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Article

Fabrication of Thermo-Responsive Molecular Layers from Self-Assembling Elastin-Like Oligopeptides Containing Cell-Binding Domain for Tissue Engineering

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Abstract: Novel thermo-responsive elastin-like oligopeptides containing cell-binding epitope (Arg-Gly-Asp-Ser sequence); arginine-glycine-aspartic acid-serine (RGDS)-elastin-like peptides (ELP) and RGDS-*deg*-ELP; were newly prepared as building blocks of self-assembled molecular layer for artificial extra cellular matrix. A detailed analysis of the conformation of the oligo(ELP)s in water and their self-assembling behavior onto hydrophobic surfaces were performed by using circular dichroism, Fourier transform infrared spectroscopy (FTIR), atomic force microscopy and water contact angle measurements. The experimental results revealed that both oligo(ELP)s self-assembled onto hydrophobic surfaces and formed molecular layers based on their thermo-responsive conformational change from hydrous random coil to dehydrated β-turn structure. Effective cell adhesion and spreading behaviors were observed on these self-assembled oligo(ELP) layers. In addition, attached cells were found to be recovered successfully as a cell-sheet by temperature-induced disassembly of oligo(ELP) layer. This achievement provides an important insight to construct novel oligopeptide-based nano-surfaces for the design of smart artificial extra-cellular matrix.

Keywords: self-assembly; elastin-like oligopeptide; thermo-response; cell attachment; artificial extra-cellular matrix (ECM); cell-sheet

1. Introduction

An important challenge in tissue engineering and regenerative medicine is the design of novel scaffolds for cell adhesion, spreading and proliferation that mimic the natural extra-cellular matrix (ECM) [1,2]. The purpose of tissue engineering is to replace failed organs with new functional tissue as a therapy. In order to realize these therapies, functional materials are needed, which can direct the growth of cells to generate new tissue by providing a well-defined biomimetic environment to surround the cells and promote specific cell interactions. Fibronectin (FN) is a predominant ECM protein that mediates the adhesion and spreading of many cell types [3]. In 1984, Pierschbacher and Ruoslahti found that the arginine-glycine-aspartic acid-serine (RGDS) sequence is the principal adhesive site of FN [4,5]. It was also proved that the RGDS sequence specifically binds to integrin receptors that are present on cell surface [4,5]. An attractive strategy to engineer the ECM-mimicking surface of synthetic functional materials is the use of polymer and such short peptide-epitopes, since polymeric materials can be easily engineered to have an appropriate mechanical strength, biocompatibility and stimuli-responsiveness. From these points of view, several types of peptide-containing block polymers have been developed as synthetic scaffolds for tissue engineering [6–9]. Thermo-responsive polymers with a lower critical solution temperature (LCST), such as poly(*N*-isopropylacrylamide) (PNIPAM), have also been used as functional surface especially in the field of cell-sheet engineering [10–13]. A pioneer work on cell-sheet engineering was reported by Okano and coworkers, who employed PNIPAM-grafted surface and accomplished the successful recovery of cultured cell as a sheet simply by temperature change [10–13]. In this case, the PNIPAM-surfaces are hydrophobic at 37 °C and therefore allow cells adhesion, but the surfaces change to hydrophilic reversibly by lowering temperature below 32 °C and to be not cell adhesive due to rapid hydration of the grafted polymer. The reversible LCST-like behavior has been observed not only for such synthetic polymers but also for natural biopolymers like elastins and elastin-like peptides (ELPs). ELPs are stimuli-responsive polypeptides that consist of repeats of the penta-peptide sequence VPGXG (V: valine, P: proline, G: glycine, X: any amino acid except P) and undergo a phase transition depending on the kind of X [14–16]. Below the phase transition temperature (T_t), ELPs are soluble in water and exist as hydrated random coils, but when temperature is elevated above their T_t, the chains fold to β-turn by hydrophobic dehydration, leading to desolvation and aggregation of the polypeptide. The T_t of ELPs can be precisely tuned between 0 and 100 °C by replacement of X [14], which is difficult to achieve with other synthetic polymers such as PNIPAM. Furthermore, the ELPs potentially offer several advantages over PNIPAM such as biocompatibility and biodegradablility characters. Based on such interesting properties, ELPs have already been employed as useful molecular tools for biological applications such as protein purification, drug delivery and artificial ECM [17–26]. In most of these studies, genetically engineered ELPs with high molecular weights were used on the subjects. However, as for the recombinant protein expression method, complicated and specialized

operations are sometimes required, and this method does not easily allow to introducing non-natural functional groups into the target protein. Our focus has therefore been on an artificial peptide with short chain-length because of its ease of chemical synthesis and modification with various functional groups. In previous studies, we have shown that poly(ethylene glycol) (PEG)-modified oligo(ELP)s perform self-assembly into spherical micelle-like aggregates in water in response to the conformational switches of oligo(ELP) segment [27]. The oligo(ELP)-coated gold nanoparticles have been also successfully prepared and selectively aligned onto the 2D-patterned hydrophobic substrate guided with the oligo(ELP) character [28]. More recently we have reported the unique thermo/pH dual-responsive materials from oligo(ELP)-shelled poly(amido-amine) dendrimer [29] and oligo(ELP)-grafted linear polyions [30].

In this study, we designed and synthesized novel thermo-responsive elastin-like oligopeptides containing cell-binding epitope, RGDS-ELP and RGDS-*deg*-ELP, as building blocks of self-assembled molecular layer for artificial ECM. Temperature-regulated conformation and self-assembling behavior onto hydrophobic surfaces of these oligo(ELP)s were examined by means of circular dichroism (CD), Fourier transform infrared spectroscopy (FTIR), atomic force microscopy (AFM) and water contact angle measurements. In addition, cell attachment and recovery experiments were carried out by using the resultant thermo-responsive peptide layers. These studies should provide simple and essential strategy for the design of novel oligopeptide-based smart surface.

2. Experimental Section

2.1. Peptide Preparation

The elastin-like oligopeptides containing cell-binding domain (RGDS-ELP and RGDS-*deg*-ELP) used in this study were prepared from solid phase peptide synthesis using 9-fluorenylmethoxycarbonyl (Fmoc) chemistry. These oligopeptides were synthesized on a Fmoc-Gly-attached CREAR-acid (cross-linked ethoxylate acrylate resin, Peptide Institute, Inc., Osaka, Japan), by using Fmoc-L-amino acid derivatives (Fmoc-Val, Fmoc-Pro, Fmoc-Gly, Fmoc-Arg(Pbf), Fmoc-Asp(OBu^t), Fmoc-Ser(Bu^t)) (3 equiv.), 1-hydroxy-7-azabenzotriazole (3 equiv.) and 1,3-diisopropylcarbodiimide (3 equiv) in *N,N*-dimethylformamide (DMF) for coupling, and piperidine (25%)/DMF for Fmoc removal. Fmoc-8-amino-3,6-dioxaoctanoic acid (diethylene glycol spacer (*deg*)) was employed as hydrophilic flexible linker. To cleave the peptide from the resin, the peptide-resins were treated with trifluoroacetic acid (TFA)/CH_2Cl_2 (9/1 (*v*/*v*)). All peptides were purified by reversed-phase HPLC (Bio-Rad, DuoFlow) on a COSMOSIL 5C_{18}-AR-300 (Nacalai tesque Inc., Kyoto, Japan, 10 × 150 mm) by using a linear gradient of water-acetonitrile (containing 0.1% TFA) as required. The resultant peptides were identified by matrix-assisted laser desorption ionization-time of flight MS (MALDI-TOFMS) (Bluker Autoflex III, Billerica, MA, USA) with dihydroxybenzoic acid as matrix and ^{1}H-NMR spectroscopy (400 MHz, JEOL JNM-AL-400, JEOL Resonance, Tokyo, Japan). Fluorescent-labeled RGDS-ELP containing nitrobenzofurazan (*NBD*) at the amino groups (*NBD*-RGDS-ELP) was prepared by reacting the *N*-terminus of RGDS-ELP with 4-fluoro-7-nitrobenzo-furazan in DMF at room temperature.

MALDI-TOFMS data of RGDS-ELP: 2070.0 $[M + H]^+$ (calcd. 2071.1) and RGDS-*deg*-ELP: 2220.0 $[M + H]^+$ (calcd. 2217.6). ^{1}H-NMR analyses (in DMSO-d_6, TMS) also gave satisfactory results (Figure S1).

RGDS-ELP: ^{1}H-NMR (d_6-DMSO, TMS), δ (ppm) 0.8–0.9 (Val-γ-CH_3), 1.6 (Arg-γ-CH_2), 1.7–2.1 (Arg-β-CH_2, Pro-β-CH_2 and Pro-γ-CH_2), 2.4–2.7 (Val-β-CH, Arg-δ-CH_2, overlapped with DMSO), 3.1 (Asp-β-CH_2), 3.3–4.6 (Arg-α-CH, Asp-α-CH, Ser-α-CH, Ser-β-CH_2, Gly-α-CH_2, Pro-δ-CH_2, Val-α-CH and Pro-α-CH), 7.1–8.9 (Arg-NH-, Arg-NH_2, peptide bond (main chain)-NH-, *N*-terminal-NH_2).

RGDS-*deg*-ELP: ^{1}H-NMR (d_6-DMSO, TMS) δ (ppm) 0.8–0.9 (Val-γ-CH_3), 1.6 (Arg-γ-CH_2), 1.7–2.1 (Arg-β-CH_2, Pro-β-CH_2 and Pro-γ-CH_2), 2.4–2.7 (Val-β-CH, Arg-δ-CH_2, overlapped with DMSO), 3.1 (Asp-β-CH_2), 3.25 (*deg*-NHCH_2CH_2O), 3.3–4.6 (Arg-α-CH, Asp-α-CH, Ser-α-CH, Ser-β-CH_2, Gly-α-CH_2, Pro-δ-CH_2, Val-α-CH and Pro-α-CH, *deg*-NHCH_2CH_2OCH_2CH_2O, *deg*-OCH_2CO), 7.1–8.9 (Arg-NH-, Arg-NH_2, peptide bond (main chain)-NH-, *N*-terminal-NH_2).

2.2. Circular Dichroism (CD) Spectroscopy and FTIR Spectroscopy Measurements

Circular Dichroism (CD) spectra were recorded on a J-820 spectropolarimeter (JASCO Ltd., Tokyo, Japan) equipped with a peltier type thermostatic cell holder coupled with a controller PTC-423L (JASCO Ltd., Tokyo, Japan) under a nitrogen atmosphere. Experiments were performed in a quartz cell with a 2 mm path length over the range of 190–250 nm at various temperatures. All peptides were dissolved in 2,2,2-trifluoroethanol (TFE) as a stock solution. The objective sample solutions of the peptides (TFE content: 5%) were prepared by diluting the stock solution with the 5 mM citric/phosphate buffer (pH 7.4) and stocked at 4 °C for overnight before measurements. Final peptide concentration was 50 μM in aqueous media. ATR-FTIR spectra of oligo(ELP) layers on hydrophobic substrate (polystyrene (PSt)) were measured with the Nexus 470 (Thermo Nicolet Co., Madison, WI, USA) using a Mercury-Cadmium-Tellurium (MCT) detector (resolution, 4 cm^{-1}; number of scan, 1024, Thermo Nicolet Co.).

2.3. Atomic Force Microscopy (AFM) and Contact Angle Measurements

The AFM images were collected at ambient temperature on a Nanoscope IIIa (Digital Instrument, Inc., Santa Barbara, CA, USA) operated by tapping using a silicon tip (tip radius 10 nm). A hydrophobic glass plate treated with octadecyletriethoxysilane (water contact angle: *ca.* 115°) and untreated glass plate (water contact angle: *ca.* 20°) were separately immersed into RGDS-ELP aqueous solution (0.1 $mg \cdot mL^{-1}$) at 4 °C and then incubated overnight at 37 °C. After rinsing with pure water at 37 °C and drying, images were obtained by using a 10 μm × 10 μm scanner. The scanning speed was at a line frequency of 1 Hz, and the original images were sampled at a resolution of 512 × 512 points. Water contact angles for RGDS-ELP or RGDS-*deg*-ELP self-assembled layers were measured after 1 ms with a DropMaster-501 contact angle meter (Kyowa Interface Science Co., Ltd., Saitama, Japan) at room temperature (20 °C). The average of five measurements taken at different positions on each sample was adopted.

2.4. Cell Attachment

NIH/3T3 fibroblast (Cell bank of National Institute of Health Sciences (Japan)) was a gift from Professor Naoki Tanaka (Kyoto Institute of Technology). The cells were cultured using Dulbecco's modified Eagles's medium (DMEM) supplemented with calf serum (volume fraction of 10%) and

penicillin/ treptomycin (volume fraction of 1%). Cells were incubated at 37 °C under 5% CO_2. Cells grown to 80% confluence were passaged by trypsinization, diluted, and inoculated into a fresh tissue culture dish. Fibroblasts were seeded at a density of *ca.* 1000 cells cm^{-2} onto RGDS-ELP or RGDS-*deg*-ELP-self-assembled PSt-dishes in the absence of serum at 37 °C under 5% CO_2. For comparison, RGDS-free oligo(ELP) was also used. After incubating for 3, 6 and 24 h, the number of attached cells per surface area and the degree of spreading of the cells (the number of spreading cell/the number of attached cell × 100) were determined by optical phase contrast microscopy (CKX41, Olympus Co., Tokyo, Japan). Four independent cell adhesion experiments were performed, and the average values were used. Errors include all result of 4 independent experiments. Cell-sheet recovery experiment was carried out as follows. The NIH/3T3 cells (*ca.* 2000 cells·cm^{-2}) were cultured on RGDS-ELP-self-assembled PSt dish in the presence of serum at 37 °C for nine days. After these cells became confluent, the cells were washed by phosphate buffer solution (PBS) (37 °C) and then incubated in serum-free DMEM at 20 °C for 5 h. Cell viability assays for surface-attached cells were conducted using the live/dead assay kit (Promokine™, Promocell Co., Heidelberg, Germany). NIH/3T3 cells (*ca.* 1.0×10^4 cells·cm^{-2}) were cultured at 37 °C on various surfaces for 24 h. Then, the cells were stained with calcein (2 μM) and ethidium D (EthD) (4 μM) for 15 min. Fluorescence images were obtained on a CKX41-FL (Olympus Co.) equipped with a digital camera system (QIClick, Nippon Roper Co., Tokyo, Japan). The live cells correspond to green cells and the red ones to dead cells.

3. Results and Discussion

3.1. Preparation of Thermo-Responsive Oligopeptides Containing Cell-Binding Domain and Their Conformational Property in Aqueous Solutions

Novel thermo-responsive elastin-like oligopeptides (RGDS-ELP and RGDS-*deg*-ELP), in which the 20-residual oligo(ELP) with $(VPGVG)_4$ sequence was connected to cell-binding RGDS epitope directly or through flexible diethylenglycol(*deg*) linker, were designed and employed as building blocks of self-assembled molecular layer for artificial ECM (Figure 1). A pentad repeat $(VPGVG)_n$ ($n = 4$) that provides sufficient chain length to cause a conformational change in response to a thermal stimulus [28,29,31,32] was chosen as the oligo(ELP) segment. We also expected that the mobility of surface-attached RGDS-epitopes would increase by introducing hydrophilic and flexible short *deg*-linker. A control oligo(ELP) without RGDS-epitope was also used for comparison (Figure 1). These peptides were successfully and easily prepared by standard solid phase peptide synthesis using Fmoc chemistry.

Temperature-dependences of the conformation of RGDS-ELP and RGDS-*deg*-ELP in dilute aqueous solution (50 μM) were first examined by means of CD spectroscopy. Figure 2a shows the temperature-dependence of CD spectra for RGDS-ELP in citric/phosphate buffer (containing 5% TFE) at pH 7.4. At low temperature of 5 °C, the spectrum gave a typical pattern of disordered random coil structure with negative maximum at 198 nm. The [θ] value at 198 nm decreased with increasing temperature, with a smaller decrease in [θ] value at 206 to 212 nm and an increase at 222 nm. A positive peak in the region between 206 and 212 nm is characteristic of type II β-turn structure. Thus, it was found that the inherent thermo-responsiveness of the conformation of oligo(ELP) is well preserved even by conjugating to epitope segment. A similar temperature-dependent spectral change

was observed for the aqueous solution of RGDS-*deg*-ELP (Figure 2b). Figure 2c displayed the conformational transition (temperature *vs.* $[\theta]_{198}$) curves for both oligo(ELP)s under the same conditions. Similar transition curves were obtained for both oligo(ELP)s; the conformational transition from disordered random coil to β-turn occurred gradually over a wide temperature range from 5 to 60 °C and the transition ranges ($\Delta[\theta]_{198}$) were *ca.* 3000–3500 deg·cm^2·dmol^{-1}. However, the absolute $[\theta]_{198}$ values for RGDS-ELP were somewhat small compared to those for RGDS-*deg*-ELP. Therefore, it seems that the introduction of flexible *deg*-linker into the RGDS-ELP causes a slight disordering of the conformation, although the thermo-responsiveness is not significantly affected. It should be noted that these conformational changes were completely thermo-reversible for both oligo(ELP)s.

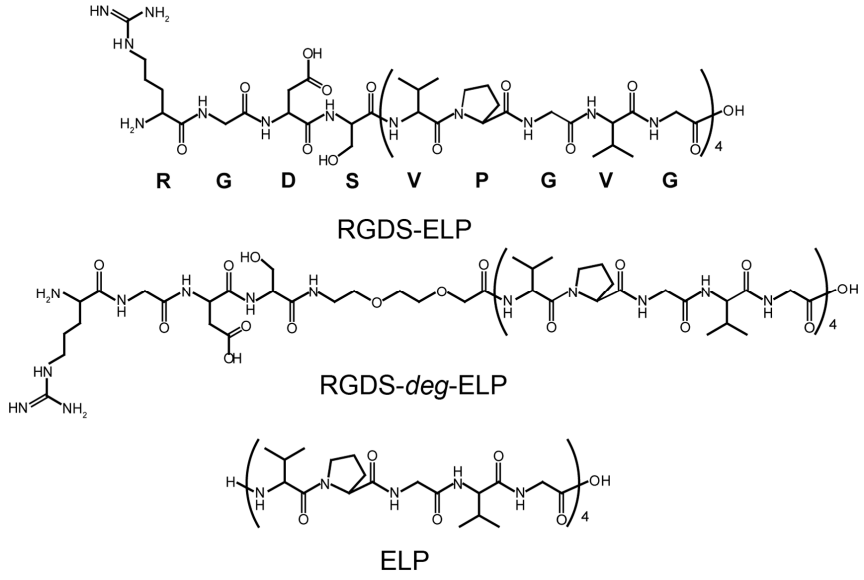

Figure 1. Primary structures of the thermo-responsive elastin-like oligopeptides containing cell-binding RGDS epitope (RGDS-ELP, RGDS-*deg*-ELP) used as building units for self-assembled molecular layers. V: valine, P: proline, G: glycine, R: arginine, D: aspartic acid, S: serine.

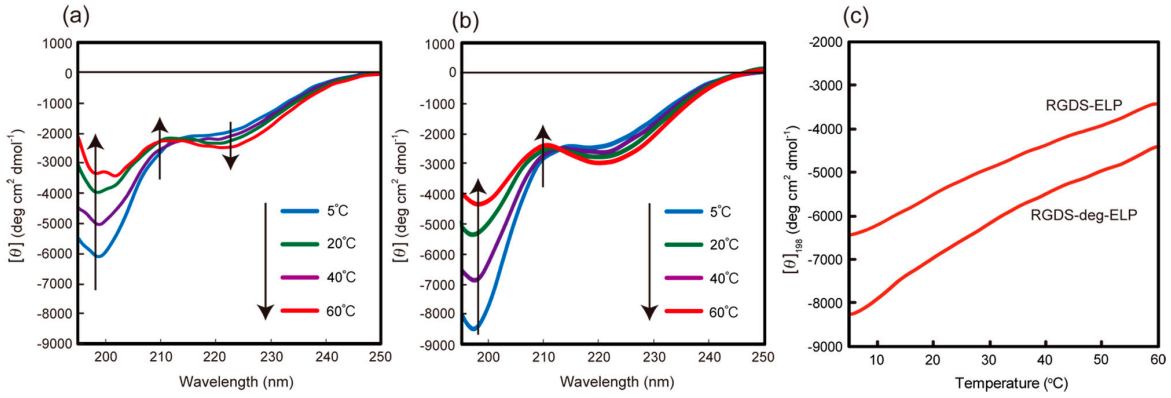

Figure 2. Temperature-dependence of circular dichroism (CD) spectra of (**a**) RGDS-ELP and (**b**) RGDS-*deg*-ELP in 5 mM citric/phosphate buffer (containing 5% TFE) at pH 7.4. [peptide] = 50 μM. (**c**) Temperature profiles of $[\theta]_{198}$ values for RGDS-ELP and RGDS-*deg*-ELP at heating process.

3.2. Thermo-Induced Self-Assembly of Oligo(ELP)s Containing Cell-Binding Domain onto Hydrophobic Surfaces

The self-assembling behavior of oligo(ELP)s with RGDS epitope onto hydrophobic substrates using the thermo-switchable conformation of the ELP segment was examined in order to fabricate the molecular layer for artificial ECM. Recently we have reported an effective and facile method for gold nanoparticle arrays guided by thermo-responsive oligo(ELP) [28]. Using such smart character of oligo(ELP) as a molecular guide, the 2D-organization of bio-functional groups will also be accomplished. To elucidate the selective adsorption of the oligo(ELP)s with RGDS epitope onto a solid substrate, we firstly prepared two glass substrates, a hydrophobic glass plate treated with octadecyltriethoxysilane and an untreated bare glass plate, providing hydrophobic (water contact angle: 115°) and hydrophilic (water contact angle: 20°) surfaces, respectively. The interaction of these glass plates with oligo(ELP)s was examined by separately immersing them into aqueous solutions of oligo(ELP)s (0.1 mg·mL^{-1}) at 4 °C and then incubating overnight at 37 °C above the conformational transition temperature, and was confirmed via visualization with atomic force microscopy (AFM). AFM is a useful technique to evaluate the 3D structural features of a surface in nano-meter scale. It is, however, well known that the convolution of the scanning tip leads to an overestimation of the sample's width. All sample dimensions were therefore estimated from the height of the surfaces in cross section.

Figure 3 shows the tapping-mode AFM images of the hydrophobic (a) and hydrophilic (b) glass surfaces obtained after immersion into aqueous solution of RGDS-ELP, respectively. It is clear that the RGDS-ELP self-assembled preferentially onto the hydrophobic surface and formed molecular layer with nearly uniform thickness. The *z*-profile (Figure 3a bottom) indicates that the cross-section of the layer ranged from 3 to 4 nm. Considering the molecular dimension of oligo(ELP) (20-mer) with β-spiral conformation (*ca.* 1.8 nm in diameter and 1.0 nm in length), it can be considered that the observed molecular layer is composed of 2–4 multi-layers of β-turn RGDS-ELP by associating with each other rather than monolayer. Similar self-assembling behavior was also observed for RGDS-*deg*-ELP, and we could successfully obtain the molecular layer of RGDS-*deg*-ELP with a thickness of 3–5 nm. In both cases, the oligo(ELP) segment mainly adopt a hydrophobic β-turn structure at 37 °C and is more likely to interact with the hydrophobic glass plate than with the hydrophilic one. Therefore, the thermo-induced conformational transition from the hydrophilic random coil to hydrophobic β-turn is considered to play an important role in aligning RGDS-ELP on a hydrophobic surface via directed assembly. Similar thermo-induced self-adsorbing behavior of oligo(ELP) was observed in our previous study [28], at which the amount of self-adsorption of oligo(ELP)(n = 20)-coated gold nanoparticle for the hydrophobic glass plate was larger than that for hydrophilic one by a factor of *ca.* 6. Note that the phase transition behavior was not observed for both oligo(ELP)s by turbidity measurement under this experimental condition (0.1 mg·mL^{-1}, 4–60 °C). In order to obtain information regarding these molecular layers, water contact angle measurements were carried out. Initial contact angle for hydrophobically-treated glass surface exhibited value of *ca.* 115°. A decrease in hydrophobicity of the substrate was observed after the formation of self-assembled oligo(ELP) layers. The obtained molecular layers of RGDS-ELP and RGDS-*deg*-ELP afforded similar water contact angles of *ca.* 83° and 90°, respectively, corresponding to a moderate

hydrophobic surface. These results indicate that the presence of flexible *deg*-linker does not significantly influence the surface property.

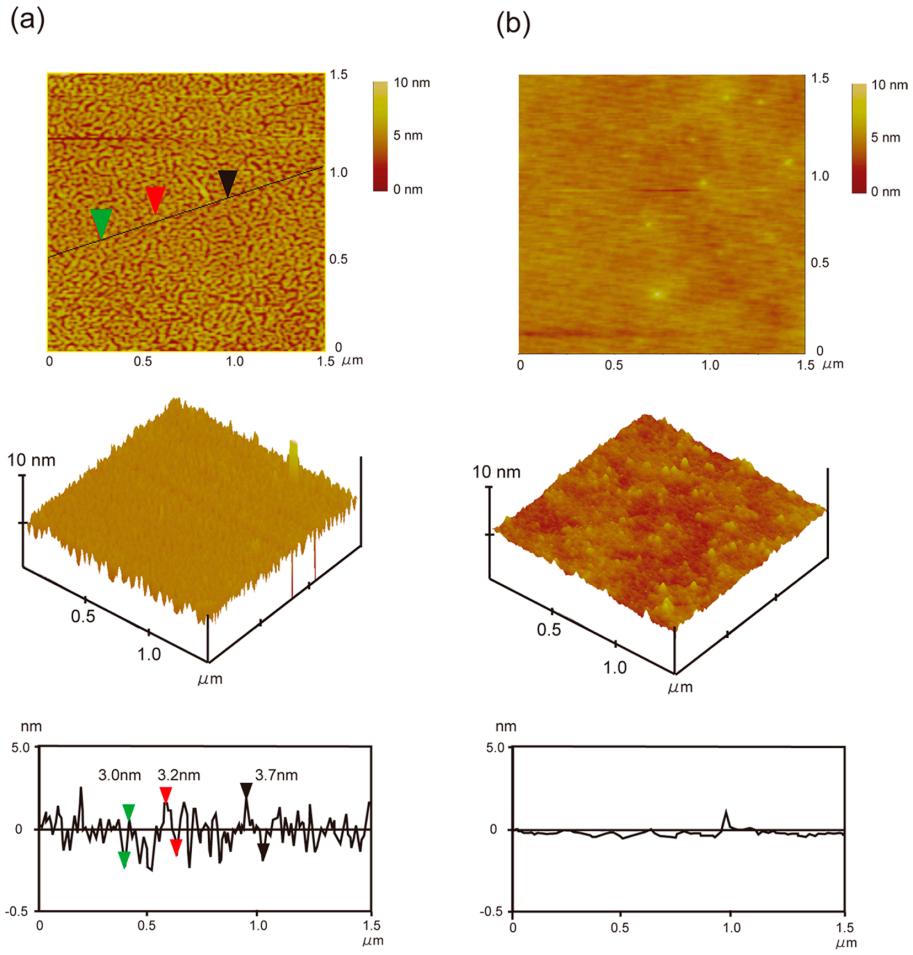

Figure 3. Tapping-mode (TM) AFM images of the (**a**) hydrophobic and (**b**) hydrophilic glass plates obtained after immersion into aqueous solution of RGDS-ELP at 37 °C, respectively. [RGDS-ELP] = 0.1 mg/mL, *z*-scale: 10 nm. Bottom figures show the detailed analysis of the heights of the surfaces.

By using such self-adsorption properties of oligo(ELP) segment to the hydrophobic surface, we also prepared the self-assembled layers of RGDS-ELP and RGDS-*deg*-ELP onto a polystyrene (PSt) dish (water contact angle: *ca.* 90°). The preparation procedure was the same as that on the hydrophobic glass plate. Figure 4 shows the ATR-FTIR spectra of PSt-dishes obtained after incubation in the aqueous solutions of the RGDS-ELP or RGDS-*deg*-ELP at 37 or 4 °C for overnight and subsequent rinsing with pure water (37 or 4 °C). In both cases, the absorption peaks due to the amide I and II were observed at 1650–1630 cm^{-1} and 1540–1520 cm^{-1} under the condition of 37 °C, respectively, demonstrating the adsorption of both RGDS-ELP and RGDS-*deg*-ELP onto PSt-dish surface. On the other hand, the peak absorbances were remarkably small at 4 °C, at which oligo(ELP) segment took a hydrophilic random coil structure. These results strongly support the importance of thermo-induced conformational transition of oligo(ELP)s for their self-assembly onto a hydrophobic surface. Water contact angles for these oligo(ELP) layers prepared on PSt-dishes exhibited values between 78° and 80° similar to that on hydrophobic glass plates (80°–90°) (Figure S2).

These wettabilities of the obtained oligo(ELP) layers were convenient for cell attachment, since the many cells are known to be hard to adhere to the hydrophilic surface [10–13].

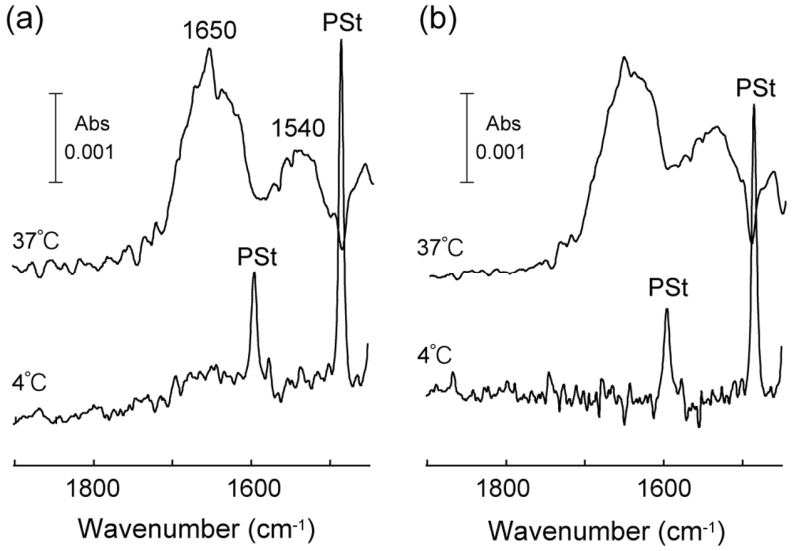

Figure 4. ATR-FTIR spectra of the self-assembled molecular layers of (**a**) RGDS-ELP and (**b**) RGDS-*deg*-ELP on PSt-dish prepared at 37 °C and 4 °C.

3.3. Cell Adhesive Properties of Self-Assembled Oligo(ELP) Layers and Cell Recovery

Subsequently, NIH/3T3 cells were seeded on the surfaces of various oligo(ELP) layers (RGDS-ELP, RGDS-*deg*-ELP and epitope-free oligo(ELP)) and cultured at 37 °C in serum-free medium, and the cell adhesion behavior and morphology were observed. As a control, the same experiment was performed for the PSt-dish. Figure 5 summarized the results of cell adhesion studies. Compared with bare PSt-dish, oligo(ELP)-covered dishes showed a much higher cell adhesive and spreading activity (Figure 5a,b). These cell morphologies were almost same as cells on a commercially available tissue culture dish (Biocoat^{TM} Fibronectin Cellware, Boston, MA, USA) (See Supporting Information). Notably, cells adhered on the bare PSt-dish probably because of a hydrophobic interaction, but did not spread at all and remained numerously round-shape even after 24 h. Enhanced cell adhesion and spreading were observed on the molecular layers of RGDS-ELP and RGDS-*deg*-ELP, as compared to the epitope-free oligo(ELP) layer. It is therefore suggested that cell adhesion on the oligo(ELP) layers that contain RGDS sequence was facilitated by integrin-specific interactions. Furthermore, the existence of flexible *deg*-linker in the peptide was found to promote cell spreading slightly, although it did not promote cell attachment unfortunately. The viability of attached cells on the RGDS-ELP- and RGDS-*deg*-ELP-covered PSt-dishes after 24 h cell culture was also investigated by using the live/dead assay kit (Figure S3). Fluorescent, green cell images were clearly observed for both oligo(ELP) layers, suggesting the high viability of the attached cells. Since the chemical structure of epitope domain can be easily modified in our method such as a density and a combination with another synergistic epitope, this oligo(ELP)-based self-assembling system will enable the fabrication of functional bio-interfaces with tunable cell adhesive property, and is applicable to versatile supported-materials such as not only plates described here but also fibers and porous materials.

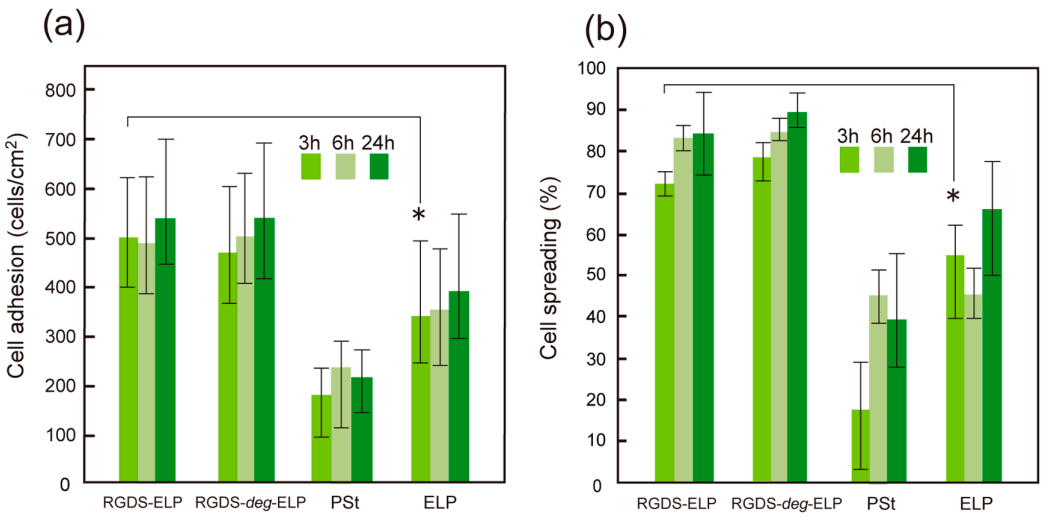

Figure 5. (**a**) Cell adhesion and (**b**) degree of cell spreading of NIH/3T3 onto molecular layers of RGDS-ELP, RGDS-*deg*-ELP, epitope-free oligo(ELP) and un-coated PSt at 3, 6, 24 h. Statistical analyses were performed with a Student's *t*-test. * $p < 0.05$.

Finally, cell recovery experiment was carried out. The NIH/3T3 cells were seeded on RGDS-ELP-covered PSt-dish and cultured at 37 °C for nine days. After these cells became confluent (Figure 6a), the cells were washed by PBS (37 °C) and then incubated in serum-free DMEM at 20 °C. Interestingly, cells cultured on RGDS-ELP-covered PSt-dish were detached from the dish surface as a cell-sheet by lowering temperature. The detachment of cell-sheet was visually detectable without microscope (Figure 6b). At this temperature, the reverse conformational changes of ELP segments are occurred from a hydrophobic β-turn to hydrophilic random coil structure, which causes a disassembly of the oligo(ELP) layer from the PSt-surface. In fact, the existence of RGDS-ELP in the cell-sheet was confirmed by using fluorescent *NBD*-labeled RGDS-ELP. When the fluorescence spectrum of the detached cell-sheet, which prepared on *NBD*-RGDS-ELP layer, was measured (λ_{ex} = 470 nm), fluorescence emission based on the *NBD* group was clearly observed at around 540 nm. It should be noted that the recovered cell-sheet can be fixed on the hydrophobic PSt-dish and cultured again at 37 °C, suggesting the cell survival after detachment. The viability of cells in the cell-sheet was also confirmed by calcein staining assay (Figure S4). Thus, this oligopeptide-based stimuli-responsive nano-surface provides an effective and facile strategy for cell-sheet engineering.

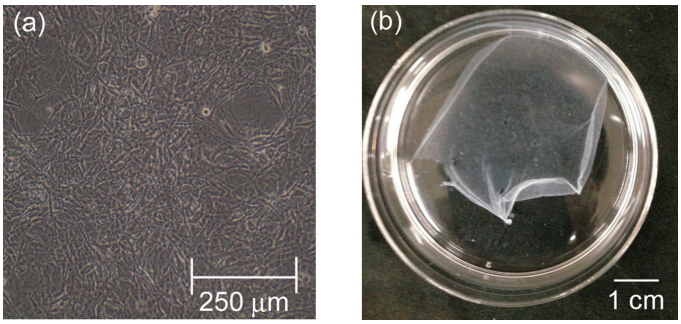

Figure 6. (**a**) Phase-contrast microscope image of NIH/3T3 cell on RGDS-ELP-layer at 37 °C. (**b**) Photograph of cell-sheet recovered from the confluent cell (as shown in Figure 6a) by cooling to 20 °C.

4. Conclusions

In this work, novel thermo-responsive and self-assembling artificial oligopeptides were synthesized, and their potentials for artificial ECM were examined in detail. Using the temperature-induced conformation change from hydrophilic random coil to hydrophobic β-turn, the RGDS-functionalized oligo(ELP)s were successfully aligned onto the hydrophobic substrates, as confirmed via AFM, FTIR and contact angle measurements. We evidenced that the resultant self-assembled molecular layers of oligo(ELP)s were effective as a scaffold for NIH/3T3 cells. In particular, the introduction of RGDS sequence into oligo(ELP)s was found to promote cell adhesion and spreading. More interestingly, attached cells were found to be recovered successfully as a cell-sheet by temperature-induced disassembly of the oligo(ELP) layer. This unique stimuli-responsive molecular layer is formed by self-assembly of simple oligopeptide whose structure and function can be easily manipulated, and therefore is attractive candidate for the design of smart artificial ECM.

Supplementary Materials

Supplementary materials can be accessed at: http://www.mdpi.com/2073-4360/7/1/134/s1.

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Author Contributions

Tomoyuki Koga designed and supervised the experimental work and wrote the manuscript. Kazuhiro Nakamoto, Koji Odawara and Tomoo Matsuoka co-designed and conducted experiments and co-authored the manuscript. Nobuyuki Higashi supervised the research project.

Conflicts of Interest

The authors declare no conflict of interest.

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