reversed-phase chromatography and ion exchange chromatography. With newly developed charged,thermoresponsive column, those angiotensin peptides were effectively separated.

We have considered that the thermoresponsive polymer chain extension and aggregation through temperature dependent hydration/dehydration changes of PIPAAm molecules attached on the silica matrixes should be utilized to modulate ligand-receptor affinity control. Thus at high temperature, target molecules with receptor sequences can easily be interacted with surface immobilized ligand molecules because the co-grafted PIPAAm molecules are in dehydrated, and aggregated conformation. By lowering temperature, then hydration and expansion of PIPAAm molecules interfere the affinity binding of target molecules and surface immobilized ligand molecules, and eventually forced-releasing the substances without the addition of binding inhibitor molecules.[15), 16)]

We have thus been continuing the researches on the development of novel chromatography matrices with thermoresponsive PIPAAm and its derivatives as modifiers to realize new systems for effective separation of bioactive substances while maintaining their structure and functions using an aqueous mobile phase.

4 Creating Tissues from Cells

Let us now return to the subject of regenerative medical researches utilizing the thermoresponsive culture dishes for tissue formation. Variety types of the cells that have been seeded on thermoresponsive culture dishes are adhered, divided, and proliferated all over the surfaces of the culture dishes in the same way as with commercially available tissue culture polystyrene dishes (TCPS).[9)] With the cells on TCPS, digestive trypsin treatment is carried out to both dissolve the extracellular matrix (ECM) proteins and to chelate and remove the calcium ions. Without trypsin treatment, one cannot recover the cells from the culture dishes. Alternatively, a scraper can be used to physically peel them off the dish surfaces. With trypsin treatment, each of the individual cells is recovered as in suspension. When a scraper is used the cells can only be recovered as irregularly shaped and unstable tissue fragments. With thermoresponsive PIPAAm modified culture dishes, however, the cells are detached and can be recovered fairly easily in noninvasive fashion only by reducing the temperature from the culture temperature of 37°C to 20°C, the temperature at which the PIPAAm hydrates. Not only do the cells retain their intercellular connections, but the ECM proteins on the bottom of the cells is also released as a monolayer of contiguous tissue.[17), 18)] Furthermore, ECM proteins also act as an "adhesive" when transferring the cell sheets to a new culture dish or when forming multiple layers of tissue. For successful cell culture and recovery of the cell monolayers from the thermoresponsive surfaces, the previously mentioned PIPAAm graft amounts (or thicknesses) are extremely important and with a grafting density of 1.5~2.0μg/cm^2, at 37°C the cells adhere and can then be spontaneously recovered once the temperature drops below the transition temperature of PIPAAm molecules. At grafting densities of any less than this range, the cells will adhere and proliferate, but these cells will not be remained adhered even through the culture temperature was decreased below 32°C. Conversely, if the grafting density exceeds 2.0μg/cm^2 then the seeded cells will not adhere at all even at 37°C where the surface grafted PIPAAm molecules are in dehydrated and aggregated conformation. It is no exaggeration to say that this is the key to surface design that achieves cell adhesion and detachment control accompanying changes in temperature. With hepatic parenchymal cells and glia cells, irreversible cell damage occurs as a result of trypsin treatment, resulting in a major decrease in survival rate and cell function. On the other hand, it is of worth noticing that no such cell damage occurs when tissue is detached and recovered from the thermoresponsive culture dishes using low temperature processing. Furthermore, it has also been discovered that apoptosis does not occur during low temperature processing (from a few minutes through to approximately 2 hours at 20°C) with this method.[19)]

Considering "cell sheets" manufactured in this way as a single unit of tissue formation, using the strategy shown in **Fig. 3**, we are also investigating single layer tissue, multiply stratified tissue formed from the same cells and multiple layer tissue formed from more than 2 different kinds of cell sheets.

Single Layer Tissue: Epidermis cell sheets and corneal epithelial cell sheets are already being provided for transplantation immediately after recovery from culture dishes using low temperature processing in clinical applications, such as for treating burns and alkali burns. Usually, with corneal transplants only one cornea can be removed from each donated eyeball and stitches are necessary for transplantation of corneal tissues. By contrast, with transplants using corneal epithelium cell sheets, only a few square millimeters of limbal tissue are required to produce a single corneal epithelial cell sheet. Furthermore, as ECM proteins exist

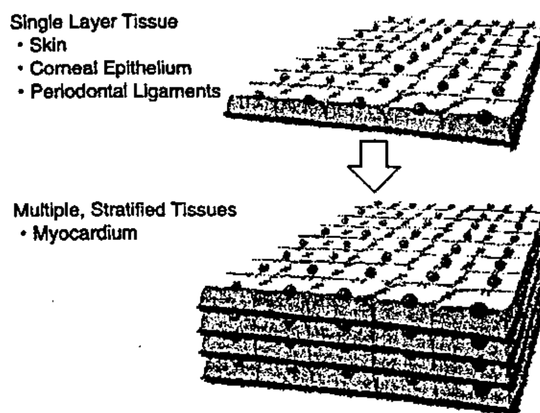

Fig. 3 Schematic drawings of the new concept of regenerative medicine to construct monolayer tissue and stratified tissues based on "cell sheet engineering" using thermoresponsive culture dishes

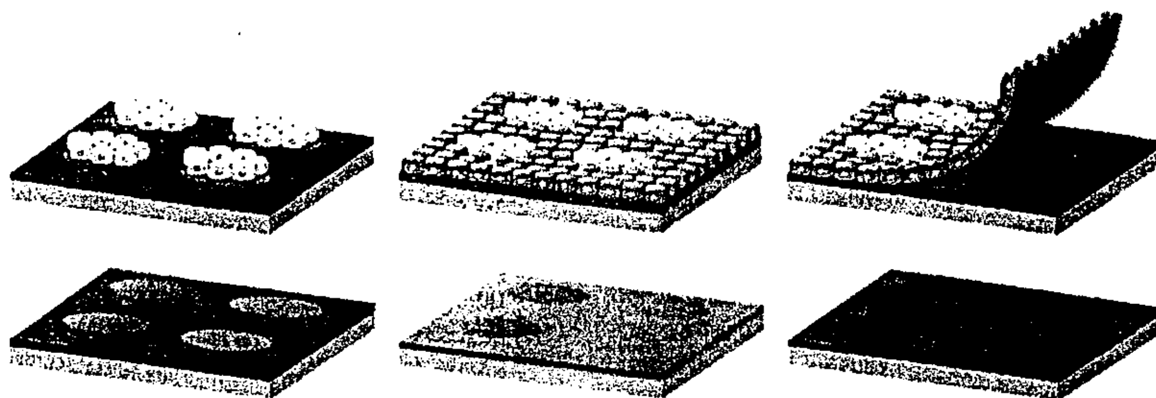

Fig. 4 Patterned dual thermoresponsive culture dishes for patterned co-culture of heterotypic cell types on one-dish surfaces

Two types of thermoresponsive polymers with different transition temperatures are fixed in an organized pattern. Cells are selectively attached to the hydrophobized domains at a temperature between the transition temperatures of the two polymers (left). Next, another cell type is seeded at a temperature higher than the transition temperature of both domains (center). Finally, a single co-cultured cell sheet is released and recovered at a lower temperature than either of the transition temperatures (right).

on the basal side of the cell sheets, it only takes about five minutes to complete the transplantation process without any stitches.[20] In addition, with cell sheet transplantation, as intercellular connection is maintained, this acts as a highly effective barrier immediately after transplantation.

Periodontal disease, such as alveolar pyorrhea, is a major problem in an aging society. At present, it is extremely difficult to regenerate gum tissue, meaning that the reliance is still on symptomatic treatment. We cultured gum tissue cells known as periodontal ligaments and made them into a single cell sheet. When this was transplanted to the gums, favorable regeneration was achieved with no periodontal pockets being formed.

Multiple Layer Tissue: We are considering the regeneration of the myocardiac tissue as an innovative treatment for heart attack victims. It is already possible to culture myocardial cells that have the ability to beat by themselves in synchronized fashion. If single cell sheets of these cells are formed into multiple layers, it is possible to regenerate heart tissue-like constructs to such a degree that one can see the cell sheets beating with the naked eye. Gap junctions are formed between multiple layers of myocardial cell sheets and we confirmed that the heart beat of multiple cell sheets was completely synchronized.[21), 22)]

When multilayered myocardiac cell sheets were transplanted onto the surfaces of the heart as a cardiac muscle patch, it started to beat in time with the heart of the host rat with myocardial infarction. Furhtermore, a remarkable improvement in heart function is achieved with tansplanted double-layered myocardial tissues.

This is sharp contrast with tissue transplantation techniques that have so far been investigated, such as injecting cardiac muscle cells in suspension, the proposed myocardial tissue transplantation may be useful as a new form of treatment with higher success rate and without cell rejection by the host.

Stomach and intestine tissue are currently used for the regeneration of the bladder in clinical situation, however, complications arise due to the secreting characteristics of epithelium of such tissue. However, when a bladder epithelium cell sheet was transplanted on to stomach tissue flaps from which the epithelium had been removed, none of the above-mentioned complications arose and the cells regenerated into tissue that is very similar to actual bladder tissue.[23]

Cell Patterning and Co-Cultured Cell Tissue: Electron beams used in the surface fixation of PIPAAm can easily be shut out using an extremely thin film of plastic or glass cover slips. Using such materials we fixed the PIPAAm molecules in a specific pattern and succeeded in culturing two different kinds of cells by controlling the culture temperature.[24), 25)] This kind of co-culture does not occur simply by seeding two different kinds of cells at the same time but by first seeding and culturing one kind of cell on a specific area and then seeding the second cell types. Applying such co-culture systems, we prepared patterned dual thermoresponsive surfaces with two kinds of thermoresponsive domains with different transition temperatures by copolymerization of a hydrophobic monomer to PIPAAm.[26] Co-cultured tissue was thus formed by culture each cell types in selective domains through temperature control and the kind of cells seeded (see **Fig. 4**, article accepted for publication). The co-cultured tissue was recovered into a single sheet at low temperature below the transition temperature of both domains.

It was noteworthy that the patterned co-cultured hepatic parenchymal cells showed significant functional increments in production of more albumin and ammonia metabolism to release urea as compared with those of monoculture hepatocytes.

This was made clear for the first time when we used patterned thermoresponsive culture dishes and we are currently analyzing this matter in more detail.

5 Conclusion

In this article we have provided an overview of part of our approach to tissue regeneration and the formulation of a separation system using a matrices modified with thermoresponsive polymers. Those thermoresponsive polymers greatly change their interaction with water in response to changes in temperature. The proposed new tissue regeneration technique, achieved by controlling both the graft densities and amounts of the thermoresponsive polymers, and the chromatography system that achieves high efficiency separation only in an aqueous environment, will become increasingly more important. Currently we are leading further researches aimed at achieving even further development. Although we have developed a new technique for activating cell function and carrying out tissue formation by utilizing polymer modification technology to achieve strict control on the nanometer level of polymer grafting and immobilization of bioactive sequences, we have decided to provide an explanation of such matters at a different time.

Researches we have introduced in this article is the results of the extensive research works of a large number of dedicated staff at this research institute, including Associate Prof. Masayuki YAMATO and Assistant Prof. Tatsuya SHIMIZU. The achievements mentioned in this article are the result of joint research with Prof. Motohiro NOZAKI and Research Assistant Prof. Kazutaka SOEJIMA of Plastic Surgery Department, Tokyo Women's Medical University regarding skin transplantation, Prof. Yasuo TANO and Assistant Prof. Koji NISHIDA of Osaka University regarding corneal grafting, Dr. Masaki HASEGAWA and Prof. Atsushi ISHIKAWA of Faculty of Dentistry, Tokyo Medical and Dental University for periodontal tissue regeneration, Dr. Shigeru MIYAGAWA, Associate Prof. Yoshiki SAWA and Prof. Hikaru MATSUDA of School of Medicine, Osaka University for cardiomyocyte sheet transplantation, and Dr. Yoshiyuki SHIRAYANAGI and Prof. Hiroshi TOMA of the Urology Department of Tokyo Women's Medical University for bladder regeneration, to all of whom we are exceptionally thankful.

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Nanostructured designs of biomedical materials: applications of cell sheet engineering to functional regenerative tissues and organs

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Abstract

Biomaterials surface design is critical for control of cell–materials interactions. Materials surface characteristics important to cell–materials interactions are the following: (a) nonfouling surfaces where cells cannot interact; (b) surfaces that interact with cells but do not alter cell morphology or metabolism (passive adhesion processes); and (c) surfaces that strongly interact with cells and cell–surface receptors to alter cell shape after metabolic interactions (active adhesion). In this paper, we briefly discuss the relationship between materials surface characteristics and cells for biomaterials designs in these categories. We have extensively investigated the thermoresponsive polymer, poly(*N*-isopropylacrylamide) (PIPAAm), as grafted surfaces allowing recovery of confluent cell monolayers as contiguous living cell sheets for tissue engineering applications. Cellular interactions with PIPAAm-grafted surfaces can be regulated vertically using the thickness of the PIPAAm-grafted layers in nanometer-scale levels, as well as laterally (spatially) using nano-patterned PIPAAm chemistry on various other surface chemistries. PIPAAm-grafted surfaces with 15–20-nm thick layers exhibit temperature-dependent cell adhesion/detachment control, while surfaces with PIPAAm layer thicknesses of more than 30 nm do not support cell adhesion. These changes in cell adhesion are explained by the limited mobility of the surface grafted polymer chains as a function of grafting, hydration, and temperature.
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Keywords: Cell-adhesive surfaces; Cell non-adhesive surfaces; Cell detachment; Hydrophilic surfaces; Nanophase separated surfaces; Thermoresponsive polymer; Cell sheet engineering

1. Introduction

Over several decades, an enormous array of materials has been produced for possible applications in biomedical research and clinical devices. Each material exhibits numerous, often uncontrolled interactions with proteins and cells both in vitro and in vivo, leading to various conclusions and determinations regarding

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materials "biocompatibility". Protein adsorption and subsequent cell adhesion behavior are frequently disadvantageous for desired functions of these materials. In biosensor devices, protein adsorption significantly reduces sensing abilities, leading to fibrosis, inflammatory complications, decreasing sensing efficacies and duration, with users often requiring implantable sensor replacements within several day periods. Furthermore, blood-contacting materials like artificial hearts and artificial vessels currently used in clinical treatments are limited to temporary, replaceable applications and/or replacement only of arterial vessels with relatively large diameters. Thus, most blood vessels with diameters smaller than 3 mm are replaced only with the natural vessels taken from cadaver donors, or as autografts. In these limited examples, but also nearly every application of materials in medicine, interfacial performance between materials and biological components, particularly cells and proteins, remains an unsolved problem and limitation to improved clinical response.

Cell–materials interactions are schematically illustrated in Fig. 1. Three categories of cell–materials interactions are defined. The first regards nonfouling surfaces where cells fail to interact with the surface over desired periods of time determined by each application. Such surface design is witnessed repeatedly in multiple reports of highly hydrophilic surfaces modified with poly(ethylene glycol) (PEG) [1–3], and polyacrylamide (PAAm) [4,5] as typical examples. The second category of interactions is "passive adhesion", in which interfacial response is controlled by physicochemical interactions between the material surface, adsorbed proteins and adhering cells. Surfaces in this category inhibit cellular metabolic changes from passive to active, receptor-mediated adhesion, but adherent cells remain intact and are readily detached from these surfaces with minimal or negligible damage [6–9]. This cell–surface interaction occurs reversibly and without metabolic signaling within the cell. A final interaction is categorized as "active cell adhesion" followed by passive adhesion, where cells resident on surfaces spontaneously activate receptor-mediated processes, change their morphology to spread and commence signaling processes typically of attachment-dependent phenotypes. Distinct metabolic processes using ATP and initiating cell signaling pathways are characteristic of this response. Spread cells rarely detach spontaneously from these surfaces without reversing events associated with morphology changes from spread to round cell shapes and release of surface-engaged receptors. Thus, enzymatic digestion of extracellular matrix proteins (e.g., using trypsin) and chelation of divalent cations as Ca^{2+} ions to de-activate cell integrin receptors are typically used to detach actively adherent cells. Several physicochemical characteristics of materials affecting cell-materials interaction are summarized in Table 1.

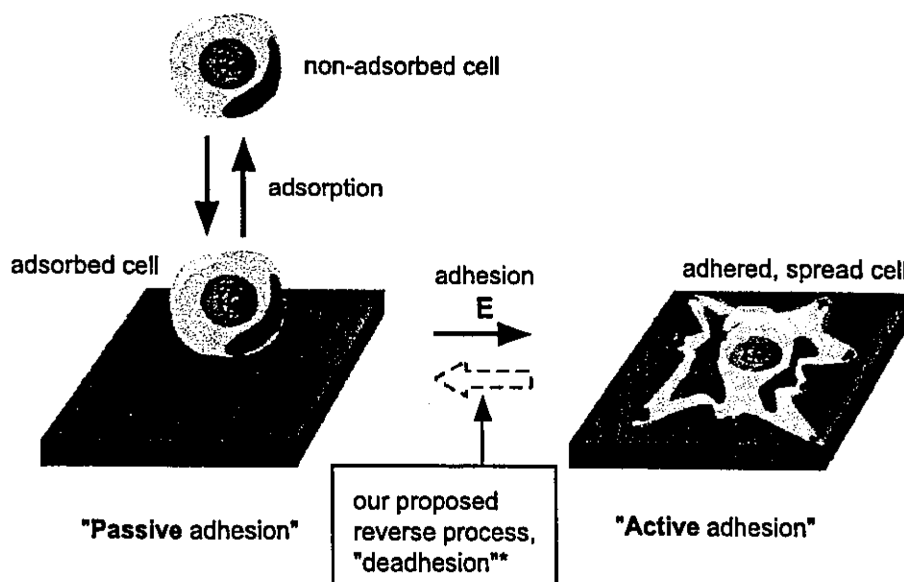

Fig. 1. Schematic diagrams for possible interactions of materials surfaces with cells.

Table 1
Physicochemical characteristics of materials affecting cell–materials interactions

• Surface roughness	
• Hydrophilic	PEG, PAAm, PVA
• Hydrophobic	PSt, PTFE
• Charges: positive	Poly(ethylene imine)
Negative	PAAc, Polystyrene sulfonate
• Nano-, micro-phase separation:	
Hydrophilic/hydrophobic	PHEMA–PSt–PHEMA [6,64,65], Pluronics [66]
Charge/non-charges	PHEMA-g-polyamine [67–69]
Crystalline/amorphous	Polyether-segmented polyamide [70–72]
Soft/hard	Polyether-segmented polyurethaneurea [73–77]
• Combination of above characteristics	[1,18,78]

In 1990, we found that many attachment-dependent cell types adherent on culture surfaces modified with nanometer-thick poly(*N*-isopropylacrylamide) (PIPAAm)-grafted chains at 37 °C can be spontaneously detached from these surfaces simply by reducing culture temperature below 32 °C for 30 min [10]. Such cell adhesion–detachment modulation on the surfaces grafted with thermoresponsive PIPAAm chains is a novel concept—no enzymes or chelators are required. Passive–active surface adhesion processes in cells can be experimentally activated using mild applications of cycling culture temperature. Over the past decade, we have extensively investigated the mechanisms of cell detachment from these surfaces [11,12], and reported possible applications in tissue engineering using the "cell sheet engineering" concept for several tissue-like cultures [13–15].

In the present paper, we briefly review the above-mentioned three categories of cell–materials interactions. Then, temperature-controlled cell adhesion/detachment behavior on the PIPAAm-grafted surfaces is reviewed in this cell–surface context, and finally opportunities to exploit this technology in tissue engineering applications will be summarized.

2. Cell non-adhesive surfaces

Cell non-adhesive surfaces, or nonfouling surfaces, have long been sought for clinical applications, especially for blood-contacting materials including artificial blood vessels, valves, and artificial heart devices, blood preservation bags, and catheters, but also in contact and intraocular lenses, shunts, implantable sensors, and many other device categories. These biomaterials seek largely to provide a clinical function while remaining largely 'inert' to adverse biological reactions. Attempts to attain these specific favorable characteristics on materials surfaces, highly hydrated, nonionic polymers are frequently immobilized, forming hydrophilic, diffuse layers on materials interfaces. Hydrophilic nonionic polymers used for this purpose include poly(ethylene glycol) (PEG) [1–3,16], poly(acrylamide) (PAAm), poly(*N*,*N*-dimethylacrylamide) (PDMAAm) [4,5,17], and poly(vinyl alcohol) (PVA) [4,18]. Nonfouling characteristics are often demonstrated in both in vitro

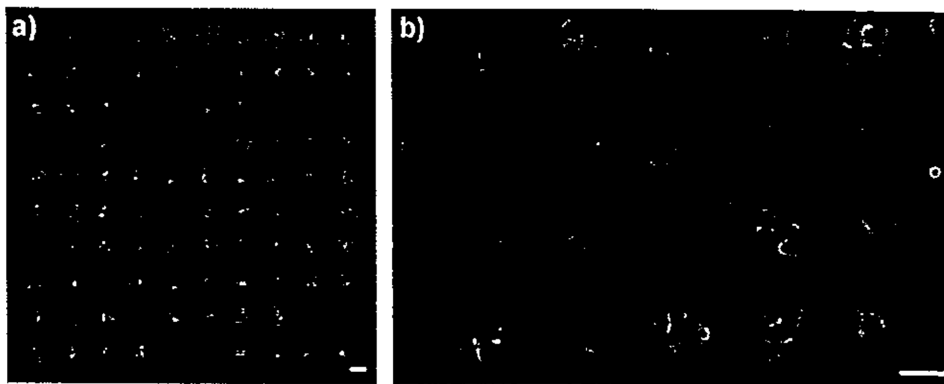

Fig. 2. Microscopic observations of selective cell adhesion on hydrophobic domains created on PAAm-grafted surfaces: (a) low magnification; (b) high magnification. Scale bar: 50 μm.

and in vivo experiments. However, it is difficult, if not impossible, to maintain long-term cell-resistant properties, especially when these surfaces are placed in static compartments (e.g., subdermal), or small veins where blood flow is very slow. Performance problems may derive from surface imperfections, insufficient density of grafted hydrophilic polymer chains, and gradual accumulation of small adsorbent molecules on the base surface. Thus, alternative methods for reliable, high density polymer grafting should be developed.

Utilizing cell nonfouling characteristics with grafted hydrophilic polymers, we recently developed new patterned cell culture dish surfaces [19]. Thermoresponsive PIPAAm-grafted surface domains grafted into desired shapes were fabricated using AAm monomer solution spread onto PIPAAm-grafted surfaces and followed by electron beam irradiation through a mask. Such modification allows us to obtain patterned surfaces for seeding cultured cells and subsequently generate cell sheets with appropriate gross morphology. Furthermore, we also modified solid surfaces with grafted PAAm followed by limited laser ablation to obtain surface patterns for creating cell arrays [20]. Fig. 2 shows a typical example of cell arrays made on PAAm grafted surfaces using limited excimer laser ablation. As seen in this figure, cells seeded on patterned surfaces adhered only within the ablated surface domains with no cells resident on non-ablated, hydrophilic PAAm surfaces. Such surfaces may be exploited to investigate basic biology of cell attachment, and drug screening in drug development and discovery.

3. Exploiting surface nano-topologies to prevent cell transitions from passive to active adhesion

We have extensively investigated the effects of surface microphase separation (cf. actually nanophase separation) of block copolymer surface coatings on their interactions with cells and biomolecules [6–9,21,22]. Triblock ABA types of the block copolymer, poly(2-hydroxyethyl methacrylate) (PHEMA)-b-polystyrene (PSt)-b-PHEMA was synthesized either by coupling bifunctional PSt and monofunctional PHEMA, or by living anionic polymerization of hydroxyl-protected HEMA and styrene (Fig. 3a). Block copolymer-coated surfaces display lamellae-type phase-separated structures with nanometer-scale domain widths (Fig. 3b). Such nanodomain sizes and morphologies can be regulated by the composition of two polymer units and their molecular weights. On these PHEMA–PSt–PHEMA surfaces, platelets adhere from platelet-rich plasma; however, adherent platelets do not change their morphologies or

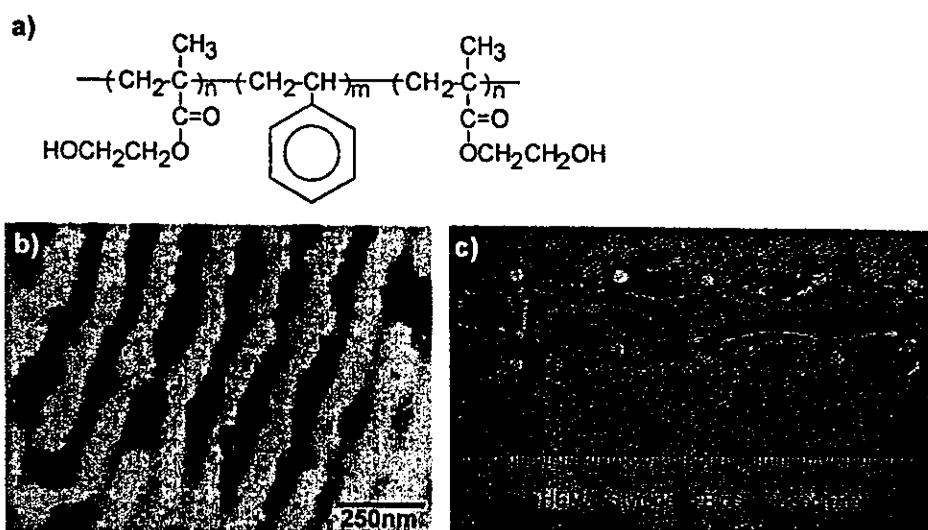

Fig. 3. Nanophase separated triblock copolymer surfaces with antithrombogenic characteristics. (a) Structural formula of the triblock copolymer, PHEMA-b-PSt-b-PHEMA. (b) Nanophase separation of the cast polymer observed by transmission electron microscopy. (c) Appearance of artificial vessels on the triblock copolymer-coated Dacron® graft 3 months after implantation into canine carotid artery.

degranulate to release reaction products, serotonin and 5-HT. Microscopic observations of these platelets on the PHEMA–PSt–PHEMA triblock copolymer surfaces showed that adherent platelets retain active motion-rolling, detachment, oscillatory vibrations, and directional changes—on these surfaces over controls [9]. These cell movements continued for more than 20 min and were distinct from those for inanimate PSt latex model particles. Treatments of platelets with an ATP synthesis inhibitor, sodium azide, or a membrane skeleton disrupting agent, dibucaine, significantly reduce platelet movement, indicating that platelets on PHEMA–PSt–PHEMA surfaces require metabolic process consuming ATP, and actively recruit the structural dynamics of their membrane cytoskeleton. Such energy-consuming movements may explain the observed low platelet activation on the triblock copolymer surfaces.

A triblock copolymer-coated 3-mm-diameter Dacron® graft was then evaluated in ex vivo experiments in canine carotid arteries [23]. On control PEG-grafted Biomer® surfaces, thrombus formation was apparent after 1-month implantation, while PHEMA–PSt–PHEMA surfaces were clear with no thrombus even after 3-month duration (Fig. 3c). These small diameter triblock copolymer-coated vessels were patent for more than a year. By transmission electron microscopic observations, multi-layer adsorption and conformational disruption of plasma proteins were apparent on PEG-grafted Biomer® surfaces. In contrast, only a monolayer of adsorbed albumin was present on triblock copolymer-coated surfaces, maintaining a native-like protein conformation [7,8]. Furthermore, this monolayer of adsorbed serum protein was maintained for extended periods.

We maintain that the unique surface nanostructure of PHEMA–PSt–PHEMA triblock copolymer surfaces dynamically modulates both serum protein composition and adsorption as well as adherent cells to produce nonthrombogenic characteristics in vivo.

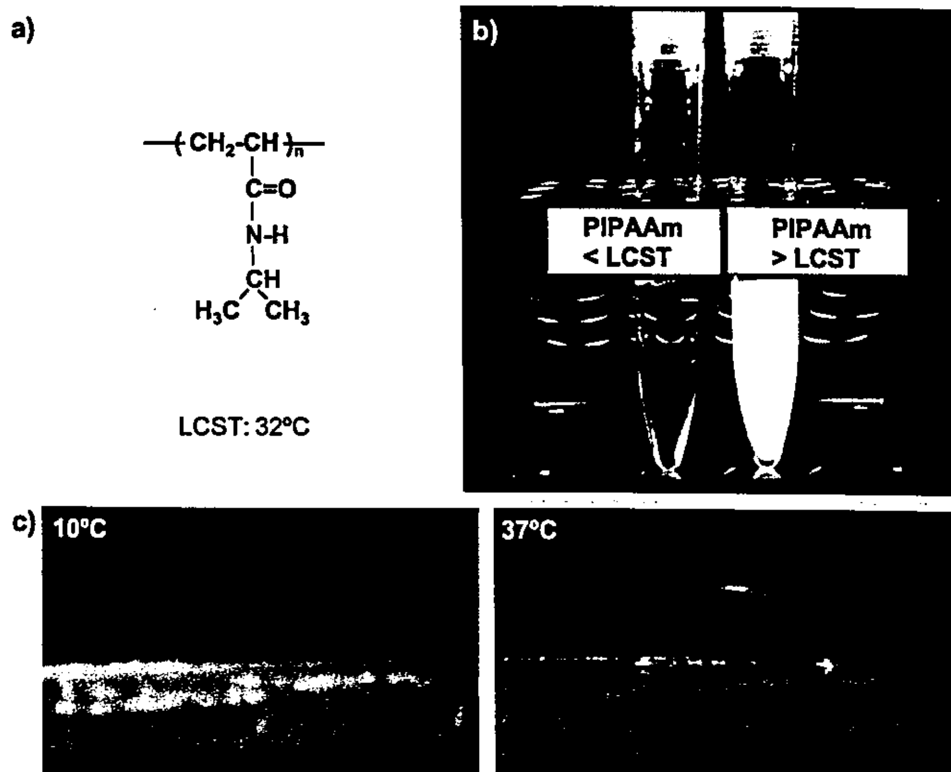

Fig. 4. Structure and properties of thermoresponsive PIPAAm in solution and on surfaces. (a) Structural formula for PIPAAm. (b) Thermoresponsive soluble/insoluble changes for PIPAAm in aqueous solution. (c) Temperature-dependent wettability changes for PIPAAm-grafted surfaces at 10 and 37 °C.

4. Using intelligent surfaces for reversible cellular adhesion and detachment

PIPAAm is now well-known to exhibit temperature-responsive soluble/insoluble changes in aqueous solution [24,25] (Fig. 4). This property can also be useful for PIPAAm-grafted surfaces. We have focused on temperature-responsive surfaces that demonstrate controlled hydrophilic/hydrophobic alterations, namely PIPAAm-grafted surfaces, as 'intelligent surfaces' controlled by external modulation of temperature [26,27]. Typical water contact angles on such surfaces change reversibly with temperature as shown in Fig. 4c. At temperatures below 32 °C, PIPAAm molecules are highly hydrated, and thus PIPAAm-grafted surfaces are hydrophilic. Above 32 °C, extensive PIPAAm dehydration occurs, resulting in an abrupt transition to hydrophobic surfaces. This change is completely reversible with temperature. Such surface characteristic changes have been exploited for new aqueous separations of bioactive compounds, including steroid hormones [28,29], polypeptides and proteins [30–33], and nucleic acids [34]. In addition to such regulation of molecular interaction with thermoresponsive polymer-modified surfaces, we have also achieved temperature-modulated cell adhesion detachment control [10]. In this system, commercially available tissue culture grade polystyrene (TCPS) dish surfaces are graft-modified with PIPAAm. Briefly, appropriate amounts of 2-propanol solutions of IPAAm monomer are spread uniformly over TCPS dish surfaces, followed by electron beam irradiation with a dose of 0.3 MGy at 150 kV. During this procedure, IPAAm monomer is both polymerized and simultaneously grafted covalently onto the TCPS surfaces [35]. These PIPAAm-modified surfaces demonstrate temperature-dependent wettability changes, being hydrophilic at low temperatures below 32 °C, and became hydrophobic above this temperature. Cells in culture respond to these surface changes.

Various types of cultured cells adhere and proliferate on PIPAAm-grafted culture dish surfaces under normal culture conditions at 37 °C with 5% CO_2 [10–12,36–41]. These adhesion–proliferation processes are comparable to that on conventional TCPS dishes. Such processes correspond to metabolically "active adhesion" detailed in Fig. 1, where cells actively consume ATP, move receptors on their membrane surfaces, and trigger cell signaling to change their morphologies from rounded to flattened, both for survival and to express phenotypic metabolic functions (gene and protein expression). Active cell adhesion and proliferation on the PIPAAm-grafted surfaces are observed on surfaces modified with PIPAAm amounts ranging from 1.5 to 2.0 μg/cm^2 [35,42]. On PIPAAm-grafted surfaces with PIPAAm lower than 1.5 μg/cm^2, cells adhere and proliferate appropriately, but these cells never detach from the surfaces by lowering the temperature below 32 °C unless a digestive enzyme (trypsin) is used. Likewise, cells do not adhere to surfaces with more than 2.0 μg/cm^2 PIPAAm or with a graft thickness of more than 30 nm [42]. This cell behavior can be correlated with similar surface presence of the cell-adhesive protein, fibronectin (FN) on the PIPAAm-grafted surfaces with different PIPAAm graft amounts. Because these modified surfaces are completely covered with grafted PIPAAm molecules as determined by angular-dependent X-ray photoelectron spectroscopic (XPS) analyses [43], retention of cell adhesion on PIPAAm-modified surfaces with lower amounts of grafted PIPAAm below 32 °C is not due to exposure of cell-adhesive TCPS PSt substrates, but to intrinsic PIPAAm interaction with adherent cell-adhesive components. Additionally, the observed failure of cultured cells to adhere to surfaces grafted with PIPAAm at high density at 37 °C cannot be attributed to a different graft chemistry. PIPAAm simply cast onto these TCPS surfaces often leaves several mg/cm^2 of PIPAAm molecules on these surfaces. However, such surfaces do not support cellular adhesion even though the seeded cells are highly adherent like fibroblasts (unpublished results). Thus, the grafting density of these chains is critical to performance in this context; the mechanistic ability of PIPAAm-grafted density to influence cell adhesion/detachment changes in culture is better considered in terms of its reversible alteration of surface wettabilities and grafted PIPAAm chains molecular mobility.

Surface wettabilities have been determined by captive bubble methods in thermostated Milli-Q water, and these data are summarized in Table 2 [42]. As seen in the table, all PIPAAm-modified surfaces show hydrophilic/hydrophobic property alterations with temperature changes between 37 and 20 °C. However,

Table 2
Physicochemical properties of PIPAAm-grafted surfaces with different graft densities

	Graft amount (μg/cm²)[a]	Graft thick (nm)[b]	Contact angle (θ)[c]		Cell adhesion at 37 °C[d]	Cell detachment at 20 °C
			37 °C	20 °C		
PIPAAm-1.4	1.4±0.1	15.5±7.2	77.9±0.6	65.2±1.2	yes	yes
PIPAAm-2.9	2.9±0.1	29.3±8.4	69.5±1.2	60.0±0.06	no	–[e]
PIPAAm-50	1080	5000	49.6 (40 °C)	11.5 (10 °C)	no	–[e]

[a] n=4, mean±S.D.
[b] n=4, mean±S.D.
[c] n=3, mean±S.D.
[d] Endothelial cell adhesion after 24-h culture.
[e] Not determined.

the higher grafted PIPAAm amounts produce lower aqueous contact angles at both temperatures. This implies that high-density PIPAAm layers retain larger amounts at both temperatures, meaning that their dehydration/hydration dynamics are reduced in a temperature cycle, producing less dramatic hysteresis effects on surface properties and on adherent proteins and cells. Such changes also are affected by the resulting chain mobility of PIPAAm-grafted surfaces as a function of density.

We reported previously that the graft chain configuration and thus the chain mobility [44] have a significant influence on the observed surface wettabilities of PIPAAm-grafted chains with temperature [26,27,45]. Multi-point grafted, and cross-linked hydrogel grafted surfaces show limited wettability changes with temperature, while the largest wettability transition was seen on single-point attached, freely mobile grafted PIPAAm surfaces. Furthermore, we previously found that the swelling changes for hydrophobized PIPAAm hydrogels copolymerized with n-butyl methacrylate (BMA) exhibit a sudden increase when the swelling fronts approaching the dry network core from both sides met each other [46]. We then compared the swelling behavior of single-side fixed, cross-linked thin and conventional network cross-linked PIPAAm hydrogels with identical thicknesses of 0.5 mm [47]. As expected from chain mobility arguments, fixation of hydrogels has a significant influence on swelling behavior: nonfixed hydrogels were highly swollen with a swelling ratio of more than 15, while the swelling ratio of the

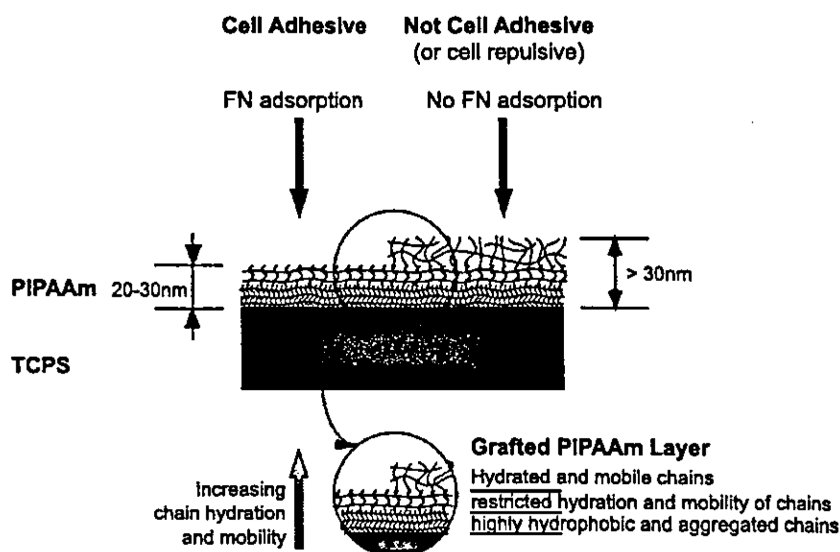

Fig. 5. Influence of PIPAAm-grafted polymer densities on cell-adhesive characteristics.

single-side fixed hydrogels was less than 1 even when the hydrogels were immersed in water at 10 °C. Furthermore, the swelling ratio for single-side fixed gels with 0.5 mm thickness was less than half of that for fixed gels with 1.0 mm thicknesses. Such differences derive from the different molecular mobilities in each cross-linked polymer chain system. Fixation of the polymer gels on glass surfaces limits chain molecular mobility in the vicinity of the glass interface, producing extensive hydrophobic aggregates and limited chain hydration. Such limited molecular mobility has a greater influence on the general network polymeric chains responses to external influences: these polymer chains are mutually connected through multiple three-dimensional cross-linking points so hindrance of hydration on one end hinders general network response. Thus, the outermost PIPAAm chains have more degrees of freedom, ability to respond to stimuli, hydration and mobility characteristics within both fixed and cross-linked PIPAAm hydrogels. This rationale can be further extended to limited chain mobility and amphiphilic nature of PIPAAm-grafted surfaces. Fig. 5 schematically illustrates the different chain mobilities possible for grafted chains of PIPAAm layers at the physiological temperature of 37 °C. At the TCPS substrate interface, grafted PIPAAm chains are highly hydrophobic due to strong hydrophobic interaction with the hydrophobic PSt interface and entropic resistance to hydration, and thus these chains aggregate and are immobilized with extended dehydration on TCPS surfaces at 37 °C. This nature of the grafted PIPAAm chains affects the second 'layer' of PIPAAm-grafted chains, which are then also restricted in their mobility, and thus hydration with water molecules is also limited. PIPAAm-grafted surfaces with such restricted chain mobility and limited hydration capabilities are likely to have temperature-regulated cell adhesion and "deadhesion" properties. PIPAAm-grafted surfaces with grafted polymer densities ranging from 1.4 to 2.0 μg/cm^2 (graft thicknesses of 20–30 nm) belong to this category. The third 'layer' of PIPAAm grafts consists of relatively hydrated and less restricted PIPAAm chains. On such surfaces with graft thicknesses of more than 30 nm, no cells, nor cell-adhesive proteins, interact strongly enough to influence cell attachment. PIPAAm surfaces with unlimited hydration and relatively unrestricted mobilities are unlikely to facilitate cell attachment. Therefore, controlling PIPAAm surface graft density is a critical issue to reliably produce temperature-responsive cell adhesion/non-adhesion behavior.

5. Cell manipulation for "cell sheet engineering"

As described in the previous section, PIPAAm-grafted surfaces with 20–30 nm graft layers support adhesion of a variety types of adherent cell types at 37 °C. Those adherent cells spread and proliferate normally to reach confluency. Confluent cell densities are comparable to those from unmodified TCPS surfaces. Single cells adherent on PIPAAm-grafted surfaces at 37 °C change morphologies from flattened to rounded morphologies similar to most cells just after cell seeding. Additionally, they proliferate normally to confluency at similar rates, and express normal phenotypic markers for each cell type, yet can be removed off PIPAAm hydrophilic surfaces after incubation at 20 °C. This last distinguishing feature is in sharp contrast from normal TCPS culture on which no cell morphological changes are observed by lowering culture temperatures.

Significantly, confluent cells on PIPAAm-grafted surfaces can be recovered as contiguous intact cell monolayers by lowering the culture temperature from 37 to 20 °C by avoiding use of digestive enzymes and chelating agents. An important difference in confluent cultures of cells versus single cells is the formation and maintenance of cell-to-cell connections in confluent cultures. Use of enzymes (e.g., trypsinization) for cell harvest destroys these cell–cell junctions, producing single cell suspensions. Use of PIPAAm allows temperature reduction harvest, retaining cell–cell junctions and permitting cell confluency maintenance post-harvest in large area sheets. Furthermore, these monolayers of cells maintain basal surface extracellular matrix proteins after detachment.

During temperature-dependent cell detachment from hydrated PIPAAm surfaces, cell metabolic changes occur [12,48]. Maximum cell recovery efficiencies for cultured endothelial cells on PIPAAm-grafted surfaces incubated at various low temperatures for 30 min and an additional re-incubation at 25 °C. Maximum cell recovery were

observed for endothelial cells first incubated at 20 °C, while hepatocytes pre-incubated at 10 °C showed a maximum recovery after re-incubation at 25 °C. These results imply that cell detachment behavior is governed by active energy-requiring metabolic processes. We also used several chemical reagents to alter cellular metabolism to investigate mechanisms of temperature-dependent cell detachment behavior. Cells treated with sodium azide, an ATP synthesis inhibitor, and with genistein, a tyrosine kinase inhibitor, did not change their morphologies and remain adherent on thermoresponsive PIPAAm surfaces even at temperatures below the surface transition temperature. The protein synthesis inhibitor, cycloheximide, slightly enhanced cell detachment from the surfaces. Furthermore, treatments with an actin filament stabilizer, phalloidin, and its depolymerizer, cytochalasin D, resulted in inhibition of cell detachment. These results suggest that cell detachment from the hydrated PIPAAm-grafted surfaces is mediated by intracellular signal transductions and reorganization of the cytoskeleton [48].

As cells adhered to PIPAAm-grafted dish surfaces proliferate to confluency, we have been able to recover confluent cultured cells as contiguous cell sheets by low temperature incubation only. Cultured cells reaching confluence in 37 °C culture are transferred to another incubator at 20 °C, prompting surface swelling and detachment. Cell morphologies were investigated periodically under a light microscope during this transition. Cells begin to detach spontaneously from the periphery of the dish surfaces, similar to the opening of curtains, upon lowering culture temperature (Fig. 6). During cell detachment, cell-to-cell connections are maintained, producing some sheet contractility upon surface release. To prevent cell sheet shrinking, we have used rigid support membranes for cell sheet recovery and manipulation, namely transfer membranes composed of chitin membranes [13], poly(ethylene terephthalate) (PET) membranes [13], and hydrophilic poly(-vinylidene difluoride) (PVDF) membranes [14,19]. Cell sheets interact with these support membranes through physicochemical interactions produced by interfacial tension, cell–surface protein adsorption to the membranes, and mechanical effects from edges of cell adsorbed to the support membranes. As the basal surface of the recovered cell sheets retains native extracellular matrix (ECM), these ECM proteins act as adhesive to new surfaces [49]. This intrinsic adhesion has proven useful for spontaneous cell sheet adsorption to carriers, but also for adhesion of sheets to other cell cultures and native tissues as explained below. With many cell types, we have now shown consistent

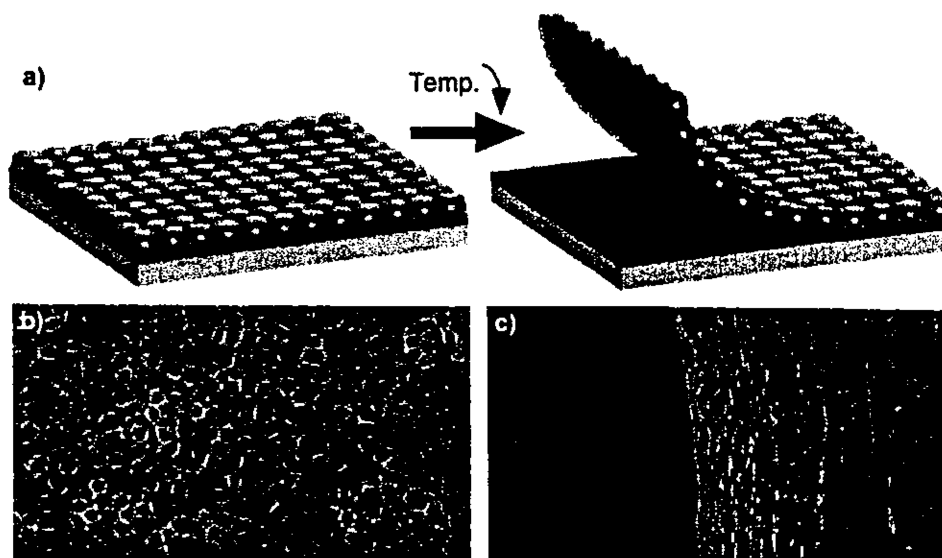

Fig. 6. Cell sheet engineering using PIPAAm-grafted surfaces. (a) Schematic illustration for temperature-induced recovery of intact monolayer cultures. (b) Confluent culture of endothelial cells on PIPAAm-grafted dishes at 37 °C. (c) Detaching endothelial cell sheet by lowering culture temperature to 20 °C.

cell sheet harvest performance for confluent cultured cell monolayers on grafted thermoresponsive culture surfaces. Cell sheets with high retention of phenotype and viability are routinely completely detached and recovered onto support membranes, then transferred to new surfaces of culture dishes or onto other cell monolayer constructs.

We term this new cell manipulation technology as "cell sheet engineering", and have utilized the technique to prepare a variety of types of cell sheets explained in further detail below. Recovered epidermal keratinocyte sheets from thermoresponsive dishes [39] are currently used clinically to treat burns and bruises. Very recently, in collaboration with Nishida et al. [41], we developed a clinically useful corneal epithelial cell sheet culture and have shown that corneal epithelium sheet transplantation to the cornea is an effective treatment for patients suffering from alkali burns, or from the Stevens–Johnson syndrome. In such cases, however, corneal epithelium for cell sheet construction is obtained from donated eye tissues, and only one patient eye can be treated with one donated eye. To overcome this limitation, we have exploited other methods. Corneal stem cells are known to exist in the limbal region of the cornea. Thus, we took 2 mm^2 biopsies of limbal tissue and cultured it on thermoresponsive culture dishes. Proliferated corneal epithelial cell sheets were recovered by low temperature treatment, and then these sheets were transplanted to human patient eyes. Retention of cell basal surface ECM proteins facilitated spontaneous transplanted corneal tissue sheet adherence to corneal stroma of the patient's eye within 5 min; suturing was not required for transplant stabilization. We have now treated 12 human patients with these corneal epithelial cell sheet transplants obtained on thermoresponsive culture dishes since December 2002, and all patients have dramatically improved their eyesight short- and long-term (unpublished data).

We have also utilized thermoresponsive culture dishes to prepare multi-layer tissue constructs with both homotypic [50] and heterotypic cells [51]. Cardiomyocytes obtained from neonatal rats were cultured on thermoresponsive dishes and grown to confluency. During low temperature detachment of the cardiomyocyte cell monolayers, we observed a spontaneous, continuous, large, pulsatile cell sheet contractile movement (i.e., oscillation) in cardiomyocyte sheets. This spontaneous beating distinguishes the cardiomyocyte phenotype and is retained upon harvest and sheet manipulation. Each sheet exhibits a distinctive and unique beat frequency [52]. Of great interest, stratified tissues comprising double, overlayered cardiomyocyte sheets completely synchronize electrically and morphologically, shown to be correlated with the presence of connexin 43 between the double-layered cell sheets. Multi-layered cardiac sheet grafts subcutaneously implanted into nude rats exhibit sheet-synchronized pulse frequencies independent of the recipient heartbeat. Implanted subcutaneously, these sheets retain these autologous beat frequencies and, over time, angiogenesis within the implanted tissue sheets is also observed [53]. Such vascularization will support grafted tissue viabilities and functioning within recipient animals, particularly for thicker cell sheets required to actually alter heart mechanics through topical application and intrinsic pulsation. Sawa and Matsuda, our colleagues at Osaka University, recently transplanted double-layered cardiomyocyte sheets into infarcted hearts of model rats, and found that heart stroke capacity was recovered up to ca. 70%, while untreated rats suffered from insufficient cardiac stroke output (unpublished data).

For heterotypic cell stratification, cultured endothelial cell sheets on PIPAAm-grafted dish surfaces were recovered by low temperature incubation, and then transferred onto confluent hepatocytes culture [51]. Generally, hepatocyte monoculture is maintained for only a week in vitro, with significant loss in both cell numbers and albumin synthesis within hepatocytes. In sharp contrast, hepatocytes layered with endothelial sheets were viable for over 40 days, secreting albumin during this extended co-culture. Thus, this new co-culture method is valuable for constructing and maintaining highly functioning stratified tissues for regeneration of function.

6. Functionalization of thermoresponsive surfaces

To provide more specific bioactive cues on culture surfaces to modulate cell interactions, we synthesized a new IPAAm derivative with reactive carboxylate functionality, 2-carboxyisopropylacrylamide (CIPAAm) [54]. This compound has a similar structure as the IPAAm monomer with thermal

transition temperatures of their copolymers remaining constant regardless of copolymer composition [55]. We then introduced CIPAAm onto TCPS dish surfaces through electron beam polymerization, producing surfaces with thermal-responsive properties but also reactive functional groups. Many types of cells were cultured on the resulting surfaces at 37 °C, and these cells detach spontaneously after incubation at 20 °C, analogous to PIPAAm surfaces. Cell detachment rates from CIPAAm copolymer-grafted culture dishes were accelerated, probably due to the existence of relatively hydrophilic CIPAAm carboxylate anions at pH 7.4 that induce rapid hydration changes of the surface-grafted polymer chains [56]. More importantly, these carboxyl groups on CIPAAm sequences can be utilized to immobilize the cell-adhesive tetrapeptide, Arg–Gly–Asp–Ser (RGDS), via amide bond formation [57]. RGDS-immobilized thermoresponsive surfaces support cell adhesion and detachment even in the absence of fetal bovine serum (FBS) [57,58]. While FBS is ubiquitously used in cell culture, its use in clinical applications is now being avoided because of risk of exposure to animal-derived pathogens. Alternative culture methods avoiding FBS for cell therapies involving humans are desired. Using CIPAAm surfaces at low temperature where the surface-grafted polymer chains were hydrated, interactions between cell adhesion peptide molecules and cell membrane integrin receptors are disrupted both spatially and mechanically. This was confirmed by using hydrophilic and freely mobile poly(ethylene glycol) (PEG) chains as spacers between the CIPAAm thermoresponsive copolymers and the RGDS molecules. This PEG-based system suppressed cell detachment even at low temperature incubation. Modification of the thermoresponsive surfaces with cell growth factors is also now possible.

Another surface functionalization method involves micropatterning cell culture surfaces to modulate site-selective cell adhesion and detachment with temperature cues [59,60]. Partial patterned grafting of PIPAAm selectively onto TCPS surfaces enabled us to pattern cells in co-culture through differential temperature modulation. Metal masks with hexagonally arranged circular holes (diameter of 500 μm or 1 mm) were used to pattern grafting of PIPAAm during electron beam polymerization. Thus, only PIPAAm-grafted domains showed temperature-responsive characteristics while the unexposed TCPS matrix remained unmodified. Cells plated and cultured on the PIPAAm-pattern grafted surfaces adhered at 37 °C. At 20 °C, only cells on the TCPS regions remain adherent cells on hydrated PIPAAm-grafted domains readily detach using gentle pipetting. Resetting the temperature to 37 °C allows the vacated PIPAAm-grafted domains to become cell-adhesive again. Subsequently, other cell types can then be added to selectively adhere to PIPAAm surface locations without previously resident cells (remaining only on TCPS areas). Two cell types can thereby be simultaneously co-cultured in determined spatial arrangements resulting in viable patterned co-cultures not readily available by other means [59]. This method has been used to co-culture hepatocytes and endothelial cells [59].

In related work, we have also prepared patterned dual thermoresponsive surfaces for co-culture of heterotypic cells and then recovered them as a contiguous cell sheet [61]. To accomplish this, the surface must have two patterned surface chemistries with two distinct thermal phase transitions. For these patterned dual thermoresponsive surfaces, the transition temperatures for surface-grafted polymers were modulated using copolymerization of IPAAm with hydrophobic *n*-butyl methacrylate [62]. We had shown previously that PIPAAm aqueous thermal transition temperatures can be highly varied using copolymerization with hydrophilic and hydrophobic co-monomers [63]. Polymer incorporation of small amounts of BMA permits cell adhesion and detachment at a lower temperature than that observed on PIPAAm homopolymer-grafted surfaces [62]. These properties permitted reliable surface modification of culture dish surfaces using patterned dual thermoresponsive polymer grafting. Co-monomer BMA in 2-propanol solution was spread over PIPAAm-grafted surfaces, followed by immediate electron beam irradiation through the patterned metal hexagonal mask. The resultant surface-grafted domains comprised PBMA co-grafted onto the underlayer of previous PIPAAm-grafted surface. Thus, grafted chains were not strict copolymers of IPAAm and BMA, although the doubly grafted surfaces exhibit temperature-responsive wettability changes at lower

temperatures than PIPAAm's transition temperature, indicating some modification of the exposed pure PIPAAm with secondary BMA grafting [62]. Angle-resolved X-ray photoelectron spectroscopic analyses of the PBMA-co-grafted domains revealed that PIPAAm sequences were concentrated near air–substrate interfaces while BMA sequences were buried more near underlying polystyrene surfaces (data not shown). Thus, the co-grafted domains maintain their thermoresponsive wettability changes; however, the original PIPAAm chain hydration and mobility was somehow restricted by the co-grafted BMA sequences, decreasing the transition temperature of the co-grafted domains. Surfaces prepared in this way exhibit three distinctive thermoresponsive surface wettability alteration states with temperature changes: (1) partly hydrophilic/hydrophobic, (2) completely hydrophobic, and (3) completely hydrophilic, observed all on the same surface. Utilizing grafted co-planar patterns of these distinct copolymer surfaces and their intrinsic property alterations with temperature, patterned co-cultures of hepatocytes and endothelial cells have been previously achieved [61]. At the interfacial boundary zones between these two cell types in planar co-culture, phenotypic hepatocyte functions—albumin secretion and urea synthesis by ammonium metabolism—were increased compared to homotypic cultures [57]. Such metabolic functions could also be increased with decreasing patterned domain sizes (hence, increased interfacial contact zones between planar co-cultured cell monolayers) without changing the ratios of the two cultured cell types. Similar confluent patterned planar co-cultures were finally exposed to low temperature incubation to detach all co-cultured cells from the patterned culture surfaces. These co-cultures spontaneously detached as single cell sheets without disrupting the connections between hepatocyte domains and surrounding endothelial cell matrix regions, indicating that hepatocyte–endothelial cell interactions are maintained during cell sheet detachment. As the co-cultured endothelial cell is considered suitable to induce angiogenesis within implanted tissues, these co-cultures and dual cell-type sheet constructs will be utilized to fabricate more complicated tissue mimics with vasculogenesis and, possibly, induced blood supplies in the future.

7. Conclusions

Well-controlled surface chemistry is essential for rational design of materials surfaces for modulating the cell adhesion and culture behavior. We distinguish three different categories of surfaces that interact with cells and proteins: (i) nonfouling surfaces such as PEG-grafted surfaces to which generally cells do not adhere under short-term culture, (ii) surfaces that interact with cells primarily through passive adhesion forces, on which cells adhere without cell receptor engagement or signal transduction, remaining morphologically unactivated; and (iii) active surfaces where cell signaling and ATP-dependent metabolic processes are active, using transmembrane signal processes to modulate cell adhesion/detachment. We have introduced our methods to achieve all three surfaces using surface polymer grafting, and described their cell interactions in culture. Nanometer-thick thermoresponsive polymer grafts on polystyrene cell culture surfaces are reliably fabricated, amenable to large scale processing, and exhibit versatile surface properties (co-planar domain patterning, varied thermal properties, variable cell adhesion properties) through polymer chemistry. On these surfaces, various types of cells adhere and proliferate normally to reach confluency at 37 °C. Reducing temperature triggers thermal transitions in the grafted polymer overlayers that alter the interfacial energies, wetting, hydration, mobility, and mechanics, thereby radically changing the cell–surface micro-environment. Adherent cells then spontaneously detach from these surfaces with decreasing temperature below PIPAAm's transition temperature. This property can be exploited in numerous different ways to achieve selective area cell removal and patterned culture, co-culture and contiguous intact cell sheet removal without the need for enzymes or culture additives that might damage cells or make them unsuitable for human use. Surface co-existence of two or more different chemistries, spatially controlled on the micron and sub-micron scales, has been shown to affect both cell and platelet adhesion processes, signaling and metabolic activation. New biomaterials surfaces that can be reliably and rationally designed to interact with cells and tissues in a responsive manner are of significant importance to obtain and maintain appropriate cellular functions in culture, and significantly, in co-culture, and possibly tissue-like properties for transplant use.

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―特別講演Ⅱ―

細胞シート工学を基盤とする再生医療

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1．再生医療テクノロジー

細胞工学，タンパク質工学，遺伝子工学の発展により，低分子物質を薬とすることから，タンパク質や遺伝子を薬として利用する新しい治療が大きく発展しようとしている。この時代の流れは，組織工学の登場により細胞や組織を利用する再生医療の局面を切り開き21世紀の医学を先導する新領域の確立に向けて大きく飛躍しようとしている。

20世紀は大量生産と大型のシステムが追求され，世界の産業は効率化と省力化のテクノロジーを経て画一から多様へ，マクロからミクロへ，構造から機能へと徐々にその対象を移行させてきている。これを可能にした最先端テクノロジーを産業分野のみならず医療分野に積極的に応用することによりこれまで以上に高度な医療技術の確立が可能になるものと期待される。21世紀に突入した現在，医療分野に超微小システム，ナノテクノロジー，バイオマテリアル，細胞操作テクノロジーなどの最先端テクノロジーを導入し，高度な医学，医療を達成することに大きな期待が寄せられている。

人工臓器研究は，人工心臓，人工腎臓に代表されるように，ほぼ完成期に到達し，医療に大きく貢献した。さらにこのような物理的・機械的な機能代行から人工肝臓，人工膵臓，人工神経などの高度な機能代行に向かう中で人工材料の生体適合性，抗血栓性，高度な機能性の実現が必要となってきている。このような状況の中で細胞を人工材料と組み合わせて利用するハイブリット型人工臓器，組織工学の研究が大きく飛躍することが期待されている。

一方，移植医療は免疫機能の制御技術が進む中でより多くの患者の治療の実現が期待されているものの圧倒的なドナー不足となっている。このような中で1990年代に入ってハーバード大 Vacanti J 教授と MIT の Langer R 教授が tissue engineering（組織工学）の系統的な研究推進の重要性を提案した。生体内で分解する乳酸—グリコール酸コポリマーでヒトの耳の形を作り，この足場の中に軟骨細胞を導入し，マウスの背中の皮下に埋め込んだ。ヒトの耳がマウスの背中にでき，衝撃的な写真を世界に示し，tissue engineering の重要性を主張した。これにより世界的な研究のブームを作り出した。生分解性高分子を足場にし，これに細胞を導入，成長因子の存在下で組織を誘導しようとする概念が示され，世界の研究者がこのコンセプトに基づいて組織再生に取り組むこととなった。

筆者はこの Vacanti の考え方で軟骨や骨などの比較的シンプルな構造の組織再生はできるものの，心臓，肝臓，腎臓などの複雑な構造の再生には新しい基本コンセプトが必要であると早くから考えていた。生体のどの組織や，臓器が細胞シートの重なりによって構築されていることに注目した。通常，培養皿上で単層に細胞を培養し，細胞シートを作製できる。しかし，培養皿上での細胞シートの構造と機能を損なうことなく剥離し，重層化する技術は存在せず，その技術開発に着手した。温度（32°C）で水和/脱水和の構造変化するポリ（N-イソプロピルアクリルアミド）を均一にしかもその微細構造を制御して共有結合で培養皿上に固定した。この表面は 37°C で疎水性（細胞接着性）であるのに対し，32°C 以下では親水性（細胞非接着性）に変化した。これにより 37°C で細胞を培養し細胞シートとした後に 20°C に温度を下げるだけでその片面の接着タンパク質を保持させて細胞シートを剥離することに成功した（図1）。