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

	Graft amount ($\mu g/cm^2$)[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

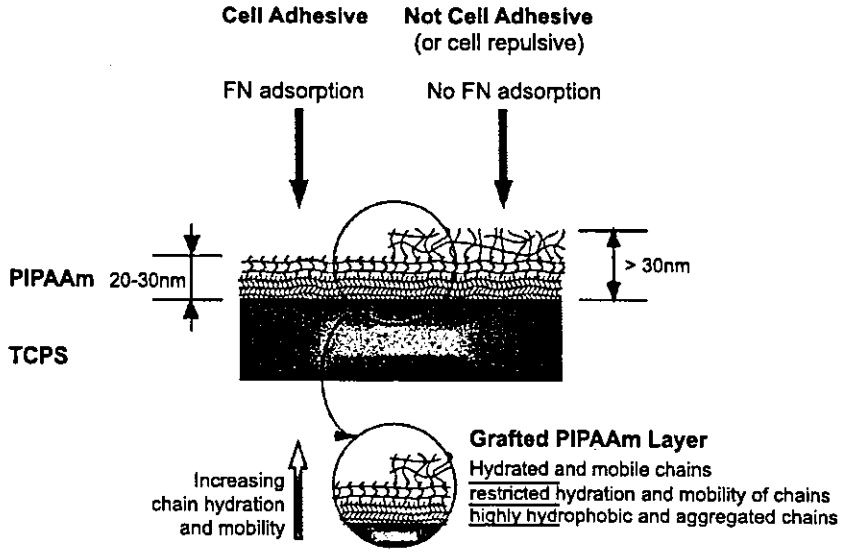

Fig. 5. Influence of PIPAAm-grafted polymer densities on cell-adhesive characteristics.

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 $\mu g/cm^2$ (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

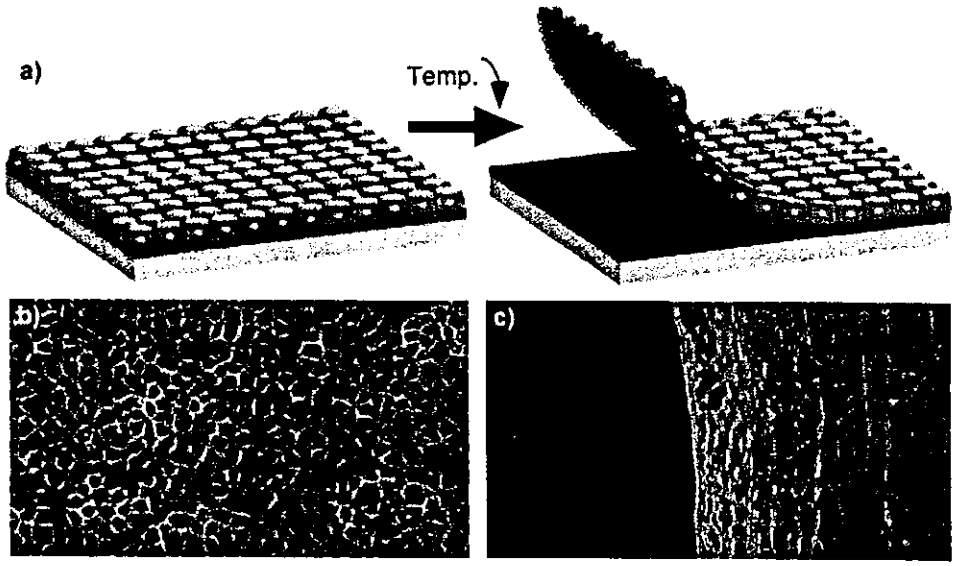

Fig. 6. Cell sheet engineering using PIPAAm-grafted surfaces. (a) Schematic illustration for temperature-induced recovery of intact monolayer cultures. (b) Confluent culture of endothelial cells on PIPAAm-grafted dishes at 37 °C. (c) Detaching endothelial cell sheet by lowering culture temperature to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

岡野　光夫

東京女子医科大学先端生命医科学研究所所長・教授

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剝離し，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剝離することに成功した（図1）。

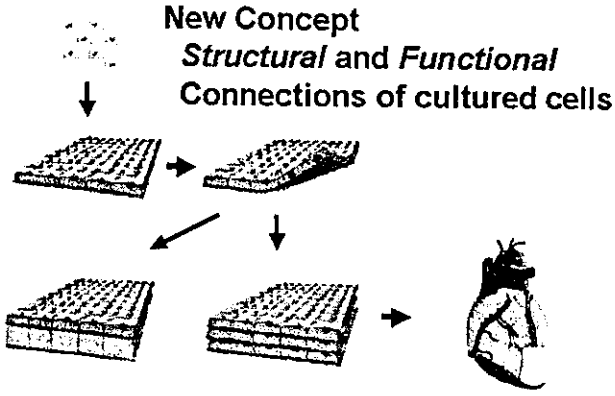

図1　Cell Sheet Engineering.

我々は培養床の表面をナノテクノロジーを基盤とする方法でインテリジェント化し，温度で表面構造を変化させ，細胞を培養した後に 37°C から 20°C に温度を下げるだけで細胞や細胞シートをその構造と機能を保持したままで剝離・回収することを世界に先駆けて成功させた。温度に応答して構造を変化させるポリ（N-イソプロピルアクリルアミド）（PIPAAm）を 20～30 nm の厚さで表面修飾し，これにより表面が親水性/疎水性の可逆的変化を示すことで細胞の接着/脱着を実現している。2 mm の角膜上皮細胞から直径 20 mm の細胞シートを作製し，移植させることができた。昨年12月より，大阪大学眼科田野教授，西田講師との共同で臨床が開始され革命的な治療が成功した。従来の角膜移植に対しこの再生医療は，より安全に，より効果的に，より簡単に治療することができ，世界的な注目を集めている。また，細胞シートを重層化させ，構造のみならず機能的にも連結させることができ，シャーレの中で拍動し続ける心筋組織を作ることができた。この重層化心筋細胞シートを拡散型心筋症あるいは心筋梗塞の治療に利用できる局面を作り，臨床に向けて研究を進めている。

東京女子医科大学先端生命医科学研究所は，医師と基礎研究者が共同で再生医療を追究しており，角膜，網膜，皮膚，膀胱，尿管，気管，血管，心筋，歯根膜，骨膜，軟骨，肝臓，腎臓，などの再生についてきわめてユニークな成果を上げている。細胞組織の作製は，毒性，薬物毒性や薬効の評価を動物試験に代わって行う新しい可能性があり診断システムの革新的な技術の開発が期待されている。

本年よりハイテクリサーチセンター「細胞シート工学研究センター」と COE「再生医療センター」がスタートし，今後，益々臨床家の研究への参加が必要になっている。大学院，先端生命医科学専攻への医師と工学研究者の参加を加速させ，再生医療の世界的な拠点形成を目指した研究活動を続けていきたい。

2．パターン化細胞表面の作製とその医療への応用

細胞は集合して組織・臓器を作り高度な機能を発現している。このとき，細胞間の直接的な結合と同時にサイトカインを介するコミュニケーションの役割を知り，それを制御する方法は細胞から組織・臓器を構築するためにきわめて重要である。

異種の細胞を 2 次元表面上でパターン化して培養する方法は，ただ単に異種の細胞を混合して培養することではできなかった新しい共培養システムを達成する点で興味深い。例えば，血管内皮細胞と肝実質細胞をパターン化することによって共培養が初めて可能になる。この方法により，細胞—細胞間の相互作用がより明確になることが期待される。化粧品や薬物の開発に，動物を利用することが次第に制限されてきている中で，機能のユニット構造，いわゆる細胞が集合して組織や臓器の機能を発揮する最小構造をアレイにした細胞チップは次世代型の診断・検査を実現するであろう。毒性試験や薬の機能試験に利用すれば 4 万の動物実験に匹敵する検査を一枚の細胞チップで行うことが可能となるであろう。

3．UV エキシマレーザーを用いた超微細加工

エキシマレーザーによるレーザーアブレーションは現在ではプリント基板の穴加工に用いられているほか，マイクロマシン分野でも広く利用されている光加工技術である。波長が長いレーザーでは発生した熱による変性が主となるが，波長を 200 nm 近くにまで下げると，炭素—炭素間の結合を選択的に切断し，副作用のない微細加工が実現できる。このようなレーザーアブレーションは基本的に非線形であり条件設定が難しいものの，

後述するように適当な条件を決めることができれば，他の方法では得ることのできない超微細加工がきわめて容易に実現できる。

我々はArFガスのUVエキシマレーザー（193 nm，5 ns）を用いて超微細加工を行っている。加工対象は数 μm～数 10 nm であるため操作はすべて顕微鏡の対物レンズ下で行う。XY 方向を独立に制御できる可変マスクと XY ステージとレーザー発振を同時にマイクロプログラムで制御するコンピューターを用いて，半自動の加工を実現した。

電子線照射により数 nm の厚さで固定化した高分子層を，約 100 nm の深さでレーザーアブレーションすることで，基盤として用いた高分子を再露出させる（図**2**）。

ここで基盤として用いる高分子と電子線照射により共有結合的に固定化した高分子の化学的性質を異なったものにしておくことで，化学的性質の異なるマイクロパターンが形成できる。

UVエキシマレーザーの照射条件を最適化することで，高分子の変性といった副作用を生じることなくマイクロパターンを形成することが可能である。ToF-SIMS を用いて，このような副作用が生じないことを確認している（図**3**）。本方法ではマイクロパターン化表面の洗浄などが必要なく，生体高分子を用いてパターン化グラフト層を作製することもできる。

このマイクロパターン化表面上に細胞を播種すると，マイクロパターンに応じた細胞接着が確認された（図**4**）。細胞は化学的性質の差異（ここでは高分子の親水性と疎水性の差異）を認識して特異的な接着挙動を示した。アブレーションの深さは 100 nm であり，このような化学的性質の差異がない状態では選択的な接着は生じないことに注意すべきである。

初代肝実質細胞を播種する系では，1辺を 30 μm にした正方形のドメインには1細胞が占拠し，1辺を 50 μm 以上にすると2～3個の細胞が同時に1つのドメインを共有することも明らかになった。

現在，本方法で作製したマイクロパターン化表面を用いて，細胞利用型バイオセンサーの作製を行うと共に，1細胞生物学，オリゴ細胞生物学とでも呼ぶべき新領域の創成に向けて努力している。これらの成果についても別の機会に紹介した

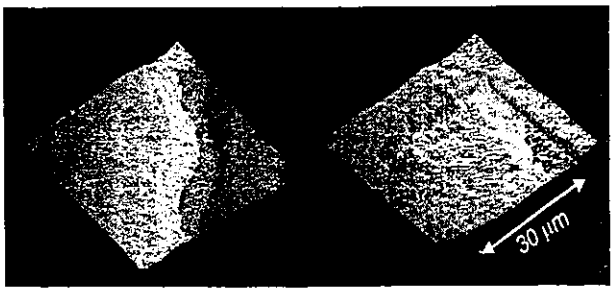

図2　作製したマイクロパターン化表面の3次元プロファイル。約**100 nm** の深さで均一にアブレーションされていることが分かる。

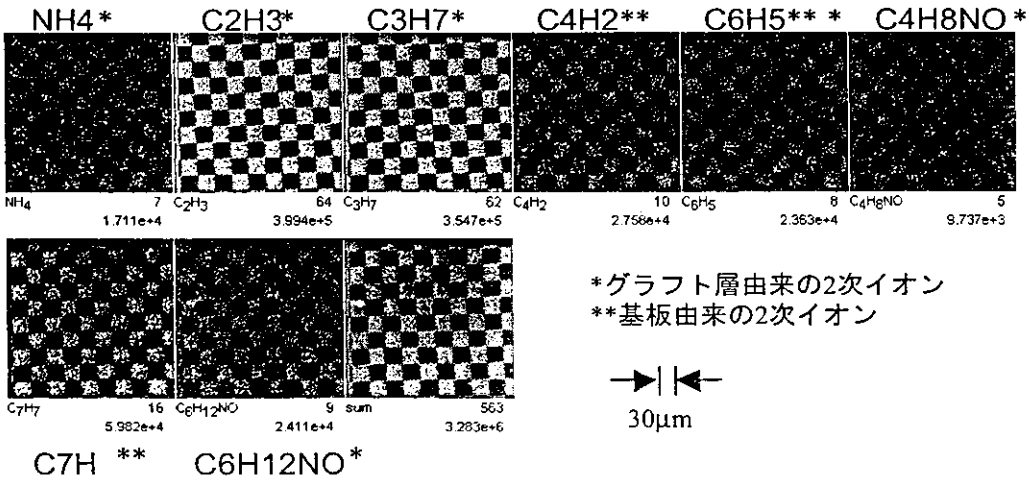

図3　作製したマイクロパターン化表面の **To-FSIMS**。両イオンが完全に相補的になっていることが分かる。

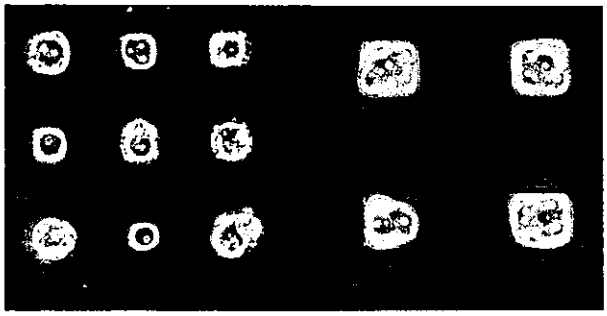

図4　作製したマイクロパターン化表面上に播種した肝実質細胞。

い。

光加工技術はウェットエッチングなどに比べ，はるかに低侵襲性でありバイオとの親和性は非常に良い。今後，さまざまな光加工技術が先進バイオテクノロジーの領域にますます進出していくことを期待している。

4．温度応答性パターン化表面と共培養細胞シートのマニピュレーション

下限臨界溶液温度（LCST）の異なるドメインを有する表面を作製した。ポリ（N-イソプロピルアクリルアミド）（PIPAAm）表面に円状でサイズの異なるマスクを通して電子線を照射し，疎水性のブチルメタクリレート（BMA）との共重合ドメインを作るとPIPAAmが32°Cに，共重合ドメインが25°C付近にLCSTを持つようにすることができる。BMA組成によってLCSTは制御可能であるので，自由にLCSTの異なるドメインをPIPAAm上に作ることができる。

このパターン化表面で温度制御により表面の疎水性を変化させ，それぞれのドメインに異なる細胞を播種，培養することができた。すなわち，ラットプライマリ肝細胞を27°Cで培養すると肝細胞はIBドメインのみに選択的に接着した。続いて内皮細胞を播種し，培養温度を37°Cにすると内皮細胞は疎水性化したPIPAAmドメインのみに接着し，肝細胞ドメインを内皮細胞の連続層が覆うような共培養を実現できた（図**5-A**）。その後培養温度を20°Cに下げ両ドメインを親水性化すると，共培養細胞層を1枚のシートとして回収可能であった（図**5-B**）。共培養細胞層において，両細胞が接近する部分で細胞機能の亢進が示されたばかりでなく，ドメインサイズを1 mmφから0.5 mmφに小さくすることで肝細胞は2倍以上のアルブミン合成活性を示した。このようにパターン化温度応答性表面を用い，細胞機能を亢進させた共培養組織の構築とシート状での回収ができることが明らかになった。回収共培養シートの重層化によって，より高次な機能を発現する組織を形成，移植することが可能になると考えられる。

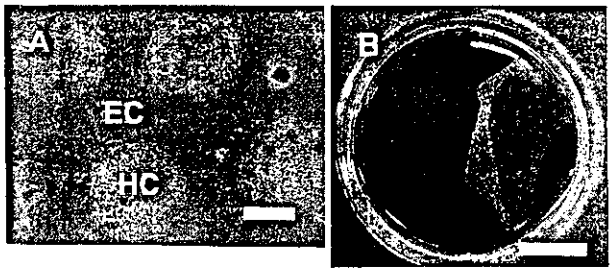

図5　**A: Patterned co-culture of hepatocytes (HC) and endothelial cells (EC) on patterned thermo-responsive surface. Scale bar＝0.5 mm. B: Lifting co-cultured cell sheet from pattemed thermoresponsive surface. Scale bar＝1 cm.**

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The use of patterned dual thermoresponsive surfaces for the collective recovery as co-cultured cell sheets

Yukiko Tsuda[a,b], Akihiko Kikuchi[b], Masayuki Yamato[b], Aiko Nakao[c], Yasuhisa Sakurai[a], Mitsuo Umezu[a], Teruo Okano[b,*]

[a] *Graduate School of Science and Engineering, Waseda University, 3-4-1 Okubo, Shinjuku, Tokyo 169-8555, Japan*
[b] *Institute of Advanced Biomedical Engineering and Science, Centre of Excellence (COE) Program for 21st Century, Tokyo Women's Medical University, CREST, Japan Science and Technology Agency, 8-1 Kawadacho, Shinjuku, Tokyo 162-8666, Japan*
[c] *Beam Application Team, Advanced Development and Supporting Centre (AD&S Centre), RIKEN, 2-1 Hirosawa, Wako, Saitama 315-0198, Japan*

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Abstract

Heterotypic cell interactions are critical to achieve and maintain specific functions in many tissues and organs. We have focused on patterned structure surfaces to enable co-culture of heterotypic cells and recovery of patterned co-cultured cell sheets for applications in tissue engineering. Thermoresponsive polymers exhibiting different transition temperatures in water comprise both poly(*N*-isopropylacrylamide) (PIPAAm) and *n*-butyl methacrylate (BMA) co-grafted as side chains to PIPAAm main chains. These copolymers were surface-grafted in patterns to obtain patterned dual thermoresponsive cell culture surfaces using electron beam polymerisation method and porous metal masks. On patterned surfaces, site-selective adhesion on and growth of rat primary hepatocytes (HCs) and bovine carotid endothelial cells (ECs) allowed patterned co-culture, exploiting hydrophobic/hydrophilic surface chemistry regulated by culture temperature as the sole variable. At 27°C, seeded HCs adhered exclusively onto hydrophobic, dehydrated P(IPAAm–BMA) co-grafted domains (1-mm∅ area), but not onto neighbouring hydrated PIPAAm domains. Sequentially seeded ECs then adhered exclusively to hydrophobised PIPAAm domains upon increasing culture temperature to 37°C, achieving patterned co-cultures. Reducing culture temperature to 20°C promoted hydration of both polymer-grafted domains, permitting release of the co-cultured, patterned cell monolayers as continuous cell sheets with heterotypic cell interactions. Recovered co-cultured cell sheets can be manipulated, moved and sandwiched with other structures, providing new useful constructs both for basic cell biology research and preparation of tissue-mimicking multi-layer materials through overlaying co-cultured cell sheets.

Keywords: Thermoresponsive surfaces; Surface patterning; Poly(*N*-isopropylacrylamide); *n*-Butyl methacrylate; Co-culture; Cell culture; Cell sheet

1. Introduction

Living tissues comprise multiple cell types wherein heterotypic cell-to-cell interactions influence and maintain the development of characteristic physiological functions and activities. Monocultures of cells rarely, if ever, manifest the diverse biological properties of living tissue. To mimic such heterotypic cellular interactions in vitro, a wide variety of surface modification technologies are utilised to produce surfaces that locate cells into predetermined arrays in culture. Patterned cell cultures are further applied in cell biology and biotechnology [1]. Surface-modification technologies used in array formation include soft lithography [2,3], photolithography [4–6] and inkjet printing [7]. Cellular functions, including growth, differentiation, and apoptosis may be controlled with the size and shape of cell adhesion areas in culture [2,3,8].

Patterned surface modification techniques are also utilised for co-culture of different cell types. Previous

*Corresponding author. Institute of Advanced Biomedical Engineering and Science, Centre of Excellence (COE) Program for 21st Century, Tokyo Women's Medical University, CREST, Japan Science and Technology Agency, 8-1 Kawadacho, Shinjuku, Tokyo 162-8666, Japan. Tel.: +81-3-3353-8112x30233; fax: +81-3-3359-6046.
E-mail address: tokano@abmes.twmu.ac.jp (T. Okano).

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reports [1,9] have described enhanced cell functions in heterotypic co-culture over homotypic cell cultures. Capabilities for harvesting and recovering heterotypic co-cultured cell monolayers should be both interesting and useful not only in cell functional assays but also for reconstruction and duplication of complex tissue and organ architectures for regenerative medicine and tissue engineering applications.

Bhatia et al. [1] succeeded in co-culture of two cell types in coordinated patterns of collagen-immobilised glass surfaces prepared by photolithography techniques. Their co-culture procedures, though, were rather complicated to coordinate cells in appropriate domains. Recently, we proposed a simpler patterned surface-modification technique using area-selective electron beam polymerisation of thermoresponsive poly(*N*-isopropylacrylamide) (PIPAAm) into domains on tissue culture grade polystyrene dishes. Using these patterned PIPAAm-grafted dishes, heterotypic co-culture of two cell types was achieved by modulating culture temperatures [10,11]. Maintaining phenotype and/or improving cell function can be achieved through this co-culture technique by selecting appropriate combinations of two cell types to be co-cultured. Novel surface designs of cell-adhesive chemistry patterned to facilitate surface site-selection of adhesive proteins and cells from media permit patterned co-culture. Analogously, surfaces patterning with thermoresponsive chemistry that exhibits significant surface property alterations with small changes in culture temperature can also be used to patterned co-cultured cell sheets and their application to tissue engineering research.

We have developed new thermoresponsive culture dishes for recovering various types of confluently cultured cell monolayers in single cell sheets and then applied to tissue engineering [12–14]. The thermoresponsive polymer, PIPAAm, shows temperature-dependent soluble/insoluble changes at its lower critical solution temperature of 32°C in aqueous media [15,16]. PIPAAm-grafted surfaces exhibit thermoresponsive hydrophilic/hydrophobic surface property alterations at 32°C, useful as novel modulating surfaces for new aqueous chromatography matrices [17–19], production of viable contiguous cell sheets [20–22], and their exploitation in constructing tissue mimics for further clinical applications [12,13,23,24].

In the present study, we introduce novel patterned dual thermoresponsive surfaces for co-culture and recovery of heterotypic cell types. We recently reported new thermoresponsive surfaces for recovering cells at pre-determined temperatures below the PIPAAm's transition temperature, 32°C, by introducing the hydrophobic monomer, *n*-butyl methacrylate (BMA) into PIPAAm-grafted dishes using electron beam-irradiated copolymerisation [25]. In this study, BMA was polymerised in patterns on PIPAAm-grafted dish surfaces to form patterned dual thermoresponsive surfaces. Temperature-regulated patterned co-culture of heterotypic cell types are investigated using these patterned thermoresponsive surfaces.

2. Materials and methods

2.1. Materials

Commercial materials used in modification of culture dishes were obtained as follows: tissue culture polystyrene dishes, Falcon 3001 (35 mm∅) and 3002 (60 mm∅) from BD Biosciences (Billerica, MA, USA); *N*-isopropylacrylamide, kind gift from Kohjin (Tokyo, Japan), was purified by recrystallisation from *n*-hexane; BMA from Tokyo Chemical Industries Inc. (Tokyo, Japan) was purified by distillation under reduced pressure and the fraction boiling at 80°C/1 mm Hg was used; 2-propanol for high-performance liquid chromatography grade from Kanto Chemical Co., Inc. (Tokyo, Japan). Materials used in cell culture studies were purchased from these respective companies: trypsin-ethylenediamine *N,N,N',N'*-tetraacetic acid·2Na (EDTA) solution, and antibiotics (streptomycin and penicillin) were from Gibco BRL Life Technologies (Grand Island, NY, USA); fetal bovine serum (FBS) from Morgate Exports Pvt. Ltd. (Bulimba, QLD, Australia); bovine serum albumin fraction V (BSA) and Dulbecco's-modified Eagle's medium (DMEM) from Sigma Chemical Co. (St Louis, MO, USA); rabbit anti-rat albumin polyclonal antibody and fluorescein-5-isothiocyanate (FITC)-conjugated goat IgG fraction to rabbit immunoglobulins were from Cappel, ICN Pharmaceuticals Inc. (Aurora, OH, USA); rhodamine-conjugated phalloidin and Hoechst 33258 were from Molecular Probes Inc. (Eugene, OR, USA).

2.2. Preparation of patterned thermoresponsive surfaces

Patterned dual thermoresponsive surfaces comprising P(IPAAm–BMA) co-grafted islands of 1 mm∅ within a PIPAAm-grafted surface matrix were prepared as schematically illustrated in Fig. 1. PIPAAm thin grafted layers were initially prepared on TCPS by electron beam irradiation (EB) (0.3 MGy, 150 kV, 10^{-5} Torr) using an Area Beam Electron-Processing System (Nissin-High Voltage Co. Ltd., Kyoto, Japan) [20,26]. PIPAAm-grafted dishes were extensively rinsed with cold distilled water, and then dried at 25°C under vacuum. In the second sequential process, a 2 w/w% BMA solution dissolved in 2-propanol (30 μl) was then spread uniformly over previously prepared PIPAAm-grafted surfaces. Aluminium masks with 1 mm-diameter hexagonally arranged patterned circular holes were placed onto the BMA spread PIPAAm surfaces,

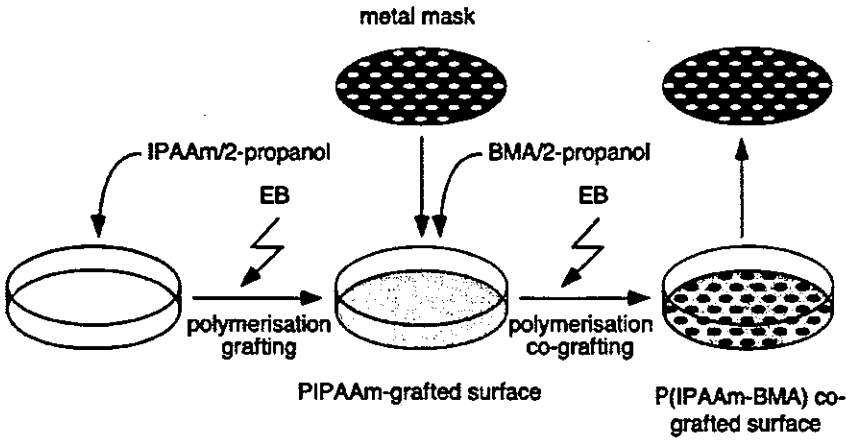

Fig. 1. Schematic diagram for preparation of patterned dual thermoresponsive polymer-grafted culture dishes.

followed by EB irradiation through the masks under identical irradiation conditions. Patterned surfaces were immersed in methanol overnight at 25°C to remove ungrafted BMA, washed with cold distilled water, and dried overnight at 25°C under reduced pressure. Dried dishes were sterilised with ethylene oxide gas prior to cell culture experiments. In addition, analogous homogeneously co-grafted P(IPAAm–BMA) TCPS surfaces were prepared without masks for surface analyses by ATR-FTIR and XPS.

2.3. Quantification of grafted polymers on TCPS surfaces

Grafted PIPAAm on each surface before and after co-grafting BMA was determined from infrared (IR) spectra using an attenuated total reflection Fourier-transform infrared spectrophotometer (ATR-FTIR, Valor-III equipped with an ATR-500M attachment, Japan Spectroscopic Co., Tokyo, Japan) [20,25]. Polystyrene as a base material of the modified surfaces (TCPS) has a strong absorption band attributed to mono-substituted aromatic rings at 1600 cm^{-1}. An absorption for amide carbonyl (amide I) from PIPAAm appears in the region near 1650 cm^{-1} [20]. The peak intensity ratio ($I_{1650/1600}$) was used to determine the amount of PIPAAm or co-grafted polymer on each surface using a calibration curve prepared for a known PIPAAm amount cast on TCPS dishes. An absorption band attributed to an ester carbonyl of BMA appears at 1750 cm^{-1}. However, no distinct absorption was acquired in this region for P(IPAAm–BMA) co-grafted surfaces, probably due to the minute amounts of BMA incorporated. Consequently, the amount of copolymer grafted onto TCPS dishes was correlated to the identical amount of pure PIPAAm in the present research.

2.4. XPS Analyses

P(IPAAm–BMA) co-grafted domains were characterised by angle-resolved X-ray photoelectron spectroscopy (AR-XPS) analyses. Angular-dependent chemical compositions on these surfaces were obtained using ESCALA 250 (Thermo Electron Corporation, West Sussex, England) with an AlKα-X-ray source (1486.6 eV, 300 W). Identification of elements present on the modified surfaces was obtained by wide energy range survey scans at high-scan rates, and then slower high-resolution scans were performed over specific energy ranges for quantification of individual elements. Survey spectra with a binding energy ranging from 0 to 1200 eV were collected at a take-off angle (measured from the substrate) of 90°. High-resolution acquisitions of carbon 1s (C1s) region was conducted with take-off angles ranging from 15° to 75° with respect to the surface to examine distribution of BMA within P(IPAAm–BMA) co-grafted domains in depth profiling.

2.5. Cells and cell culture

Bovine carotid artery endothelial cells (ECs) at passage 14 were purchased from Japan Health Science Foundation (lot no. 121495, Osaka, Japan). The cells were maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C under a humidified atmosphere of 5% CO_2 and 95% air. Cells were used between passages of 17 and 25 for all cell culture and cell detachment experiments. ECs were harvested with 0.25% trypsin and 0.02% EDTA in Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline (PBS) and subcultured at a 1:4 split ratio once a week. For co-culture study, rat primary HCs were isolated from 5 week-old male Wistar rats by the modified method of Seglen [27] as previously described using collagenase for cell dissociation [28]. The HCs

isolated in this way always proved to have more than 95% viability as determined by trypan blue dye exclusion tests. Culture medium used for HCs was DMEM supplemented with identical amount of FBS and antibiotics, 10 ng/ml epidermal growth factor (EGF), 10 mmol/l nicotinamide, 0.2 mmol/l L-ascorbic acid 2-phosphate, and 1% dimethylsulphoxide [29]. Cell morphology was monitored under a phase contrast microscope (Eclipse TE300; Nikon, Tokyo, Japan) equipped with a digital camera (FinePix S1Pro, Fujifilm, Tokyo, Japan).

2.6. Temperature-dependent cellular adhesion areal changes

ECs were plated on patterned surfaces at a cell density of 1.0×10^5 cells/cm^2 and cultured at 37°C or 27°C with a humidified atmosphere of 5% CO_2 for 3 days. Culture temperature was then changed, and cell adhesion behaviour of areal changes was monitored as a function of temperature.

2.7. Patterned co-culture and recovery of co-cultured cell sheets

Temperature-regulated pattern co-culture of the two cell types was investigated as follows: rat primary HCs were firstly seeded onto patterned dual thermoresponsive surfaces at 1.0×10^5 cells/cm^2 at 27°C after pre-incubation of dishes with DMEM including 10% FBS overnight at 27°C. After 5-h culture at 27°C, non-adherent cells were removed by gentle exchange of the culture medium, and then the dishes were allowed to stand at 27°C for 2 days and additionally at 37°C for another 2 days. The next cell type (EC) was then plated onto HC-cultured dishes at 1.0×10^5 cells/cm^2 and co-cultured at 37°C. All culture processes were carried out with the medium for HCs. Patterned culture dishes were transferred to a CO_2 incubator set at 20°C to examine recovery of 3-day co-cultured cell monolayers as single, continuous cell sheets.

2.8. Immunofluorescent studies on patterned co-culture of HCs and ECs

Cultured cells on the thermoresponsive surfaces were washed three times with PBS at 37°C and fixed with 4% paraformaldehyde in PBS for 20 min. Solutions were pre-warmed at 37°C to avoid hydration of grafted polymers on dish surfaces. After permeabilisation with 0.5% Triton X-100 in PBS for 2 min, cells were blocked with 0.1% BSA in PBS for 1 h and reacted with a 1:500 dilution of rabbit anti-rat albumin polyclonal antibody (5 mg/ml) at 4°C overnight, followed by incubation with a 1:1000 dilution of goat anti-rabbit antibody labelled with FITC (40 mg/ml) at 25°C for 2 h and finally washed with 0.05% NaN_3 in PBS. For F-actin staining, cells were double-stained with a 1:100 dilution of rhodamine-conjugated phalloidin (200 U/ml) at 25°C for 2 h. For cell nuclear staining, cells were triple-stained with a 1:500 dilution of DNA-binding dye, Hoechst 33258 (1 mg/ml) at 25°C for 5 min. These stained cells were observed under a fluorescence microscope (TE2000-U; Nikon) utilising equipped filter blocks. Wavelengths of each excitation (λ_{EX}) and emission (λ_{EM}) spectra for Hoechst 33258, FITC and rhodamine-conjugated phalloidin were λ_{EX}: 365 ± 10 nm, λ_{EM}: 400 nm for Hoechst 33258, λ_{EX}: 450–490 nm, λ_{EM}: 520 nm for FITC and λ_{EX}: 510–560 nm, λ_{EM}: 590 nm for rhodamine, respectively, using appropriate light filter units.

3. Results and discussion

3.1. Surface characterisation

Recently, we showed that cell adhesion/detachment control was achieved at lower culture temperature on PIPAAm derivatives copolymerised with hydrophobic monomer, BMA, than that on PIPAAm-grafted surfaces [25]. In this study, patterned dual thermoresponsive surfaces consisting of P(IPAAm–BMA) co-grafted islands and PIPAAm-grafted surface matrix were prepared by EB polymerisation using metal masks to establish patterned co-culture surfaces exploiting hydrophilic/hydrophobic changes from each domain chemistry. On the prepared surfaces, pattern formation of P(IPAAm–BMA) co-grafted islands and PIPAAm matrix was visible immediately after immersion in cold water due differences in surface wettabilities of each domain as seen in Fig. 2.

Fig. 2. Macroscopic view of the prepared patterned dual thermoresponsive surfaces immediately after washing with cold water. Scale bar: 1 cm.

In our previous reports [30,31], amounts of grafted PIPAAm on TCPS dishes was shown to influence thermal regulation of cell adhesion behaviour. On PIPAAm-grafted surfaces with low densities of PIPAAm (less than 1.5 μg/cm^2), cells adhere and proliferate as those on TCPS. However, cells also remain adherent: no cell detachment occurs even at 20°C, probably because grafted polymer hydration and subsequent surface property alterations are not sufficient to induce cell detachment at low PIPAAm graft density. On the other hand, cells do not show either adhesion or proliferation on surfaces grafted with more than 2 μg/cm^2 of PIPAAm. Amounts of grafted PIPAAm, P(IPAAm–BMA) co-grafted and P(IPAAm-*co*-BMA) (where IB5 represents 5% BMA mole fraction in monomer feed) were determined by FTIR-ATR measurements and are summarised in Table 1. The FTIR detection limit was 1.5–3.5 μg/cm^2 from FTIR standard curve. All surfaces are grafted with approximately 2 μg/cm^2 of PIPAAm, co-grafted P(IPAAm–BMA) or IB5 indicating no FTIR-observable differences in amounts of grafted PIPAAm before and after BMA co-grafting. This suggests that initially grafted PIPAAm on TCPS is not destroyed by a second EB sequential irradiation to co-graft BMA segments.

Table 1
Grafted amount of PIPAAm on prepared surfaces determined by ATR-FTIR

Surface	Amount of grafted PIPAAm (μg/cm^2)[a]
PIPAAm	1.81 ± 0.05
P(IPAAm–BMA)	1.80 ± 0.04
IB5	2.00 ± 0.02

[a] Data are expressed as the mean of three samples with standard error of the mean.

Surface elemental analyses of P(IPAAm–BMA) co-grafting were assessed by AR-XPS (detection limit ~0.1 at%), an effective method to determine surface compositional depth profiles in the copolymer film surfaces without etching them [32,33]. Angular-dependent chemical compositions are summarised in Table 2. The π–π^* shake-up peak originating from the base TCPS polystyrene substrate is apparent from 292 to 296 eV. This spectrum is a composite of the boundary layer between P(IPAAm–BMA) co-grafted layers and polystyrene. Since the π–π^* shake-up peaks were detected by AR-XPS between 45° and 75° take-off angles, data obtained at 15–30° take-off angles were used to analyse surface chemical compositions for P(IPAAm–BMA) co-grafted dishes. Only three (i.e., C, N and O) elements were detected, indicating that patterned, grafted domains consist of IPAAm and BMA units on the polystyrene base substrate and other substances do not contaminate the EB grafting process. Distribution of IPAAm and BMA within the P(IPAAm–BMA) co-grafted domains in depth profiling was determined by high-resolution C1s signals. The amide carbonyl originating from IPAAm and ester carbonyl from BMA side chains exhibit different C1s binding energy. The chemical compositional fraction of each monomer unit in the grafted layers is identified through the C1s peak deconvolution at each take-off angle. Depth profiling of P(IPAAm–BMA) co-grafted surfaces revealed that IPAAm sequences were concentrated near the air–substrate interface while BMA sequences were higher abundance near the polystyrene interface. This phenomenon is quite interesting since relatively hydrophilic IPAAm but not hydrophobic BMA sequences are distributed more to the air side. Samples were dried under vacuum after extensive water wash, which should influence the obtained XPS results.

Table 2
Angle-dependent compositional changes of IPAAm and BMA on P(IPAAm–BMA) co-grafted thermoresponsive surfaces by angle-resolved XPS analyses

Angle (deg)	Relative depth (sin θ)	C (%)	O (%)	N (%)	C1s peak composition			π–π^* shake-up
					CONH[a]	COO[a]	BMA/IPAAm	
90	1	82.1	11.1	6.3	n.d.[b]	n.d.[b]	—	n.d.[b]
75	0.97	82.2	10.7	7.0	0.12	0.04	—	0.02
60	0.87	82.2	11.2	6.6	0.13	0.02	—	0.02
45	0.71	80.2	12.6	7.3	0.12	0.02	—	0.02
30	0.50	78.7	12.4	8.9	0.17	0.09	0.53	n.d.[b]
25	0.42	79.3	12.5	8.2	0.18	0.03	0.17	n.d.[b]
20	0.34	78.6	12.2	9.2	0.18	0.06	0.33	n.d.[b]
15	0.26	78.4	12.2	9.4	0.19	0.04	0.21	n.d.[b]
Calcd.[c]		74.6	13.1	12.4				—

[a] Determined by peak deconvolution of C1s.
[b] n.d.: not detected.
[c] Calculated surface atomic composition for co-grafted polymers in bulk.