Mini Review

Bio-functionalized surface designs necessary for applications in regenerative medicine

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Modification of cell culture substrates and scaffolds with bioactive molecules is frequently used to enhance and/or regulate cellular functions and metabolism during culture. These biomaterials have contributed to an advanced understanding of cell biology and materials designs in biomedical applications. In contrast, we have proposed a new technology -- *cell sheet engineering* -- for realization of tissue/organ reconstruction with structural and functional regeneration. Using cell sheet engineering, confluently cultured cells are harvested as viable, contiguous cell monolayers applied for fabrication of three-dimensional biomimetic tissues or cell sheet-utilized therapies. We have recently introduced bio-functionalization of thermoresponsive surfaces with cell adhesive peptides, e.g., RGDS, and/or cell growth factors, e.g., insulin (INS), for rapid fabrication of cell sheets. Surface-immobilized RGDS peptides promote initial cell adhesion, while INS immobilization accelerates proliferation of adherent cells. More pronounced and synergistic influences on cell growth are observed on RGDS-INS co-immobilized thermoresponsive surfaces even under the serum-free culture conditions. Here, we briefly review the current findings of our bio-functionalized thermoresponsive surfaces for rapid, effective cell sheet fabrication and non-invasive harvest as tissue monolayers for further applications in regenerative medicine.

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Introduction

Much attention is dedicated to rational designs of biomaterials for cell culture and further utilization in tissue reconstruction and regenerative medicine. Materials in regenerative medicine must enhance and/or regulate cellular metabolism and functions associated with cell differentiation, proliferation, signaling, or

even apoptotic induction during culture. One simple approach for improving conventional biomaterials is their modification with bioactive proteins/peptides through adsorption or covalent immobilization[1-8]. Various materials have been developed for applications not only as cell culture substrates[1-6], but also as tissue engineering scaffolds and implants[7,8].

We recently established a new concept -- *cell sheet engineering*[9] -- as an alternative to conventional tissue engineering methods applied for regeneration of metabolically high functional tissues/organs. Exploiting our previously developed nanometer-thick thermoresponsive poly(*N*-isopropylacrylamide) (PIPAAm)-grafted surfaces, interactions between cells and materials surfaces can easily be modulated through temperature-dependent alteration of hydrophilic/hydrophobic surface properties without contamination or interference by proteolytic enzymes[10,11]. On PIPAAm-grafted surfaces, various cell types adhere and proliferate at 37℃. However, by reducing culture temperature to 20 ℃, below the PIPAAm's transition temperature, adherent cells spontaneously detach, and recovered cells like neurons or vascular endothelial cells can be subjected to transplantation without loss of their functional and metabolic activities. Confluently cultured cells are also harvested non-invasively as viable tissue monolayers[12] complete with their deposited extracellular matrix (ECM) proteins[13]. Harvested cell sheets can be applied for fabrication of three-dimensional biomimetic tissues by layering or stratifying cell sheets[14,15] or direct cell sheet-utilized therapies such as in reconstructing corneal tissues[16,17]. Some other engineered tissues such as periodontal ligament tissues[18] and esophageal tissues[19] are also available in animal models, and will likely soon be applied for clinical trials.

For further clinical applications of engineered tissue constructs, elimination of animal-derived materials and culture components will be an advantageous approach. We recently investigated immobilization of biomolecules onto thermoresponsive surfaces. Newly developed IPAAm analogue with carboxyl functional groups, 2-carboxy-*N*-isopropylacrylamide (CIPAAm), is introduced and copolymerized with IPAAm for covalent immobilization of popular RGDS peptides[20,21], the growth factor insulin (INS)[22], and their combination RGDS-INS[23], respectively. Immobilized RGDS peptides facilitate cell adhesion onto thermoresponsive surfaces, while INS immobilization accelerates growth of adherent cells. Much less immobilized INS is necessary to induce effective cell proliferation than with the addition of soluble INS to the culture media on unmodified thermoresponsive surfaces. Cells grown on these bio-functionalized thermoresponsive surfaces can also be recovered spontaneously by reducing culture temperature to 20℃, releasing the adherent cell sheets.

Here, we briefly review our new concept of cell sheet engineering and our current findings from bio-functionalized thermoresponsive surfaces for their utilization in rapid, effective cell sheet fabrication and non-invasive harvest. Influence of surface-immobilized biomolecules on cell adhesion, proliferation, and thermally induced cell detachment is also summarized here.

Modification of biomaterials with bioactive proteins and peptides

Modification of biomaterials with bioactive molecules for stimulated cell adhesion and growth has been investigated and reported with numerous findings in the fields of molecular cell biology and materials science. In particular, the cell recognition motif RGD is one of the most widely applied for improved biomaterials[24]. In this section, we review the background and the current findings of biomolecule-modified materials for biomedical applications.

1) Bio-functionalized cell culture surfaces

Gümüşderelioğlu et al.[2] synthesized poly (vinyl ether)-based hydrogels immobilized with RGD or INS and evaluated human skin fibroblast adhesion and proliferation behavior under the serum-free culture conditions. RGD-immobilized hydrogels showed higher cell adhesion, while INS immobilization demonstrated significant cell growth up to 2.5-fold more than RGD-immobilized hydrogels. Ito et al.[3] reported that INS-immobilized poly (acrylic acid)-grafted polystyrene films induced long-lasting activation of phosphatidylinositol-3 kinase, a downstream messenger of INS receptor in growth signaling, while signaling with soluble INS was transient. They also confirmed similar behavior using epidermal growth factor (EGF)[4]. Such continuous cell stimulation without ligand internalization is known as "membrane-anchored signal transduction"[25]. Growth factor-immobilized surfaces should artificially induce similar effects, leading to effective cell proliferation.

Surface patterning technologies have also been investigated, with photolithographic micropatterning as one of the most widely available techniques. Ito[26] reported micropatterned immobilization of biomolecules by coupling ligand molecules with photoreactive azidophenyl-derivatized poly (allylamine). Micropatterned EGF molecules accelerated proliferation of Chinese hamster ovary cells, while cultured cells on unmodified areas were unaffected. Whitesides' group[27,28] introduced the popular, innovative microfabrication technique, "soft lithography", using elastomeric poly (dimethylsiloxane) stamps or channels for pattern transfer or surface modification. Using this technique, pat-

terned self-assembled films of proteins on gold surfaces were generated[27,28]. Effects of adherent cell morphology on cell function and survival on these well-defined micropatterned surfaces varying in micron dimensions were also examined[29,30]. Furthermore, microfluidic channels were also obtained with similar procedures, and were successfully used for laminar flow cell patterning[29,30].

2) Bio-functionalized scaffolds and implants

Influence of biomolecules immobilization to three-dimensional scaffolds and implants has also been reported. Ho et al.[7] prepared RGDS-immobilized chitosan scaffolds and evaluated the effect of RGDS modification on cell adhesion and *in vitro* mineralization. Rat osteosarcoma cells densely adhered on RGDS-immobilized scaffolds compared with unmodified and negative control RGES-immobilized substrates. von Kossa staining showed enhanced mineralization of cultured osteoblastic cells on RGDS-immobilized scaffolds. These results suggest that immobilization of suitable biomolecules to scaffolds facilitates short-term osteoblast-like cell adhesion, proliferation, and following bone-like tissue formation. Ferris et al.[8] prepared RGD-modified titanium implants and evaluated short- and long-term cell attachment, morphology, and function *in vitro* and new bone formation *in vivo*. RGD modification is functionally stable *in vitro* and significant increases in new bone thickness around RGD-modified surfaces at 2 and 4 weeks were observed *in vivo*. Results suggest that peptide-modified metal implants may be used to enhance implant integration *in vivo*. Healy et al.[31,32] introduced RGD-modified interpenetrating polymer networks (IPNs) of poly (acrylamide/ethylene glycol-*co*-acrylic acid) on titanium (Ti) and investigated osteoblast behavior *in vitro*[32]. IPN-Ti and negative control RGE-IPN-Ti inhibited adhesion and growth of primary rat calvarial osteoblast cells in the serum-containing media, while RGD-IPN-Ti supported osteoblast attachment and spreading. von Kossa staining also clearly showed promoted *in vitro* mineralization on RGD-IPN-Ti despite exhibiting lower levels of proliferation than positive control tissue culture polystyrene (TCPS) surfaces, significant difference ($p < 0.0002$) was observed between RGD-IPN-Ti and unmodified Ti, IPN-Ti, RGE-IPN-Ti. They conclude that this approach may be continued pursuit of biomimetic surface modification for engineering osseous implant surfaces.

Thus, various techniques are used for modification of biomaterials to improve, enhance and/or regulate cell-based functions and activities, respectively. However, for applications of these biomaterials in regenerative medicine, several problems remain unsolved. Conventional cell culture recovery methods using proteolytic enzymatic digestion are not suitable for direct clinical use because harvested cells (their surface proteins and receptors) can be damaged, and some cell types (e.g., stem cells) are extremely sensitive to proteolytic degradation. Moreover, the architecture of many functional tissues/organs such as heart, liver, or kidney, consists of closely associated cells with comparatively little associated, interstitial ECM. For regeneration of such complicated functional tissues/organs, we propose that engineered tissue constructs should effectively recapitulate biomimetic cell-cell densely connected structures and functionally synchronous networks within host sites after implantation[33].

Bio-functionalized thermoresponsive culture surfaces

We have already reported our cell sheet engineering technology applied for biomimetic tissue reconstruction or cell sheet-utilized therapies[14-19]. In this section, we introduce our concepts of creating more advanced thermoresponsive surfaces immobilized with specific biomolecules for rapid, effective cell sheet fabrication and non-invasive harvest[20-23].

1) Preparation and characterization of bio-functionalized thermoresponsive surfaces

Newly synthesized CIPAAm[34], an IPAAm analogue with carboxyl side groups, was copolymerized with IPAAm (CIPAAm feed concentrations briefly used were 1.0 and 4.0 mol%) and grafted onto TCPS surfaces through electron beam irradiation methods[10,20-23]. Covalent immobilization of biomolecules onto these thermoresponsive surfaces was then performed via amide bond formation between surface-grafted CIPAAm carboxyl groups and N-termini of biomolecules as can be seen in Fig.1. Amounts of immobilized RGDS/INS onto thermoresponsive surfaces were quantified by immunoassay techniques in the independent experiments; data are summarized in Table 1. Immobilized RGDS and/or INS onto thermoresponsive surfaces increased with feed concentrations of both CIPAAm and RGDS/INS, respectively[23]. Consistently, percentages of reacted carboxyl groups on thermoresponsive surfaces significantly decreased with increased INS feed concentrations[23]. These results indicate that the carboxyl groups grafted onto the surfaces are not completely reacted with RGDS/INS for both IC1 and IC4 surfaces (1.0 and 4.0 mol% of CIPAAm feed concentrations, respectively). This means that some of the carboxyl groups remain unreacted due to the limited diffusion of large molecular weight biomolecules within the nanometer-thick three-dimensional polymer networks that were grafted onto the surfaces. These unreacted carboxyl groups enhance surface hydrophilicity, resulting in the suppressed

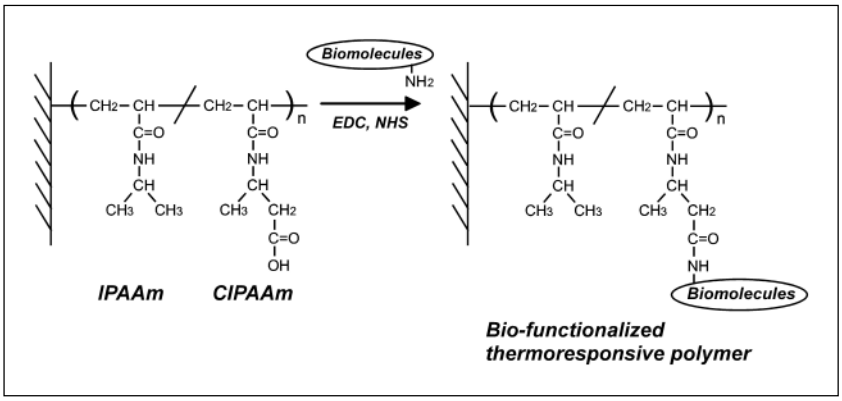

Fig.1 Immobilization of bioactive ligands onto carboxyl-functionalized thermoresponsive cell culture surfaces

Table 1 Immobilization of RGDS and/or INS onto carboxyl-functionalized thermoresponsive surfaces

Sample code [a]	Amount of grafted CIPAAm (nmol/cm^2) [b]	Grafted amount of biomolecules (pmol/cm^2) [c]	
		RGDS	INS
IC1	0.2	0	0
IC1-R174	0.2	174 ± 13	0
IC1-R96I14	0.2	96 ± 10	14 ± 1
IC1-I21	0.2	0	23 ± 1
IC4	0.8	0	0
IC4-R388	0.8	388 ± 18	0
IC4-R122I18	0.8	122 ± 9	18 ± 1
IC4-I37	0.8	0	37 ± 1

[a] Sample code ICX-RYIZ denotes poly(IPAAm-*co*-CIPAAm)-grafted dishes with CIPAAm feed concentrations (X in mol%) and immobilized RGDS (R) and INS (I) (Y and Z in pmol/cm^2, respectively).

[b] Carboxyl group densities on thermoresponsive surfaces are estimated from the experimentally determined amounts of grafted copolymers onto the surfaces (1.9 μg/cm^2 for both IC1 and IC4, respectively)[22,23] and the feed compositions of CIPAAm monomer to IPAAm monomer[34].

[c] Data are expressed as mean ± standard deviation; n = 4.

cell adhesion and the enhanced cell detachment.

Bio-functionalized IC1 and IC4 surfaces maintained their hallmark thermoresponsive hydrophilic/hydrophobic responsiveness with temperature changes, similar to PIPAAm-grafted surfaces. Co-immobilization of RGDS and INS also had minimal influence on surface thermoresponsive wettability changes between 20 and 37℃[23].

2) Enhanced cell adhesion and proliferation on bio-functionalized thermoresponsive surfaces

We investigated initial cell adhesion on RGDS- and/or INS-immobilized thermoresponsive surfaces compared with conventional PIPAAm-grafted surfaces using bovine carotid artery endothelial cells (ECs) under both fetal bovine serum (FBS)-supplemented and FBS-free culture conditions. Initial adhesion of seeded ECs on unmodified IC4 surfaces was lower than that on unmodified IC1 surfaces as can be seen in Fig.2, most likely due to the increased surface hydrophilicity. RGDS immobilization provided a much higher initial EC adhesion (Fig.2), and adherent cell numbers and spread cells both increased with density of immobilized RGDS[21,23]. Under optimized RGDS immobilization conditions, EC spreading in the FBS-free culture media was higher compared with that observed on RGDS-unmodified thermoresponsive surfaces in the FBS-supplemented culture media[21,23].

INS immobilization facilitated improved EC growth over INS-unmodified thermoresponsive surfaces. Rapid EC growth was

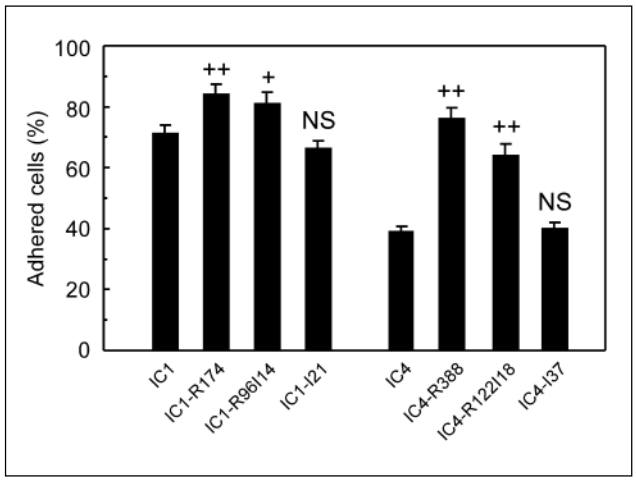

Fig.2 Initial cell adhesion on bio-functionalized thermoresponsive surfaces in the presence of FBS. Symbols; 4 h: closed bars. Seeding density; 1 x 10^4 cells/cm^2. Statistical significance of initial EC adhesion on bio-functionalized ICX surfaces compared with that on unmodified ICX surfaces were evaluated by unpaired Student's *t*-test, respectively; +: $p < 0.01$, ++: $p < 0.001$, NS: Not Significant. Bars represent standard deviation; n = 4.

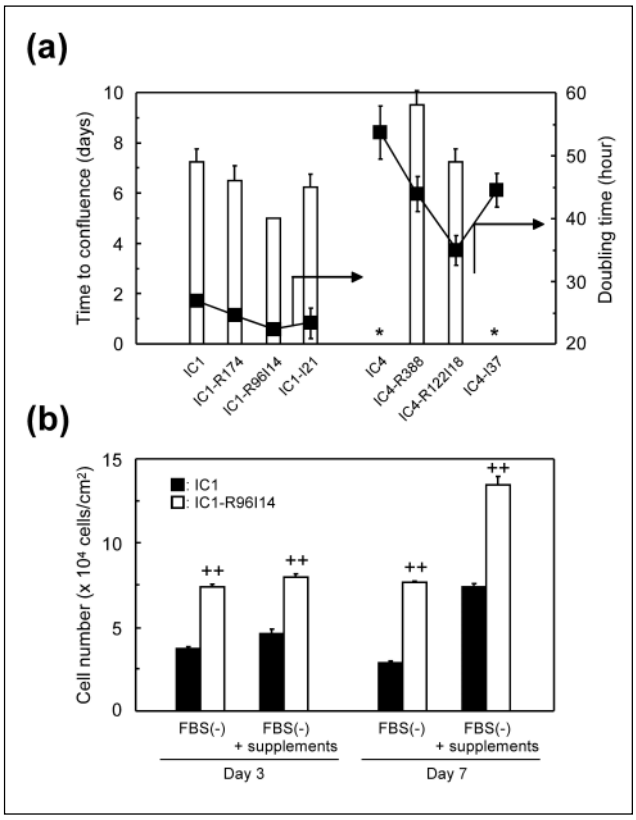

Fig.3 (a) Cell growth on bio-functionalized thermoresponsive surfaces in the presence of FBS. Symbols; time to confluence on bio-functionalized thermoresponsive surfaces: open bars, doubling time estimation on bio-functionalized thermoresponsive surfaces: closed plots. Seeding density; 1 x 10^4 cells/cm^2. * Cells did not grow to confluency on marked surfaces.
(b) Effects of RGDS-INS co-immobilization on cell growth after 3 and 7 days culture under the FBS-free conditions. Symbols; unmodified IC1 surfaces: closed bars, bio-functionalized IC1-R96I14 surfaces: open bars. Seeding density; 5 x 10^4 cells/cm^2. Statistical significance of FBS-free EC growth on bio-functionalized IC1-R96I14 surfaces compared with that on unmodified IC1 surfaces were evaluated by unpaired Student's *t*-test, respectively; ++: $p < 0.001$. Bars represent standard deviation; n = 4 for both experiments.

observed on RGDS-INS co-immobilized thermoresponsive surfaces, on which ECs became confluent within shorter time periods than those on RGDS- or INS-immobilized and unmodified thermoresponsive surfaces (Fig.3a, open bars). Estimated doubling times for cultured EC growth on bio-functionalized thermoresponsive surfaces were shortened by co-immobilization of appropriate amounts of RGDS and INS (Fig.3a, closed plots). Much lower amounts of immobilized INS were required to facilitate EC proliferation than for soluble INS addition to cell cultures on unmodified thermoresponsive surfaces, regardless of RGDS co-immobilization[22,23]. Amounts of immobilized INS decreased by co-immobilization with RGDS, inducing initial EC adhesion and supporting subsequent EC proliferation stimulated by INS[23]. Thus, effect of co-immobilization of both RGDS and INS onto thermoresponsive surfaces is apparent comparing with cell proliferation on RGDS- or INS-immobilized thermoresponsive surfaces.

We further investigated cell growth on bio-functionalized thermoresponsive surfaces without the addition of FBS in the culture media; results are shown in Fig.3b. EC adhesion and proliferation were drastically suppressed and eventually, spread cell numbers decreased with time in the absence of FBS components on IC1 surfaces[23]. Bio-functionalized IC1-R96I14 surfaces having both immobilized RGDS and INS at 96 and 14 pmol/cm^2, respectively, showed higher EC adhesion than unmodified IC1 surfaces even under the FBS-free culture conditions, although ECs did not reach confluency. Seeded ECs proliferated and reached confluency on bio-functionalized thermoresponsive surfaces after 2 weeks in culture in the FBS-free EBM-2 culture medium supplemented with specific soluble proteins for cell growth[23]. These results indicate that RGDS immobilization onto IC1 surfaces strongly supports initial EC adhesion, while sur-

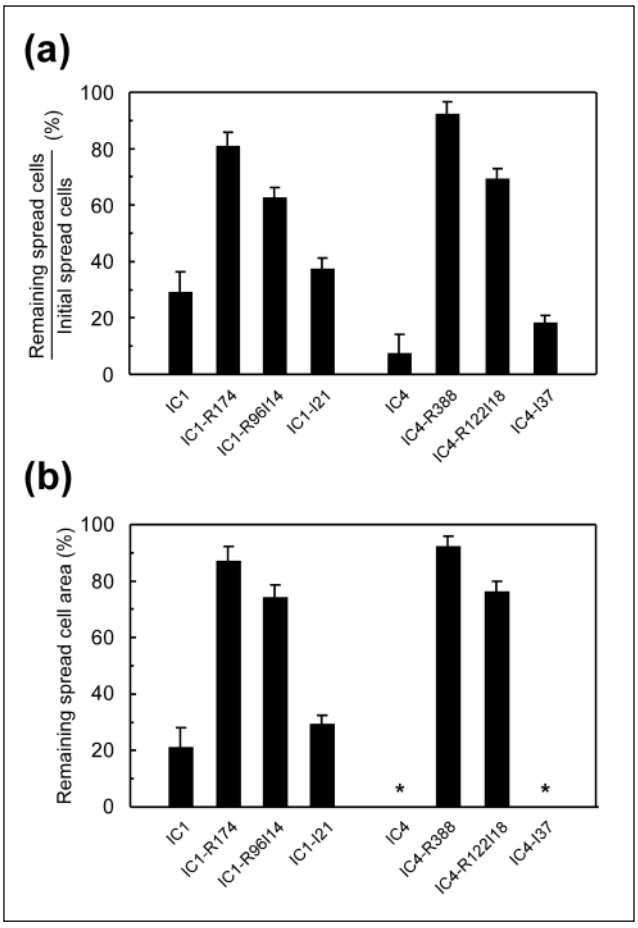

Fig.4 Thermally induced detachment of (a) single cells (24 h culture) and (b) cell sheets (additional 3 days culture after ECs reached to confluency) from bio-functionalized thermoresponsive surfaces in the presence of FBS after 0.5 h incubation at 20℃. Seeding density; 1 x 10^4 cells/cm^2 for single cell detachment, and 1 x 10^5 cells/cm^2 for cell sheet recovery, respectively. * Cells did not grow to confluency on marked surfaces. Bars represent standard deviation; n = 4.

face-immobilized INS alone leads to insufficient EC growth in the present conditions. However, appropriate amounts of soluble known growth factors added to the culture medium as supplements induce EC proliferation to confluency on bio-functionalized thermoresponsive surfaces even under the FBS-free culture conditions.

As described in the previous section, amounts of immobilized RGDS and INS onto thermoresponsive surfaces increase with feed concentrations of both CIPAAm and RGDS/INS, while increased carboxyl groups also enhance surface hydrophilic behavior, resulting in the decreased cell adhesion. We thus conclude that optimization of appropriate amounts of both surface-grafted carboxyl groups and immobilized biomolecules is essential to facilitate maximum cell adhesion, proliferation, and thermally induced detachment.

3) Non-invasive cell (sheet) recovery from bio-functionalized thermoresponsive surfaces

Single cell detachment from bio-functionalized thermoresponsive surfaces was examined after 24 h culture at 37℃ by reducing culture temperature to 20℃. Fig.4a shows that EC detachment from unmodified IC4 surfaces was higher than that from unmodified IC1 surfaces, most likely due to the increased surface hydrophilicity as discussed in Fig.2. Immobilized RGDS suppressed EC detachment from thermoresponsive surfaces with increased densities of immobilized RGDS, while INS immobilization had little influence, regardless of the amounts of immobilized INS[23]. These results suggest that the amounts of immobilized RGDS onto thermoresponsive surfaces dominate thermally induced single cell detachment, rather than immobilized INS. Harvest of cultured confluent cell-derived tissue-like monolayers from bio-functionalized thermoresponsive surfaces was then examined. Confluent cultures of ECs were conveniently recovered from RGDS-INS co-immobilized thermoresponsive surfaces as intact monolayers using thermal treatments, regardless of the amounts of surface-immobilized RGDS and INS present[23]. Cell sheet harvest from RGDS-immobilized thermoresponsive surfaces also required longer incubation time, similar to single cell detachment events (Fig.4b).

Bio-functionalized thermoresponsive surfaces for applications in tissue engineering and regenerative medicine

As discussed in the former section, co-immobilization of appropriate amounts of RGDS and INS onto thermoresponsive surfaces strongly promotes both cell adhesion and proliferation under both FBS-supplemented and FBS-free culture conditions[23]. Moreover, thermally induced PIPAAm swelling and conformational changes are sufficient to weaken bio-specific interactions between surface-immobilized RGDS/INS molecules and their receptors present on cell membrane surfaces to detach the adherent cells at least in the present immobilization ranges[20-23].

We investigated select capabilities of these bio-functionalized thermoresponsive surfaces for applications in tissue engineering and regenerative medicine. For autologous cell sheet transplantation in animal models, we are currently confirming that harvested primary lapine and porcine dermal fibroblasts adhere and proliferate on bio-functionalized thermoresponsive surfaces under the FBS-free culture conditions, similar to ECs and NIH3T3 fibroblasts, respectively (unpublished data). Using such an ap-

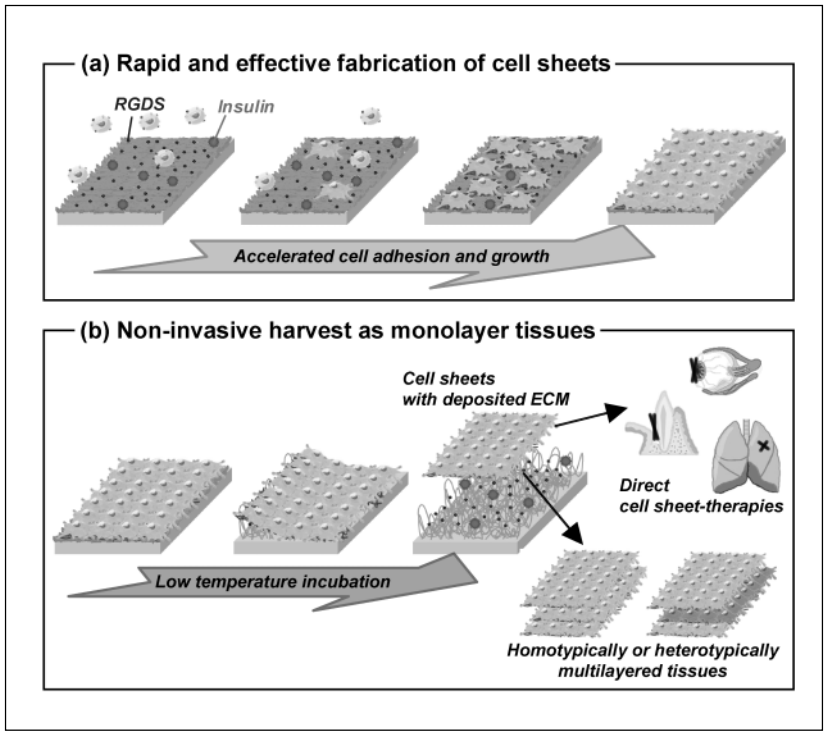

Fig.5 Advanced bio-functional thermoresponsive culture surfaces applied for (a) rapid, effective fabrication of cell sheets and (b) non-invasive harvest as monolayer tissues, followed by direct cell sheet-therapies to target dysfunctional tissues/organs or three-dimensional biomimetic tissue reconstruction.

proach, appropriately designed bio-functional thermoresponsive surfaces should prove valuable to realize xenogeneic-free culture of functional tissues and regenerative living equivalents, following clinical applications.

Throughout these studies, we succeeded in creating advanced bio-functional thermoresponsive surfaces immobilized with biomolecules for stimulated initial adhesion and subsequent growth of cultured cells. Using these bio-functional thermoresponsive surfaces, much faster and easier fabrication of metabolically high functional monolayer tissues can be achieved as illustrated in Fig.5a. In addition, engineered tissues with biomimetic cell-cell densely connected structures are also recovered non-invasively from bio-functional thermoresponsive surfaces only by reducing culture temperature, following reconstruction of three-dimensional tissues *in vitro* or direct cell sheet-therapies without loss of their activities (Fig.5b). We conclude that our bio-functional thermoresponsive surfaces would become an innovative cell culture device for facilitated tissue engineering and regenerative medicine.

Conclusions and future remarks

In this mini-review, we have briefly summarized our recent progress on bio-functionalized thermoresponsive surfaces for applications in tissue engineering and regenerative medicine. Bioactive molecules to be immobilized onto functionalized thermoresponsive surfaces can be selected based on target cell functional properties, including proliferation, differentiation, or even apoptotic induction. Cell sheet detachment without proteolytic enzymes is also sufficiently maintained even after immobilization of target cell-specific biomolecules. Bio-functionalized thermoresponsive surfaces with these properties can help avoid use of animal-derived components, proteinaceous agents, and contaminating viral agents. Improved structural and functional fabrication strategies for biomimetic tissues for further clinical applications are anticipated, exploiting the multiplexed capabilities of this surface: thermally programmed harvest of large-scale, multi-cellular constructs cued by immobilized bioactive molecules. Thus, such new surface designs with different varieties of immobilized bioactive proteins/peptides should prove versatile for a broad spectrum of tissue/organ reconstruction objectives interesting for regenerative medicine.

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