Molecular Imprinting

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# Thermo-Responsive Hydrogel Layers Imprinted with RGDS Peptide: A System for Harvesting Cell Sheets**

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Cell sheet technology is a novel approach to preparing and harvesting monolayer cell sheets by using poly(*N*-isopropylacrylamide) (PNIPAAm)-modified surfaces as thermo-responsive cell culture substrates.[1] At lower temperatures the cultured cells detach spontaneously as the surface of the substrate changes from hydrophobic to hydrophilic. In this way, an intact cell monolayer can be harvested non-invasively together with its underlying extracellular matrix (ECM). As a frequently used way to achieve scaffold-free tissue engineering, cell sheet technology holds great promise in cell-based regenerative medicine.[2]

Considering the limited availability of autologous cells and the timeliness requirement of clinical treatments, current attempts at improving the efficiency of thermo-responsive cell sheet harvest systems face a dilemma. On the one hand, the cell culture substrate must be very cell-adhesive to markedly promote rapid adhesion and proliferation of the limited autologous cells for a timely therapy. On the other hand, the same substrate should become very cell-repulsive after the formation of a confluent cell monolayer such that the mature cell sheet can be rapidly released without hurting the cells and their underlying ECM. Specifically, simple regulation of the chemical composition or the topography of the surface can only either promote cell adhesion or accelerate cell detachment[3] and these surfaces commonly have very low bioactivity.[4] Introducing cell-adhesive biomolecules by means of covalent binding or physical adsorption can improve the surface bioactivity but result in the inevitable deceleration of cell detachment[5] and serious leakage of the biomolecules,[4,6] respectively. Thus far, no single method without additional auxiliary means can effectively enhance cell adhesion during culture as well as facilitate the rapid harvest of cell sheets.

To conquer this long-standing problem, we conceive of introducing cell-adhesive biomolecules to a thermo-responsive cell culture substrate in a reversible way and modulating them through temperature-dependent interactions. In this case, biomolecules can be stably immobilized on the substrate at cell culture temperature (37 °C), while they can be released as the temperature drops (e.g., 20 °C), thus facilitating both the initial cell adhesion and the final detachment of the cell sheet. With this strategy in mind, we find that the reversible interaction known as "specific binding" in noncovalent molecular imprinting is very appealing. As is well known, polymeric receptors with tailor-made recognition sites and "specific binding" properties comparable with those of natural receptors can be easily prepared by molecular imprinting.[7] More importantly, molecularly imprinted polymers (MIPs) containing thermo-responsive recognition sites (i.e., the sites with temperature-dependent interactions between MIPs and targeted molecules) can be readily obtained using PNIPAAm-based materials.[8]

We herein report a novel system for harvesting cell sheets which relies on a PNIPAAm-based MIP hydrogel layer with thermo-responsive affinity toward specific biomolecules (Scheme 1). The commonly used cell-adhesive peptide Arg-Gly-Asp-Ser (RGDS) was chosen as the target biomolecule to demonstrate the proof-of-principle of our strategy.[9] In our design, the thermo-responsive recognition sites in the MIPs were the tactic used to achieve temperature-dependent interactions between RGDS molecules and cell culture substrate. Specifically, besides the temperature-induced change of the surface wettability, the thermo-responsive recognition sites on the MIP hydrogel layer also resulted in the stable recognition and binding of RGDS at 37 °C and the triggered release of RGDS as the temperature was decreased. In contrast to the introduction of biomolecules by means of covalent binding or physical absorption, the thermo-responsive affinity of the MIPs toward RGDS not only significantly promotes cell adhesion during cell culture (37 °C) but also facilitates the detachment of cell sheets at low temperature (20 °C). To the best of our knowledge, although MIPs have exhibited some expanded bioapplications with the emergence of various biomolecule (e.g., proteins or peptides) imprinted polymers,[10] this study is the first demonstration of molecular imprinting as a methodology to biofunctionalize thermo-responsive cell culture substrates to harvest cell sheets for potential biomedical applications.

To achieve the best affinity between RGDS and the MIP matrix during cell culture, the imprinting process was performed by redox-initiated polymerization at 37 °C in

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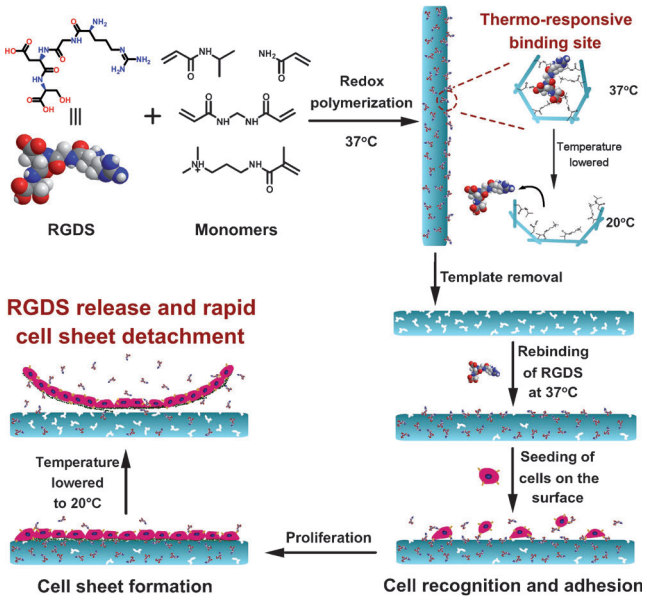


***Scheme 1.*** Strategy to introduce the RGDS peptide on a thermo-responsive cell culture substrate by means of molecular imprinting and the schematic illustration for the cell adhesion and the harvesting of a cell sheet from our system.

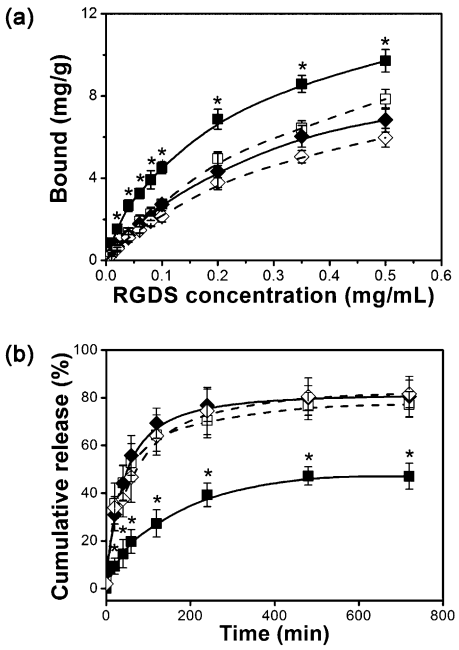


***Figure 1.*** a) Binding isotherms for the RIP (filled symbols) and CP (open symbols) hydrogels at 20 °C (diamonds) and 37 °C (squares), respectively. The hydrogels (8.8 mg) were incubated with a series of RGDS solutions ($C = 0.01$–$0.5$ mg mL$^{-1}$, 1 mL) at different temperatures for 12 h. b) Release kinetics of RGDS from RIP (filled symbols) and CP (open symbols) hydrogels (8.8 mg mL$^{-1}$) at 20 °C (diamonds) and 37 °C (squares), respectively. Equal loading (4.5 μg mg$^{-1}$) of RGDS in the RIP and CP hydrogels was determined according to the fitting curves from isothermal binding. The RGDS amount was determined by using a BCA protein assay kit. For all data, $*p < 0.05$, RIP at 37 °C versus others.

phosphate buffer solution using *N*-isopropylacrylamide (NIPAAm) as the thermo-responsive backbone monomer and *N*-[3-(dimethylamino)propyl]methacrylamide (DMAPMA), acrylamide (AAm), methylene bisacrylamide (MBAAm) as positively charged and hydrogen-bonding monomers and crosslinker, respectively (Scheme 1). Polymerization was performed between two glass plates and resulted in a hydrogel thin layer with a thickness of 0.75 mm. Following that, RGDS templates, adsorbed oligomers, and unreacted monomers on the hydrogel layer were removed by alternately washing with ultrapure water and 0.5 M NaCl solution.[8a]

The resultant RGDS-imprinted polymer (RIP) and control polymer (CP) hydrogels were then used and their temperature-dependent RGDS rebinding and release properties were studied. Similar to previously reported PNIPAAm-based imprinted hydrogels,[8a,b,11] the RIP hydrogel clearly bound more RGDS peptide than the corresponding CP hydrogel over a wide range of RGDS concentrations at 37 °C, while the "specific binding" capacity (defined as the binding difference between the RIP and CP hydrogels) almost disappeared at 20 °C (Figure 1 a and Figure S4 in the Supporting Information). Further, Scatchard analysis clearly indicated that a large number of binding sites with a markedly higher association constant ($K_a = 3.45 \times 10^4$ M$^{-1}$) (defined as "specific recognition sites") were present in the RIP hydrogel at 37 °C, but not in the RIP hydrogel at 20 °C and not in the CP hydrogel at any temperature (Figure S5 and Table S1). In addition, the RGDS release kinetics from the RIP was highly dependent upon temperature (Figure 1 b). These findings, together with the high selectivity of the RIP hydrogel towards RGDS (Figure S16) and the similar surface properties of the RIP and CP hydrogels (Figure S17 and Table S3), clearly demonstrated the presence of thermo-responsive "specific recognition sites" in the RIP hydrogel.

We then checked the cell-adhesion behavior of the RIP and CP hydrogels by seeding MC3T3-E1 cells on them at 37 °C. Figure 2 a shows the adhesion profiles of MC3T3-E1 on the RIP and CP hydrogels bound with a similar amount of RGDS after 6 h of culture. Without RGDS, the number of adhered cells on the CP hydrogel was slightly higher than that on the RIP hydrogel, probably because at 37 °C the RIP hydrogel is more hydrophilic than the control (Figure S3).[3f] However, the number of adhered cells was quadrupled for the RIP hydrogel that was imprinted with 4.5 μg mg$^{-1}$ RGDS, but less than double for the analogous CP hydrogel. This significant increase in the number of adhered cells in the RIP hydrogel should be ascribed to the markedly higher association constant ($K_a$) of the "specific recognition sites", which resulted in decreased leakage of RGDS from the RIP hydrogel compared to that from the CP hydrogel (Tables S1 and S2). It's worth noting that when the amount of bound RGDS was 6.9 μg mg$^{-1}$ or more (which exceeded the apparent maximum number of "specific recognition sites", $N_{max}$; Table S1), the number of adhered cells gradually dropped. Considering that soluble RGDS competes with surface-bound RGDS and weakens cell adhesion,[5a,b] this is easy to understand for the RIP hydrogel because a higher portion of RGDS was bound with nonspecific binding and the release of RGDS

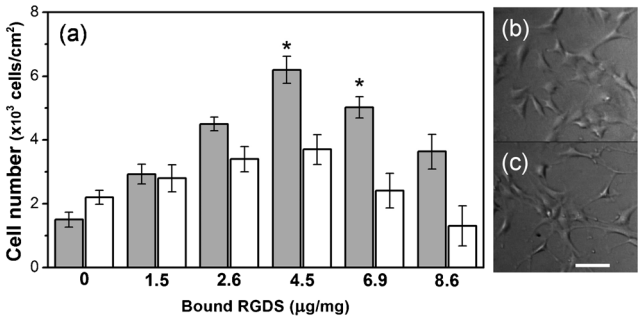


***Figure 2.*** a) Effect of the amount of bound RGDS on cell adhesion for both the RIP (gray bars) and CP (white bars) hydrogels after 6 h of culture at 37 °C. The RGDS amount was determined by using a BCA protein assay kit. For all data, $*p < 0.05$, RIP versus others. b, c) Morphology of representative adhered MC3T3-E1 cells on RIP (b) and CP (c) hydrogels after 24 h of culture at 37 °C, respectively. The scale bar corresponds to 100 μm.

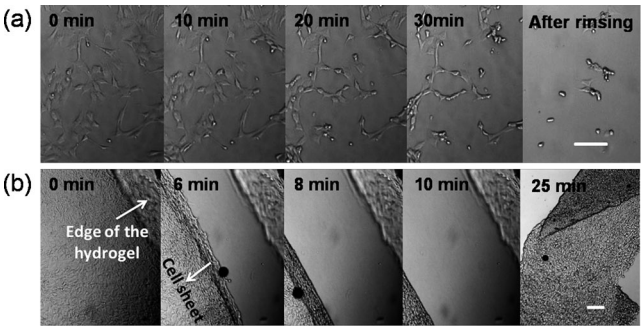


***Figure 3.*** a) Detachment of scattered cells from the RIP hydrogel after 0, 10, 20, 30 min and following rinsing at 20 °C, respectively. b) Detachment of a cell sheet from an RIP hydrogel after 0, 6, 8, 10, and 25 min at 20 °C, respectively. Note that the cell sheet migrated out of the view of the microscope after 13 min. However, a harvested cell sheet was found in the medium. For both scattered cells and cell sheet culture experiments, the hydrogels were bound with 4.5 μg mg$^{-1}$ of RGDS. Scale bars correspond to 200 μm.

into the medium increased. For the same reason, an almost destructive effect on cell adhesion was even observed for the CP hydrogel in line with the much lower affinity and more serious leakage of RGDS (Tables S1 and S2). Nevertheless, it is clear that the RGDS-bound RIP hydrogel consistently showed better cell adhesion than the CP hydrogel. In addition, cells on the RIP hydrogel had better spread morphology after 24 h of culture (Figure 2 b,c and Figure S7). Taken together, these results clearly demonstrate that the "specific binding" on MIPs can be used as a more efficient approach to immobilizing RGDS to promote cell adhesion because the leakage of RGDS is far less than that observed for the system with physically absorbed peptide bound by nonspecific interactions.

Next, the detachment of both scattered cells and a confluent cell sheet from the RIP hydrogel was examined to determine whether the release is thermo-responsive. When the temperature was decreased from 37 °C to 20 °C, a gradual transition of the cell morphology from a spread out to a round shape was clearly seen for the scattered cells (Figure 3 a and Figure S8a); for the confluent cell monolayer, an intact cell sheet peeled off continuously, starting from the edge of hydrogel (Figure 3 b and Figure S9). Moreover, the temperature-induced cell detachment was highly dependent on the amount of bound RGDS. Specifically, cell detachment was accelerated from the RIP hydrogel with a higher amount of RGDS (Figure 4 a). Again, this happened because the RIP hydrogel imprinted with more RGDS certainly released more RGDS as a competitive ligand in the medium as the temperature dropped, thus leading to accelerated cell detachment. This phenomenon is in line with the results from a previous study from Okano's group, where temperature-induced cell detachment was accelerated by the addition of soluble RGDS.[5a] But unlike Okano's method, the soluble RGDS in our system arose solely from temperature-induced self-release. Importantly, in addition to contributing competing soluble ligands to "pull out" cells from the culture surface, this temperature-induced RGDS self-release process also caused significant reduction in the amount of RGDS on the surface, which weakened the ligand–receptor interactions

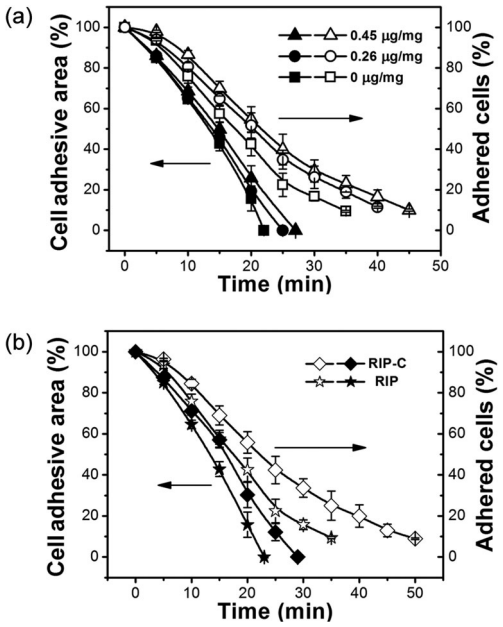


***Figure 4.*** a) Detachment profiles of cell sheets (filled symbols) and scattered cells (open symbols) on an RIP hydrogel bound with 0 (triangles), 2.6 (circles), and 4.5 (squares) μg mg$^{-1}$ of RGDS at 20 °C, respectively. b) Detachment profiles of cell sheets (filled symbols) and scattered cells (open symbols) on RIP (stars) and RIP-C (diamonds) hydrogels at 20 °C. The feed concentration for covalently immobilizing RGDS on RIP-C was 0.25 mg mL$^{-1}$, and the RIP and RIP-C hydrogels were both incubated with 0.1 mg mL$^{-1}$ of RGDS at 37 °C for 12 h. In both figures, initial number of the adhered cells or initial occupied area was defined as 100% for scattered cells and cell sheetd, respectively.

between adhered cells and the hydrogel and functioned to "push away" cells from the surface. Intuitively, this synergistic two-way action of the RIP hydrogel facilitates the more efficient harvest of cell sheets.

Further, the cell adhesion and detachment behavior of the RGDS-bound RIP hydrogel and the RIP hydrogel with

  

covalently immobilized RGDS (denoted as RIP-C) were compared. Although the cell adhesion efficiency on the noncovalently bound RIP hydrogel (4.5 $\mu g\,mg^{-1}$) was slightly lower than that on the RIP-C hydrogel (Figure S7), the cell detachment rate from the RIP hydrogel was much faster (Figure 4a and Figure S10). In contrast to the effect of increasing the RGDS amount on cell detachment from the RIP hydrogel, an increase in the amount of covalently immobilized RGDS caused apparent deceleration in the detachment of both scattered cells and confluent cell sheets (Figure S10) due to the stable covalent bonding and specific integrin–RGD interactions.[12] This, from the opposite side, proved that the thermo-responsive "specific binding" of RGDS in the RIP hydrogel facilitated cell detachment as the temperature decreased. In addition, we directly compared the detachment of scattered cells and cell sheets from the RIP and RIP-C hydrogels after incubation with RGDS under the same conditions. As expected, cell detachment from the surface of the RIP hydrogel was still facilitated (Figure 4b and Figure S8). Moreover, the detachment of cell sheets from the present RIP system (about 23 min) was much faster than from previous systems containing covalently immobilized RGD.[5a,b] These findings, together with the results from aforementioned cell adhesion study, undoubtedly demonstrate that the thermo-responsive "specific recognition sites" in a noncovalent molecular imprinting system can be used to introduce cell-adhesive peptide on a hydrogel substrate, which functions to promote not only initial cell adhesion but also final detachment of cell sheets simply by adjusting the temperature.

In summary, we have, for the first time, employed a molecular imprinting methodology to introduce the cell-adhesive peptide RGDS onto a thermo-responsive cell culture substrate, which was innovatively used as a highly efficient novel system for harvesting cell sheets. With the reversible thermo-responsive "specific recognition sites", the imprinted hydrogel could recognize and bind RGDS molecules at a temperature common for cell culture (37 °C), and then rapidly release RGDS when the temperature was lowered. Compared with simple physical absorption and covalent immobilization approaches used in previous studies, the thermo-responsive "specific binding" in the imprinted hydrogel proved to not only promote cell adhesion during cell culture, but also facilitate cell detachment during cell sheet harvest, thus ingeniously solving the long-standing problem in cell sheet technology. Moreover, the current work represents an exciting new biomedical application of MIPs. Because of the high affinity of the imprinted hydrogel polymer (comparable to that of the natural receptor)[7] and its innocuity to biomolecules (compared to covalent interactions),[4] such a novel method of introducing biomolecules by means of molecular imprinting may be a versatile and effective approach to biofunctionalization of materials.

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