the grafted polymer thickness dominates subsequent cell attachment behavior have also been found for another substrate, PIPAAm-grafted glass (PIPAAm-GC) surfaces (Fig. 19.2b) [16, 17]. By contrast to PIPAAm-TCPS, grafted PIPAAm layers 4.8 nm thick on a glass surface were found to be optimal for the temperature-dependent alteration of cell attachment and detachment. Above this optimal polymer thickness, cells did not adhere to PIPAAm-GC even at 37 °C. Such polymer thickness dependence is explainable in terms of graft PIPAAm chain mobility (Fig. 19.2a). Furthermore, the optimal graft PIPAAm layer thickness on glass for facilitating the temperature-dependent alteration of cell attachment/detachment was found to be thinner than that for TCPS, probably because the density and architecture of graft PIPAAm chains vary among these substrates [17].

Our findings regarding the dependence on the thickness of grafted PIPAAm have been recognized as part of a broader strategy to develop temperature-responsive cell culture surfaces through various methods [1, 81] (e.g., polymer coating [47], plasma irradiation [54], photoirradiation [48] and surface-initiated living radical polymerization methods, including atom transfer radical polymerization (ATRP) [45, 46] and reversible addition-fragmentation chain transfer polymerization (RAFT) [67]). Among these, surface-initiated ATRP and RAFT precisely controlled lengths of PIPAAm (molecular weight) with dense brushes. An intensive investigation of PIPAAm brush surfaces shows that the chain length and density of PIPAAm brushes and subsequent protein adsorption behavior significantly influence cell attachment behavior. When the molecular weight (M_n) and density of graft polymer chains are optimally tuned to 23,000–58,000 and 0.03–0.04 (chains/nm^2), respectively [1, 81], a PIPAAm brush surface undergoes a temperature-dependent alteration of cell attachment and detachment. More importantly, under optimal PIPAAm brush conditions, temperature-dependent changes in cell attachment and detachment were found on adsorbed fibronectin between PIPAAm brushes, and this is referred to as ternary adsorption [20, 86].

Recently, facile methods for the preparation of temperature-responsive cell culture surfaces have been developed without the use of special equipment such as EB and plasma irradiation systems. These methods are beneficial because they allow researchers to prepare temperature-responsive cell culture surfaces by themselves without using specialized equipment. Polymer coatings and visible light irradiation serve as facile alternative tools for preparation owing to their simplicity and cost effectiveness [18, 47 85].

19.2 Cell-Sheet-Based Regenerative Therapy

Alternation from a hydrophobic to a hydrophilic surface with low temperature treatment allows adhered cells to be noninvasively detached from PIPAAm-TCPS surfaces without cells being damaged while conventional cell recovery treatments using trypsin or chelate agents digest membrane proteins and ECM components of cultured cells [24, 38, 39, 93].

Cells on PIPAAm-TCPS, as well as TCPS, proliferate to confluency. After cell growth achieves confluency, temperature changes detach intact cell sheets from PIPAAm-TCPS while maintaining proteins among cell-cell bindings as well as on the cell surface. The detached cell sheet preserves the ECM component deposited on the basal side of the cell sheet. The recovered cell sheet is readily stacked onto another cell sheet or is transplantable to tissues and organs, as the ECM functions as biological glue and promotes adhesion between the cell sheet and another cell sheet or tissue [63, 94]. The deposited ECM allows the cell sheet to be transplanted and adhere to target tissues and organs without suture.

Therefore, cell sheet-based tissue engineering and regenerative therapy have attracted attention

as novel medical treatment approaches. In this section, cell sheet-based regenerative therapies for treating human diseases are described.

19.2.1 Autologous Cell-Sheet-Based Regenerative Therapy

The first trial for clinically transplanting cultured cell sheets was executed in 1981 for burn treatments by Green et al. [51]. Cultured autologous epidermal sheets were recovered by dispase treatment and were provided to patients with burn injuries. On the other hand, cell sheets that had detached from the temperature-responsive culture dish held ECMs on the bottom of the sheets due to the lack of enzymatic treatments (e.g., trypsin and dispase). This characteristic is expected to facilitate the efficient transplantation of cell sheet tissues to targets.

Table 19.1 shows clinical applications of cell sheet regenerative therapy. As epithelial cell sheet tissues, including epithelial keratinocyte sheets, corneal epithelial cell sheets, and oral mucosal epithelial cell sheets, resemble native sheet-like structures in the body, these cell sheet tissues replace damaged tissues after transplantation.

Since 2002, we have conducted collaborative research with K. Nishida on corneal epithelial regeneration treatments using cell sheets [49, 50]. From patients who had lost corneal epithelial cells due to alkali burns or drug side effects, ~2 mm^2 tissues containing epithelial stem cells at the boundary between the cornea and conjunctiva were collected. The cells collected were expanded and cultured to form corneal epithelial cell sheets [49]. In the case of binocular diseases, alternative epithelial cell sheets were prepared from oral mucosa collected from patients [50]. Both epithelial cell sheets regenerated human corneal epithelium after they were transplanted onto injured eyes, improving vision capabilities. Based on this achievement, CellSeed Inc., Japan, a venture company based out of Tokyo Women's Medical University (TWMU), initiated clinical trials in collaboration with Hospices Civils de Lyon in France in 2007 that were completed in 2011, indicating its effectiveness [9].

Collaborative research with Y. Sawa revealed that the regeneration of ischemic myocardium is induced by the attachment of skeletal myoblast sheets. Autologous skeletal myoblast sheets were generated from patient leg muscles and were stacked into three layers for transplantation. As a first clinical trial, autologous skeletal myoblast sheets were transplanted onto the heart surfaces of a patient who had experienced serious heart failure due to dilated cardiomyopathy. The patient's cardiac functioning was improved, eliminating the need for a left ventricular assist device (LVAD) and for cardiac transplantation. This recovery is attributed to angiogenetic effects of cytokines secreted from transplanted sheets in the proximity of the transplanted site and to paracrine effects inducing the recruitment of stem cells. Based on this method [59], a new medical "Heart Sheet" product for patients with ischemic heart disease was approved on September 18, 2015 and has been launched by Terumo Corporation, Japan.

In the field of esophageal research, clinical studies on the treatment of artificial ulcers accompanied by early esophageal cancer resection with endoscopic submucosal dissection (ESD) were first initiated by T. Ohki in April 2008. ESD even of large lesions is less invasive than surgical approaches. However, frequent extension using balloons is required to prevent stenosis due to artificial ulcer scarring after extensive ESD treatment. To prevent stenosis without invasive balloon extension, autologous oral mucosal epithelial cell sheets were endoscopically transplanted onto the ulcer surfaces of 10 patients. This procedure was found to promote the re-epithelialization of the esophagus and to prevent esophageal narrowing after ESD, leading to ESD therapy without lowering each patient's quality of life [53]. Additionally, collaborative clinical studies have been conducted for the treatment of 10 patients at Nagasaki University Hospital [90] and for the treatment of 10 patients suffering from Barrett's esophagus at the Karolinska Institute, Sweden. Based on these treatment outcomes, CellSeed is conducting clinical trials in collaboration with National Cancer Center Japan for the treatment of artificial ulcers.

Table 19.1 Cell-sheet based regenerative therapies using autologous cell sources

Tissue/organ	Target illness	Cell sheets	Implementation site (country)	Comments
Corneal epithelium	Limbal stem-cell deficiency	Corneal limbal-derived cell sheet	Osaka University (Japan)	Clinical research from 2002
		Oral mucosal epithelial cell sheet		
		Oral mucosal epithelial cell sheet	Les Hospices Civils de Lyon (France)	Clinical trials during from 2007 to 2011
			Collaborator: Cellseed, Inc. (Japan)	
Myocardium	Severe cardiac disease (e.g. ischemic heart disease, dilated cardiomypathy)	Myoblast sheet	Osaka University (Japan)	Clinical research from 2006
			Terumo Corporation (Japan)	Approved on September 18, 2015, and have launched Japan
			Collaborator: Cellseed, Inc. (Japan)	
Esophagus	Prevention of stenosis after endoscopic submucosal dissection of esophageal cancer	Oral mucosal epithelial cell sheet	Tokyo Women's Medical University (Japan)	Clinical research from April 2008
			Tokyo Women's Medical University (Japan)	Tissues and cell sheets were transferred between Tokyo and Nagasaki by airplane, and transplanted in Nagasaki
			Nagasaki University (Japan)	
			Cellseed, Inc. (Japan)	Clinical trial has started from 2017
			National Cancer Center (Japan)	
			Tokyo Women's Medical University (Japan)	
	Barrett's esophagus	Oral mucosal epithelial cell sheet	Karolinska Institute (Sweden)	10 cases of clinical research from 2011
Periodontal ligament	Periodontal disease	Periodontal ligament-derived cell sheet	Tokyo Women's Medical University (Japan)	10 cases of clinical research from 2011 to 2014
Cartilage	Knee cartilage injury	Cartilage cell sheet	Tokai University (Japan)	Clinical research from 2011 to 2014
Middle ear mucosa	Removed mucosa in middle ear cavity after tympanoplasty	Nasal mucosal cell sheets	Jikei University School of Medicine (Japan)	Clinical research from 2014
Lung	Closure of air leakage after pulmonary resections	Dermal fibroblast sheet	Tokyo Women's Medical University (Japan)	Clinical research from 2018

In the dental field, the transplantation of periodontal ligament-derived cell sheets has been carried out for the regenerative treatment of periodontal tissue, specifically of periodontal ligaments [28]. Periodontal disease induces the loss of periodontal tissue, resulting in tooth loss or instability. Therefore, the regeneration of periodontal tissues is essential to preventing tooth loss. Ten clinical studies were conducted from 2011 to 2014 using autologous cell sheets derived from periodontal ligaments of extracted teeth (e.g., wisdom teeth). Three-layered periodontal ligament cell sheets were attached to tooth surfaces in combination with bone prosthetic materials. Eventually, absorbed alveolar bone was regenerated, preserving teeth that were originally to be extracted [29].

In the orthopedic field, cartilage damage is typically treated via chondrocyte implantation, mosaic formation or microfracture procedures. Recently, tissue-engineered cartilage has been applied to repair articular cartilage damage using various transplanted cells [13]. Clinical research on joint repair using autologous chondrocyte sheets derived from cartilage tissue has been conducted by M. Sato since 2011. Safe outcomes, improved clinical symptoms, and the regeneration of hyaline cartilage were confirmed in all cases, showing that positive therapeutic effects were obtained. This therapeutic method will be made available with approval from the Ministry of Health, Labour and Welfare, Japan.

In the field of otolaryngology, cell sheet-based regenerative therapy has been applied to tympanoplasty in middle ear surgery. Poor mucosal regeneration in the resected area of the mastoid cavity occurs after conventional tympanoplasty, resulting in the frequent re-adhesion of tympanic membranes and the recurrence of adhesive otitis media. Our collaborative clinical research with H. Kojima and K. Yamamoto was conducted for the transplantation of autologous mucosal cell sheets onto the surfaces of exposed bone from which mucosa had been removed during tympanoplasty. As a surrogate of middle ear mucosa, autologous mucosal cell sheets were derived from an approximately 10 × 10 mm^2 piece of nasal mucosal tissue. Our clinical research shows that all of the patients tested exhibited good postoperative course without adverse events or complications and that the patients' hearing abilities remained strong after transplantation [91].

In the field of thoracic surgery, the clinical application of autologous skin fibroblast sheets for the closure of lung air leakages has been executed in collaboration with M. Kanzaki. Air leakages are observed after lung resection, causing severe complications when proper treatments are not applied. Therefore, the prevention of air leakages is essential for managing patients after lung resection. Autologous skin fibroblast sheets fabricated from 5 × 15 mm pieces of patient skin were applied to seal air leakages for a 44-year-old male patient with multiple bullae following pulmonary resections by video-assisted thoracoscopic surgery. Air leakages were fully sealed with the attachment of cell sheets. No adverse events were observed after 7 months, and the patient recovered fully [31].

In addition to the abovementioned therapeutic studies, several preclinical studies using haptic cell sheets [52], pancreatic cell sheets [55], and iPS-derived cell sheets including cardiomyocytes [44] have been ongoing.

19.2.2 Allogenic Cell Sheet-Based Regenerative Therapy

Human mesenchymal stem cells (MSCs) are a promising cell source for clinical applications [10], as the isolation from various tissues and expansion of these cells are relatively easy. In addition, it has been reported that MSC can supply growth factors and cytokines while also exhibiting immunomodulatory properties [10]. Typical regenerative treatments using MSCs have been conducted through the injection of suspensions. However, the injection method using an enzymatically treated single cell suspension presents disadvantages in terms of efficient transplantation due to the limited stability of cells at the target site. In contrast, MSC sheets facilitate attachment to tissue surfaces while maintaining cell-cell binding, leading to highly efficient transplantation.

Diabetic foot ulcers can form as a complication, and their treatment in many cases is difficult due to the occurrence of reduced blood flow and neuropathy. The progression of diabetic ulcers results in the development of gangrene, which can mostly require the amputation of the patient's foot. As a means of healing diabetic ulcers, a new therapy using allogenic MSC sheets has been developed [32]. In a preclinical study, allogenic adipose-derived MSC sheets were transplanted into a type 2 diabetic model with obesity (Zucker rats). For the wound healing model, full-thickness skin deficiencies were created on the cranial 10 × 15 mm area where cell sheets were transplanted in combination with artificial dermis. Seven days after transplantation, the degree of wound closure was significantly higher in the cell sheet transplantation group (transplant group) compared to the rats with artificial dermis (control group). The mean time to wound closure was 34.2 days for the control group, whereas it was shortened to 25.6 days for the transplant group. Through histological analyses, angiogenesis was found to be significantly present in the transplant group. Vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), epidermal growth factor (EGF) and other growth factors were also found to be secreted during *in vitro* cultivation. The transplantation of GFP-expressing MSC sheets revealed that GFP positive cells were positioned around newly generated blood vessels, suggesting that transplanted MSCs may support the formation of blood vessels. Consequently, the transplanted MSCs not only promoted wound healing by secreting angiogenic factors but also were involved in the construction of blood vessels.

MSC transplantation was also applied for the treatment of osteonecrosis due to drug side effects. Bisphosphonate (BP) is a primary choice for the treatment of osteoporosis and is used to treat cancer and diseases involving bone decline. Recently, BP-related osteonecrosis of the jaw (BRONJ) was found to occur in patients receiving BP. To limit the occurrence of BRONJ, MSC sheets were transplanted onto bone exposed after tooth extraction in BRONJ-like rats [30]. After a two-week transplantation period, osteonecrosis healing was significant in the MSC sheet-transplant group relative to those for the control group of BRONJ rats receiving sham surgery. Neovascularization was found in the transplant group due to the secretion of angiogenic factors from transplanted MSCs. Furthermore, the gene expression of receptor activators of nuclear factor κ-B ligand (RANKL), which induced the differentiation of osteoclast precursor cells into osteoclasts, was detected from MSCs. In addition to angiogenetic effects, the transplantation of MSC sheets contributed to the promotion of bone turnover.

Recently, treatments using allogenic chondrocyte sheets have been applied by M. Saito et al. at Tokai University. Typically, young patients of approximately 1 year old suffering from polydactyly were treated by removing unnecessary fingers. These fingers are ordinarily considered medical waste, but they contain chondrocytes with the potential to proliferate. Using fingers amputated from patients suffering from polydactyly, allogenic chondrocyte sheets have been prepared. Clinical research on joint repair using allogenic chondrocyte sheets has been conducted at Tokai University.

19.3 Design of Temperature-Responsive Cell Culture Surfaces for 3D Tissue Fabrication

As noted above, a few layered cell sheets have been clinically transplanted onto the superficial layers of tissues and organs due to ease of transplantation. In regard to challenging issues of the next generation of tissue engineering, much attention has been paid to creating 3D tissues and organs. One approach involves mimicking emergence processes (e.g., cell proliferation and differentiation in the developmental stage of biology). Self-organized multicellular bodies have been formed through the proliferation and differentiation of pluripotent stem cells such as ES and iPS cells under appropriate ECMs (e.g., Matrigel®) and growth factors, resulting in the formation of small tissues and organs referred to

as organoids [15]. Several researchers have generated organoids including teeth [25], liver tissue [72], optic-cups [14] and intestinal tissue [19]. In general, organoid sizes are limited to miniaturized units of tissues and organs mainly for investigating biological events *in vitro* and surrogates to test drug toxicity levels and effects.

Another approach to 3D tissue and organ generation involves cell sheet engineering, which enables one to create cell-dense assemblies and hierarchical architectures through the sequential lamination of cell sheets. Key technologies for constructing 3D structures from cell sheets and for transplanting them are described in the following sections.

19.3.1 Materials and Devices for Manipulating Recovered Cell Sheets

As single cell sheets are flexible, the development of cell sheet manipulation techniques is essential for the fabrication of cell-dense 3D engineered tissue and for the transplantation of cell sheets onto damaged tissues and/or organs. Detached cell sheets float in a medium and are manipulated with a pipette. Support membranes, such as chitin [33], porous polyethylene terephthalate (PET) [33], poly(vinylidene difluoride) (PVDF) [24], and parchment paper, are used to facilitate the manipulation of cell sheets. Prior to manipulation, a support membrane is overlain on the apical side of confluent cells, which are cultured on temperature-responsive cell culture surfaces at 37 °C. During low temperature treatments, confluently cultured cell sheets detach with the support membrane while all sheets are physically attached to the support membrane. When the deposited support membrane is peeled off, cultured cells are harvested as a cell sheet together with the support membrane. The harvested cell sheet is adhered to another TCPS surface to maintain the ECM composition of the basal side of the cell sheet. The cell sheet is in turn readily detachable from the support membrane by using an excess volume of aqueous media. However, special skill is required to manipulate cell sheets with support membranes and a pipette.

To promote the adhesion of cell sheets, cell adhesive materials, such as the polyion complex hydrogel composed of (poly(*N,N*-dimethylacrylamide-*co*-2-acrylamido-2-methylpropane sulfonic acid) (P(DMAAm-*co*-AMPS) and poly(*N,N*-dimethylacrylamide-*co*-2-acryloxyethyltrimethylammonium chloride) (P(DMAAm-*co*-AETA-Cl), fibrin, and gelatin, have been coated on the surfaces of support membranes and plunger devices [58, 79]. Tang et al. modified polyion complex hydrogels on porous PVDF membranes through cross-linking and grafting by EB irradiation [79]. They successfully transferred cultured fibroblast sheets from temperature-responsive cell culture surfaces to a collagen-coated surface using the polyion complex-coated membrane.

Sasagawa et al. developed a plunger device for cell sheet manipulation (Fig. 19.3a). Cell adhesive natural hydrogels, such as fibrin and gelatin, were coated onto the surface of the plunger [58]. The hydrogel-coated plunger was placed onto confluent cells cultured on the temperature-responsive cell culture surface. By incubating the entire plunger on the cell culture surface at a low temperature (20 °C), the cells detached from the surface while remaining attached to the hydrogel surface. By lifting the plunger, the cell sheet can be readily harvested and transferred to another cell sheet cultured on a temperature-responsive cell culture surface without damaging cells. These two cell sheets bind together to form double-layered cell sheets at 37 °C. Repetitively layering cell sheets with this device allows for the fabrication of thick cardiac tissues and hierarchically aligned structures [22, 56, 69, 84]. Using the plunger cell sheet manipulator technique, automatic apparatuses for cell sheet stacking have been developed. Five-layer cell sheets 70–80 μm thick have been automatically fabricated with this apparatus [34].

Recently, Tadakuma et al. developed a new device that enables one to rapidly transfer and

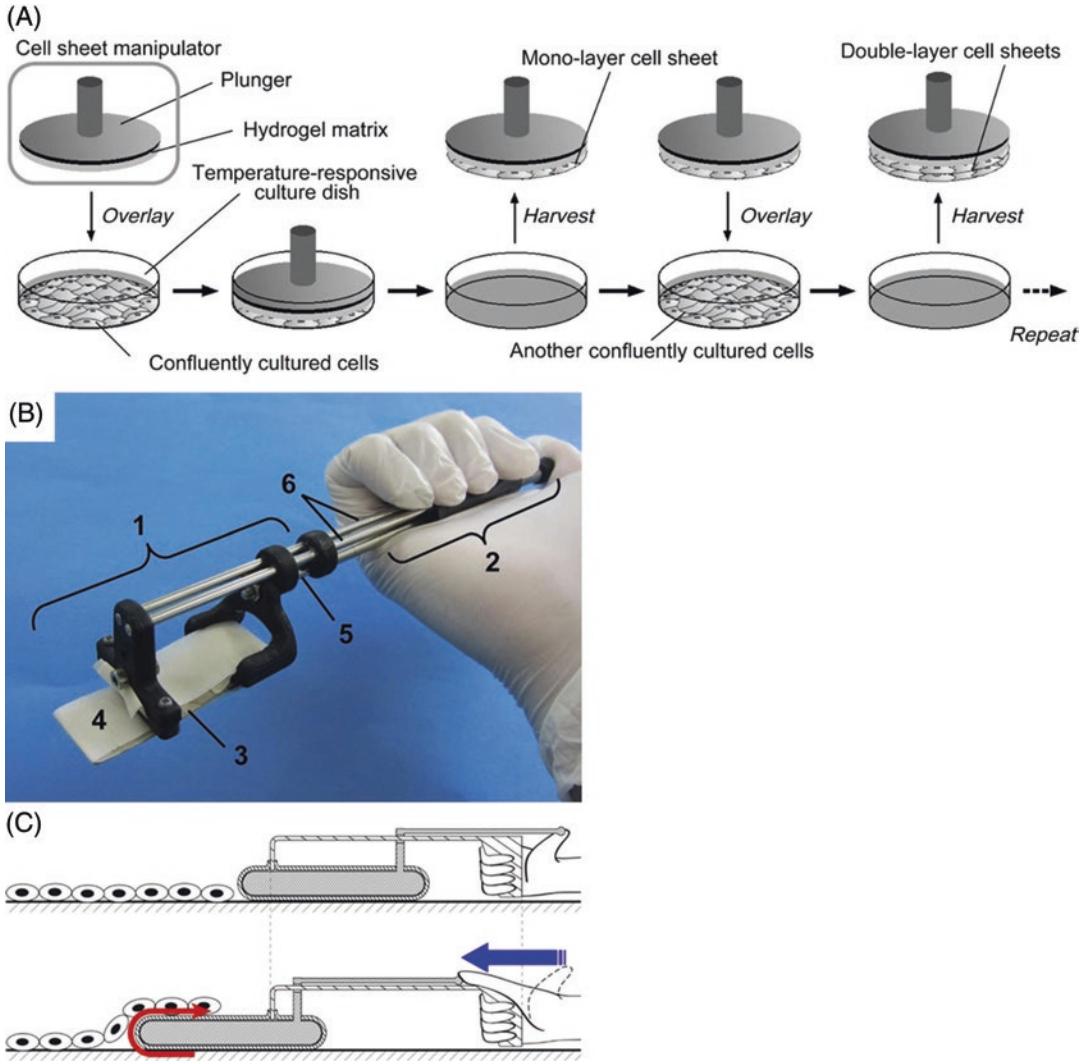

Fig. 19.3 Cell sheet layering manipulation procedures and of new cell sheet transfer/transplantation devices. (**a**) Schematics of layering cell sheets utilizing temperature-responsive culture dishes and a cell sheet manipulator. Upon repeated application of this procedure, cell sheets can be stacked. (**b**) Photograph of a new cell-sheet transfer/transplantation device consisting of several parts (a scoop (1), a handle (2), an inner plate (3), an outer movable belt (4), a pushing rod (5), and stainless rods (6)). (**c**) Schematic illustration of cell sheet scooping mechanism of the device shown in (**b**). The outer removal belt comes into direct contact with cell sheets and dish surfaces. The outer removal belt surface is polytetrafluoroethylene, which is non-adherent, low friction, and low wearing. The manipulation of the device does not affect the cell sheet when scooping and releasing the cell sheet. (Reprinted from [58, 65]) with permissions from Elsevier)

transplant recovered cell sheets onto different surfaces or tissues (Fig. 19.3b, c) [65]. The device is mainly composed of two parts: scooping and handling parts (Fig. 19.3b). Compared to conventional cell sheet transfer methods involving the use of a pipette, this device allows one to retrieve recovered cell sheets and to then transfer them in several seconds without requiring any special skills. The device also allows for the rapid transplantation of cell sheets onto soft, rugged and uneven surfaces and rat subcutaneous tissues within 30 seconds.

19.3.2 Temperature-Responsive Cell Culture Surfaces for the Acceleration of Cell-Sheet Detachment

Novel rapid cell-sheet detachment has been investigated as a means to maintain biological activity and physiological cell-sheet functioning when applied for the subsequent fabrication of cell-sheet-based tissue or transplantation. In addition, rapid cell sheet recovery techniques are anticipated to support rapid cell sheet transplantation, reducing the patient burden in clinical application. In the case of conventional temperature-responsive cell culture surfaces, grafted PIPAAm chains are gradually hydrated from the periphery of the temperature-responsive cell culture surface to the central region as the temperature decrease across the LCST [40]. Cell sheet can be quickly detached by accelerating the hydration of grafted PIPAAm chains. Several approaches developed involve grafting cross-linked PIPAAm onto porous membranes [40], incorporating hydrophilic units into PIPAAm [12, 41] and hierarchically grafting PIPAAm onto hydrophilic polymers (Fig. 19.4) [4, 35].

Porous membrane surfaces have been used as base materials to enhance water permeability [40]. Cell sheets detach more quickly from PIPAAm-grafted porous membranes than from PIPAAm-TCPS. The incorporation of hydrophilic components, such as poly(ethylene glycol) (PEG) and 2-carboxyisopropylacrylamide (CIPAAm), into grafted PIPAAm also accelerates the detachment of cells (Fig. 19.4a–c) [12, 41]. As hydrophilic components are readily hydrated, changes in the hydrophilicity of polymer-grafted surfaces are accelerated when temperatures are decreased to below the LCST. Furthermore, the introduction of a hydrophilic interface layer between PIPAAm and TCPS affects the kinetics of temperature-responsive change in cell culture surfaces (Fig. 19.4d). By successively grafting PAAm and PIPAAm, double polymeric layers and polyacrylamide (PAAm) and PIPAAm layers form on a TCPS surface (PIPAAm-PAAm-TCPS). An interpenetrating polymer network (IPN) of PIPAAm and PAAm in turn forms on the basal TCPS surface. At 37 °C, cells fail to adhere to PIPAAm-PAAm-TCPS surfaces with high PAAm content, while cells adhere to and proliferate on PIPAAm-PAAm-TCPS with low PAAm content. Under lowered temperatures, cell sheets rapidly detach from PIPAAm-PAAm-TCPS with low PAAm content. Hydrated water molecules within PAAm components are considered to be supplied to the grafted upper PIPAAm layer as temperatures decrease to below the LCST.

In addition, Tang et al. found that the grafted architecture of thin PIPAAm influences cell sheet detachment (Fig. 19.4e) [80]. The comb-type PIPAAm hydrogel contains grafted PIPAAm chains with free mobile terminals, which enhance the deswelling/swelling rates of PIPAAm hydrogels [97]. By increasing temperatures across the LCST, only free mobile terminals of grafted PIPAAm chains rapidly dehydrate and aggregate. Such dehydration and aggregation induce a more rapid deswelling of comb-type PIPAAm hydrogels compared to conventional cross-linked PIPAAm hydrogels. By contrast, with decreasing temperature across the LCST, the rapid hydration of PIPAAm chains with free mobile terminals accelerates the swelling of comb-type PIPAAm hydrogels. To accelerate swelling, comb-type thin PIPAAm hydrogels have been grafted onto TCPS [80]. This surface undergoes more rapid cell sheet recovery than PIPAAm-TCPS, which does not contain free mobile PIPAAm chains.

19.3.3 Heparin-Immobilized Thermoresponsive Surfaces for the Sustained Stimulation of Cellular Receptors and the Recovery of Cultured Cells

Stimulation with growth factors in the culture system is required to express the phenotype of a specific cell type (e.g., hepatocytes) in a differentiated state or to maintain undifferentiated embryonic stem cells. Typically, growth factors in soluble form are supplemented in the culture medium. This supplementation must occur

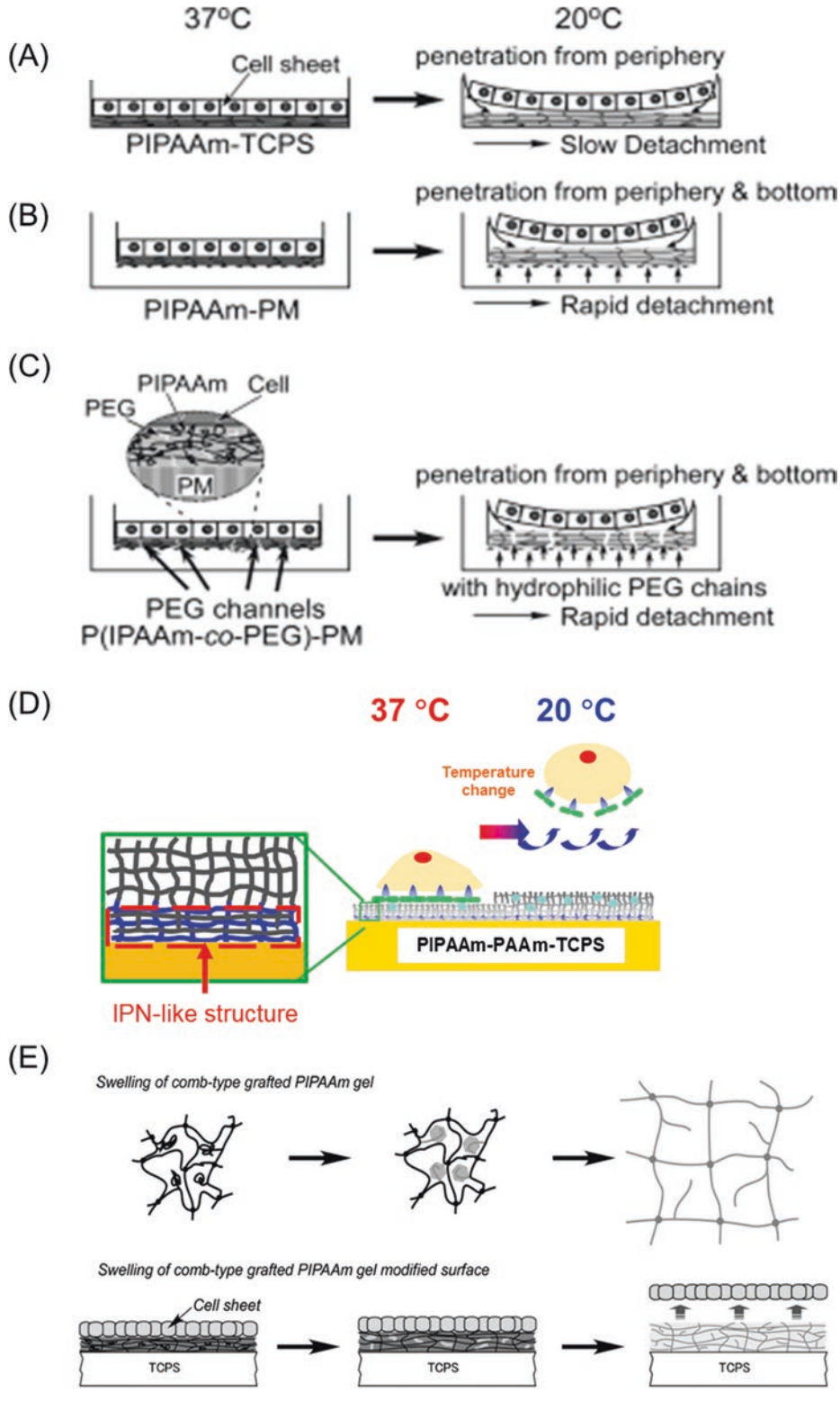

Fig. 19.4 Schematic illustration of the rapid hydration of grafted PIPAAm chains. (**a**) PIPAAm-TCPS, (**b**) PIPAAm-grafted PM, (**c**) P(IPAAm-*co*-PEG)-grafted porous membrane (PM), (**d**) PIPAAm-PAAm-TCPS, (**e**) comb-type PIPAAm (upper illustration) and comb-type PIPAAm gel-grafted TCPS (bottom illustration). (Reprinted from [4, 81]), and [80]) with permissions from John Wiley and Sons and Elsevier)

frequently, and it is difficult to maintain its effectiveness over the long term due to the down-regulation of cellular receptors. By contrast, covalently immobilized growth factors on a cell culture substrate escape receptor down-regulation, resulting in prolonged cell stimulation by immobilized growth factors [27, 37]. However, during passage with trypsin treatment, proteolytic activity induces the degradation of receptor proteins and eventually the deterioration of cellular functions.

To achieve the sustained stimulation and non-invasive recovery of cultured cells, temperature-responsive surfaces covalently conjugated with ECM molecules have been designed [36]. In particular, proteoglycan-mimicked temperature-responsive surfaces resemble a basement membrane that supplies cellular microenvironments. Negatively charged heparan sulfate chains on proteoglycans stabilize growth factors through the formation of complexes, leading to the maintenance of activity and to the suppression of growth factor diffusion. In turn, the introduction of heparin (with the same structures and functions as heparan sulfate) onto poly (IPAAm-*co*-CIPAAm)-grafted surfaces mimics ECM microenvironments [5–7]. Heparin typically exhibits an affinity with various heparin-binding proteins such as basic fibroblast growth factor (bFGF, or FGF-2), heparin-binding epithelial cell growth factor-like growth factor (HB-EGF), and VEGF.

Heparin-immobilized temperature-responsive surfaces have been used to form hepatocyte sheets with hepatic functions maintained (Fig. 19.5) [7]. Liver tissue engineering using hepatocytes serves as an attractive approach for the treatment of liver diseases. In a preclinical study, hepatocyte sheets transplanted into a subcutaneous site stably persisted over more than 200 days and secreted specific proteins [52]. In contrast, cultured hepatocytes rapidly lose their viability and phenotypic functions when isolated from the natural *in vivo* microenvironment of the liver. Soluble EGF or HB-EGF in the cell culture system is essential for the survival of hepatocytes. Thus, heparin-immobilized temperature-responsive surfaces bound with

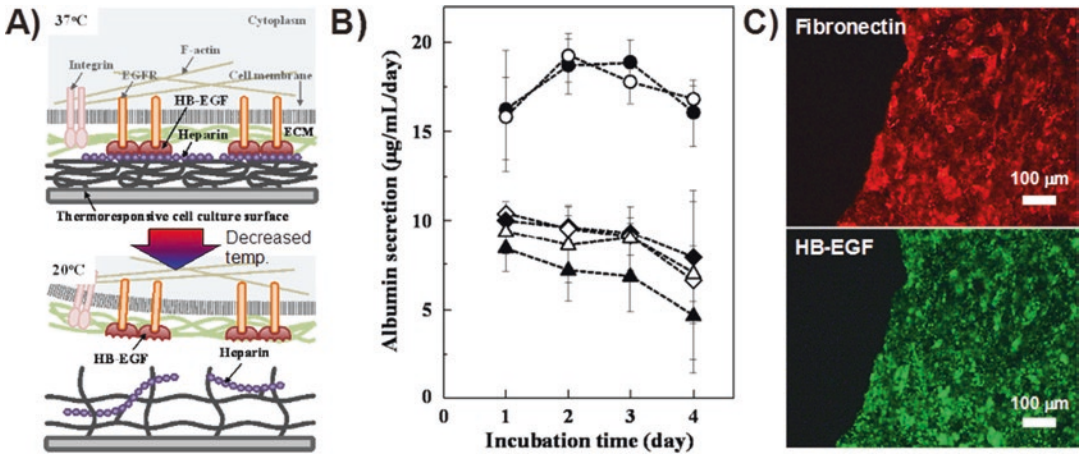

Fig. 19.5 Design of heparin-immobilized temperature-responsive surfaces for hepatocyte sheets with maintenance of their functions. (**a**) Adhered hepatocytes survive on HB-EGF-bound heparin-immobilized temperature-responsive surfaces at 37 °C (upper). Cultured hepatocytes detach at 20 °C, presumably due to a reduction of affinity between EGF receptors, HB-EGF and surface-immobilized heparin (lower). (**b**) Timing of albumin secretion from hepatocytes on heparin-immobilized temperature-responsive surfaces bound with 76.2 ± 3.8 ng/cm^2 HB-EGF (circles), PIPAAm surfaces with 100 ng/cm^2 soluble HB-EGF and 1.0 mg/cm^2 soluble heparin (triangles), and collagen-coated tissue culture polystyrene surfaces (diamonds) subjected to culturing with epidermal growth factor (EGF, 10 ng/mL, open symbols) or without EGF (closed symbols). (**c**) The cultured hepatocyte sheet was detached with fibronectin (red) and HB-EGF (green) from heparin-immobilized surfaces while lowering the temperature to 20 °C. (Reprinted from [7]) with permission from Elsevier)

HB-EGF have been used to prepare hepatocyte sheets with sustained stimulation of EGF receptors on hepatocytes. Hepatocytes survive on heparin-immobilized temperature-responsive surfaces bound with HB-EGF of 10 ng/cm^2, leading to the formation of their sheets. During cultivation, hepatocyte-specific functions (e.g., albumin secretion) were highly maintained compared with soluble HB-EGF, presumably due to the continuous activation of hepatocytes. This shows that bound HB-EGF continuously stimulates hepatocytes through EGF receptors, resulting in the maintenance of hepatic functions. It was previously reported that the viability and functioning of primary rat hepatocytes, such as albumin secretion and urea synthesis, are preserved on substrates with covalently immobilized EGF [37]. By contrast, cultured hepatocytes on heparin-immobilized temperature-responsive surfaces are detached as a contiguous sheet with fibronectin and HB-EGF when temperatures are lowered to 20 °C. Therefore, heparin-immobilized temperature-responsive cell culture surfaces facilitate the manipulation of hepatocyte sheets while maintaining hepatic functions by simply changing the culture temperature.

Furthermore, heparin-immobilized temperature-responsive surfaces promote cell proliferation and cell sheet formation [6]. Heparin-immobilized temperature-responsive surfaces bound with bFGF accelerate the proliferation of fibroblasts. After 3 days of culture, bFGF-bound heparin-immobilized temperature-responsive surfaces are able to hold 2–3 times more fibroblasts than surfaces with the same amount of soluble or physisorbed bFGF. The incubation period required for fibroblasts to reach confluence on bFGF-bound heparin-immobilized temperature-responsive surfaces is 3 days. However, cultured fibroblasts on PIPAAm surfaces with physisorbed bFGF reach confluence after 5 days. This prolonged period is considered to be due to the denaturation and/or random orientation of bFGF through physisorption, leading to decreasing bFGF activity. In contrast, binding bFGF to immobilized heparin preserves the activity and stabilizes the formation of bFGF/FGF receptor/heparin complexes. Therefore, bFGF bound to heparin-immobilized temperature-responsive surfaces promotes cell proliferation.

Finally, heparin-immobilized temperature-responsive surfaces offer two advantages with respect to cost effectiveness. First, heparin-immobilized temperature-responsive culture dishes decrease the amount of HB-EGF needed for culturing a hepatocyte sheet. No additional HB-EGF in the medium is required during the 4-day incubation period. On PIPAAm-grafted surfaces without heparin, by contrast, the medium containing HB-EGF as a soluble form is exchanged three times over 4 days. Therefore, cell culture systems using heparin-immobilized temperature-responsive surfaces have been shown to be beneficial for reducing the total cost of cell cultures associated with the amount of HB-EGF required. Second, heparin-immobilized temperature-responsive surfaces accelerate cell sheet fabrication. Shortening the time required for cell sheet preparation fabrication reduces costs. bFGF bound to heparin-immobilized temperature-responsive surfaces shortens the culturing period required to achieve the confluence of fibroblasts by 2 days. In addition, it has been found that the detachment of fibroblast sheets from heparin-immobilized temperature-responsive surfaces was faster than that from PIPAAm surfaces, presumably because surfaces are hydrated with immobilized heparin. Consequently, a cell culture system using heparin-immobilized temperature-responsive surfaces has been shown to be beneficial in terms of reducing total costs related to the growth factors and media required.

19.3.4 Design of Micropatterned Temperature-Responsive Cell Culture Surfaces for Generating Microscopically Aligned Tissues

Tissues and organs comprise heterogeneous cell species that express and maintain their physiological functions through complex interaction

between cells and ECMs. Micropatterning technologies have been applied to spatially position cells to construct biomimetic tissue *in vitro* ([8]). Micropatterned temperature-responsive cell culture surfaces are designed for fabricating microscopically aligned tissue contracts [26, 68, 69, 82–84, 96]. In particular, the use of micropatterned temperature-responsive surfaces facilitates the transfer of patterned cells without the structural destruction of cell-cell binding.

A temperature-responsive cell culture surface patterned with two different temperature-responsive polymers (PIPAAm and poly(*n*-butyl methacrylate) (PBMA)-*co*-grafted PIPAAm) has been developed by EB irradiation with a stainless mask [82]. The LCST of the co-graft polymer area is lower than that of PIPAAm. At 27 °C, primary rat hepatocytes were selectively adhered to the co-grafted domain (1.0 mm in diameter), as the PIPAAm grafted area was sufficiently hydrophilic to be non-cell adhesive [82, 83]. Sequentially, by increasing the temperature to 37 °C, bovine aortic endothelial cells were favorably attached to the grafted PIPAAm area. A co-culture of hepatocytes with bovine aortic endothelial cells enhanced the hepatic physiological functions of albumin secretion and urea synthesis relative to monocultures of hepatocytes [83]. The co-culture system was useful for the fabrication of liver tissue structures and for liver regeneration. By decreasing the temperature to 20 °C, co-cultured cells were recovered as a contiguous co-cultured cell sheet maintaining hepatocytes and endothelial cell interactions.

Micropatterned temperature-responsive surfaces wherein striped PIPAAm and brush PIPAAm-*b*-poly(*N*-acryloylmorpholine) (PIPAAm-*b*-PAcMo) domains are fabricated in combination with surface-initiated RAFT polymerization via the photolithographic method have been found to direct not only the orientation of cultured cells but also the alignment of recovered cell sheets [68]. In the case of polymer brush surfaces prepared through surface-initiated RAFT polymerization, reactive groups remain at polymer brush terminals. Additional polymer chains are grafted to the terminals of grafted polymer brush surfaces. As shown in Fig. 19.6, reactive dithiobenzoate (DTB) groups at the end of grafted PIPAAm brushes have been converted into inert maleimide groups by spatial masking with photoresist. After the removal of photoresist, another PAcMo was grafted to the terminal of PIPAAm brush surfaces, where reactive DTB

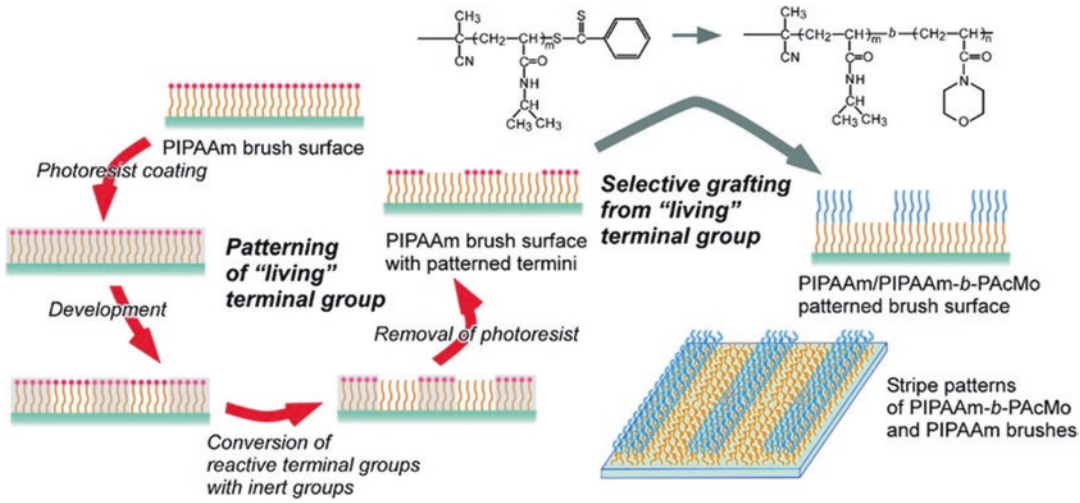

Fig. 19.6 Schematic illustration of the fabrication of micropatterned temperature-responsive PIPAAm and PIPAAm-*b*-PAcMo brush surfaces using a two-step RAFT polymerization procedure. PAcMo chains are further grafted from the terminal of PIPAAm chains *via* reactive DTB groups. (Reprinted from [68]) with permission form American Chemical Society)

remained. Normal human dermal fibroblasts (NHDFs) preferentially adhered to the microstripe patterned PIPAAm brush domain at 37 °C and were oriented in a direction parallel to the stripe patterns after 24 hours only when the PIPAAm brush domain was 50 μm in width. By contrast, NHDFs randomly adhered to PIPAAm brush surfaces without micropatterning. Further incubation caused the patterned NHDFs to migrate and proliferate on the patterned PIPAAm-*b*-PAcMo brush domain. Eventually, all of the cultured NHDFs preserved their orientations. Finally, after 5 days of cell culture, adhered cells became confluent and oriented on the line-striped micropatterned PIPAAm and PIPAAm-*b*-PAcMo brush domains. Fluorescence photographs showed that actin filaments in cultured cells on patterned domains also became oriented. By decreasing the temperature to 20 °C, NHDFs with well-oriented structures can be harvested as an anisotropic cell sheet, with different shrinking rates observed for the vertical and parallel directions of the cell sheet.

Once the anisotropic cell sheet detaches from the surface and is spontaneously shrunk, the unique oriented cell structure is lost due to cytoskeleton rearrangement. To prevent shrinkage, a cell sheet manipulator device is used to harvest anisotropic cell sheets while maintaining their unique cell orientations [69]. When this anisotropic cell sheet is translocated to TCPS using a manipulator device, its anisotropic character is structurally maintained for 7 days. Biological assays for anisotropic NHDF sheets show that VEGF secretion is enhanced relative to isotropic NHDF sheets, which comprise randomly oriented cells, while type-I collagen and transforming growth factor-β1 (TGF-β1) are independent of the orientation of culture cells. Anisotropic myoblast sheets are also applied for neuron-muscle tissue fabrication, sandwiching neurons and endothelial cells [70].

This micropatterned temperature-responsive cell culture surface is further used for the fabrication of tubular neural tissues using human-iPS cells derived from neurons and human astrocytes [71]. Astrocyte-sandwiching neurons are cultured on a micropatterned temperature-responsive cell culture surface. For striped patterns of appropriate widths, seeded astrocytes selectively align onto the cell adhesive striped pattern. Seeded neurons then spread and are oriented in the same direction as the aligned astrocyte. Finally, astrocytes are seeded again on the co-cultured and striped cells. The co-cultured cells form tubular structures wherein neurons are wrapped by astrocytes. One month of cell culture allows co-cultured cells to elongate and form tightly bundled structures that mimic neural tissue-like constructs. Patterned temperature-responsive cell culture surfaces may be useful for the fabrication of well-organized oriented cells and/or ECM as well as native tissues in the body.

19.4 Challenging Issues Related to Thick and Large 3D Tissue Fabrication

19.4.1 Vascularization

Layering multiple cell sheets allows for the fabrication of thick and functional tissue structure as described above. However, four-layered cardiomyocyte sheets undergo necrosis within the structure due to the limited diffusion of O_2 and nutrients [64]. Therefore, the formation of vascular networks is required to supply O_2 and nutrients to transplanted cell sheets. Endothelial cells (ECs) derived from the hearts of neonatal rats contribute to the formation of capillary-like networks within cardiac cell sheet tissues with stimulation by VEGF [57, 62, 66]. In the absence of endothelial cells, such cell network formation does not appear. The transplantation of triple-layered cardiac cell sheets including EC networks into rat dorsal subcutaneous tissues or rat infarcted myocardial tissues induces the migration of ECs to host tissues over a few days [60, 64]. Tubular microvessel networks form from these ECs in transplanted cell sheets and connect to the host vessel over several days. Transplanted cell sheets can survive for longer periods without necrosis. Furthermore, the transplantation of cardiac cell sheets containing EC networks improves the functioning of infracted myocardial tissues [60].

Additional transplantation of cardiac cell sheets containing EC networks facilitates the connection between the EC network and newly formed microvessels in implanted cardiac cell sheets. More specifically, the stepwise transplantation of triple-layered cardiac cell sheets allows for the fabrication of ca. 1 mm-thick cardiac tissues composed 30 layers of cardiac cell sheets with well-organized vascular networks *in vivo* [64].

Based on the insight gained from these experiments, novel bioreactor systems are developed for the fabrication of engineered cell sheet-based tissues with vascular structures formed *in vitro* [56, 61]. Two different types of vascular beds (a resected section of femoral tissues with arteries and veins preserved or collagen gel) are used in the bioreactor system. A cell culture medium with growth factors, such as VEGF and bFGF, is perfused to the vascular bed, on which cardiac cell sheets containing ECs are cultured. In both vascular bed systems, ECs in the cell sheets migrate toward the vascular bed, forming a vascular tubular structure. This tubular structure connects to vascular beds, capillaries of resected femoral tissues and microchannels in the collagen gel. Finally, the vascular structure matures to supply nutrients and O_2 to the deposited cell sheets. In the same way, thick and cell-dense tissues and multi-layered cardiac cell sheets are constructed on the vascular bed through step-by-step cell sheet transplantation as described above.

On the other hand, ECs seeded on a single myoblast sheet do not form such a vascular network. When sandwiching ECs between two myoblast sheets, ECs spontaneously form vascular networks with random orientations [70]. This shows that the 3D environments around ECs may dominate vascular network formation. More interestingly, when ECs are sandwiched between two anisotropic cell sheets, they form vascular formations oriented in the same directions as anisotropic myoblast cell sheets. ECs likely recognize the anisotropy of myoblast sheets. This result may suggest that EC vascular networks are controlled by anisotropic myoblast sheets.

Liver tissues are also composed of thick cell-dense tissues and require vascular formation in transplanted hepatic cell sheet tissues to survive and maintain liver-specific functions for long periods. In actuality, hepatic cell sheets transplanted into the vascularized mouse subcutaneous space generated by a bFGF releasing device prior to engraftment survive for more than 230 days with sustained secretion of albumin and α1-antitrypsin [52]. By contrast, hepatic cells in a biodegradable scaffold do not survive for long periods in a normal subcutaneous space. In addition, the transplanted hepatic cell sheet proliferates and grows in response to a regenerative stimulus (two-thirds liver resection for a recipient mouse). These results denote the importance of vascular network platforms for subsequent mini-liver construction.

Cell-based therapies for type 1 diabetes mellitus are developed using pancreatic islets. In clinical trials, islets are injected and transplanted into the liver through the portal vascular system. However, injected islets are not efficiently transplanted into the liver, probably due to blood-mediated and leukocyte inflammatory reactions, etc. To improve the transplantation efficiency of islets and their survival for the treatment of diabetes, islet-derived cell sheet-based therapy has been investigated based on vascularized subcutaneous spaces of diabetic-severe combined immunodeficiency (SCID) mice [55]. The nonfasting blood glucose levels in recipient SCID mice transplanted with islet-derived cell sheets decrease to a state of normoglycemia within a week, while mice without transplantation maintain a hyperglycemic state. The normoglycemia state is stably maintained for 110 days. By removing the transplanted islet-derived cell sheet, the nonfasting blood glucose levels in SCID mice immediately return to hyperglycemic levels. Histological and immunohistochemical analyses demonstrate that the islet-sheet not only newly forms a vascular network therein but also secrets insulin 4 days after transplantation into the vascularized subcutaneous space. The vascular formation releases insulin into the systemic circulation.

19.4.2 Temperature-Responsive Microcarriers for the Large-Scale Production of Cultured Cells

Much attention has been dedicated to the large-scale culture of undifferentiated cells, such as human MSCs, for therapeutic applications. Large-scale cell culture systems using microcarriers (MCs) are promising because of the large surface area to volume ratios of small particles. MC systems enables one to expand adherent cells such as stem cells to generate sufficient quantities for practical tissue engineering and cell therapy applications.

On the surfaces of MCs, cultured cells grow as monolayers suspended in a medium with gentle agitation. Subcultures of cells on MCs require ongoing trypsin treatment, causing damage to the cell membrane. Thus, non-invasive subculture systems of MCs are considered to preserve the viable and subsequent proliferative properties of recovered cells. Surface modification technologies with temperature responsiveness have been applied to MCs to collect cultured cells by lowering the temperature [75–77]. On the surfaces of chloromethylated polystyrene beads, surface-initiated ATRP has been applied to immobilize PIPAAm brushes on surfaces [75, 76]. Chinese hamster ovary (CHO-K1) cells on temperature-responsive MCs were found to expand 49.8-fold in a stirred suspension culture over 7 days. When temperatures were lowered to 20 °C, the kinetics of CHO-K1 cell detachment from temperature-responsive MCs was greatly influenced by both the grafted amount of PIPAAm and bead diameter. Typically, cell harvest efficiency increased with the bead diameter, presumably because the cell-cell junction on the surface is disrupted. The cell harvesting efficiency achieved using the optimized PIPAAm-immobilized MCs was 76.1 ± 16.3% with low temperature treatment.

Furthermore, the introduction of positively charged moieties into PIPAAm brushes improves the efficiency of cell detachment from MC surfaces while maintaining cell adhesion and proliferation [77]. Copolymerized PIPAAm brushes with quaternary amino groups show larger amounts of adsorbed anionic BSA than nonionic PIPAAm MCs. Hydration and/or repulsive electrostatic interactions on positively charged PIPAAm brushes facilitate the dispersion of MCs in cell culture medium containing CHO-K1 cells, resulting in enhanced cell proliferation. In addition, after temperatures are lowered to 20 °C, the efficiency of cell detachment for the positively charged MCs was larger than that for nonionic MCs. This higher efficiency is likely attributable to the enhancement of hydration with the introduction of positively charged moieties.

Consequently, temperature-responsive MCs exhibiting temperature-dependent cell adhesion/detachment properties can be utilized to develop therapeutic cells using large-scale cell culture.

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