JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
Communication
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Inducing Rapid Cellular Response on RGD-Binding Threaded Macromolecular Surfaces

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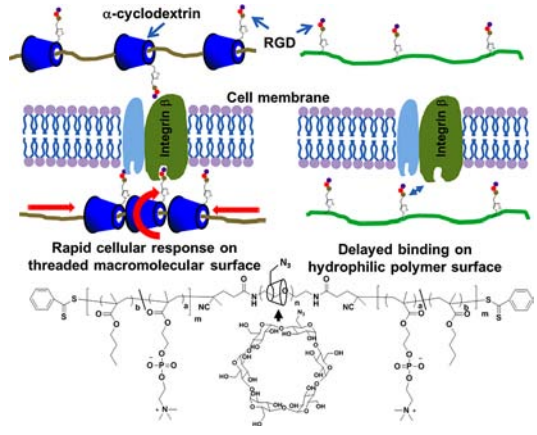
Supporting Information

ABSTRACT: The rapid response of integrin β_1 molecules to an RGD peptide on a dynamic polyrotaxane surface was successfully induced. As a result, RGD peptides introduced on a highly dynamic cyclodextrin molecule enhanced the frequency of contact with specific integrin molecules on the cell membrane at the early stage of material–cell interactions.

Regulation of the time required for cells to respond to specific ligands on material surfaces is a potentially useful technique for the development of novel analytical devices such as dynamic cell-sorting surfaces or high-performance biochips. Cell–material interactions are mediated by the binding of plasma membrane proteins to specific ligands in the extracellular matrix (ECM) present on the material surface.[1] Recently, various studies have been conducted in order to investigate the effect of the distribution of Arg-Gly-Asp (RGD, a specific cell-binding motif) on cellular responses. For instance, the maximum distance between ligands that can result in stable cell adhesion was found to be ∼60 nm. Other factors such as the RGD distribution or density, the level of ligand order/disorder, and the presence of a gradient were also shown to have significant effects on cell behavior.[2] RGD immobilized on rigid surfaces was demonstrated to regulate cellular responses such as adhesion density, directionality, and aspect ratio by specific modulation of the ligand display. Although these studies clarified the role of integrin–ligand interactions in directing cell fate, the temporal aspects of the binding were not taken into consideration in most of the published work.

Living cells are known to communicate with ECM molecules via their membrane proteins in a dynamic manner rather than a static one.[3] Therefore, surfaces that present the RGD ligand in a dynamic way should increase the frequency with which an integrin comes into contact a ligand, resulting in faster cell binding. Polyrotaxane (PRX) is a supermolecule containing mobile host molecules [e.g., α-cyclodextrin (α-CD)] threaded on a linear guest molecule [e.g., poly(ethylene glycol) (PEG)]. The α-CD molecules can move freely along the PEG backbone because of the formation of a non-covalent inclusion complex. Our previous SPR studies demonstrated a great enhancement of the ligand–receptor binding constant when a specific ligand (mannose) was introduced via the mobile α-CD molecules to its receptor (concanavalin A) immobilized on a surface.[4,5] It was clarified that the increased molecular mobility of ligands provided by using the PRX backbone was effective for enhancing the binding constant with the corresponding receptor protein. These results suggested that the same methodology could be used to develop dynamic surfaces that would enable regulation of the response time of cell–material interactions (Scheme 1).

Scheme 1. Schematic Illustration of RGD-Introduced PRX Block Copolymer and Random Copolymers



To demonstrate this theory, monoazidated α-CD (Az-CD) and propargyl-RGD peptide were first prepared. Because α-CD containing more than one azide group could induce multivalent interactions, Az-CD was synthesized to estimate the dynamic nature of PRX segment. An anchoring segment (denoted as PMB) composed of 2-methacryloyloxyethyl phosphorylcholine

Received: January 24, 2013
Published: April 1, 2013

   dx.doi.org/10.1021/ja400817q | J. Am. Chem. Soc. 2013, 135, 5513–5516

(MPC) and *n*-butyl methacrylate (BMA) at each end of the monoazidated PRX segment was synthesized by reversible addition−fragmentation chain transfer (RAFT) polymerization. A PMB segment is a well-known surface modifier that can be stably immobilized on various substrates.[6] Because the MPC unit is effective in eliminating nonspecific protein interactions,[7] the PMB anchoring segment was also anticipated to prevent nonspecific molecular interactions.[6,8] The synthesized monoazidated PRX block copolymer was then allowed to react with mixtures of propargyl-RGD and propargyl alcohol having three different compositions (100:0, 50:50, and 0:100) by a click reaction to give RGD-PRX surfaces with different RGD contents (denoted as RGD*x*-PRX, *x* = 100, 50, 0, respectively).

To confirm the dynamic nature of the PRX segment, rigid RGD surfaces (denoted as RGD*x*-Random, *x* = 100, 50, 0) were similarly prepared using a random copolymer containing 10 mol % 2-azidoethyl methacrylate. Quantitative analysis of the peptide-modified surfaces using the micro-BCA method showed that similar amounts of peptide were present on the RGD100-PRX and RGD100-Random surfaces [Figure S9 in the Supporting Information (SI)]. The dynamic natures of the RGD*x*-PRX and RGD*x*-Random surfaces were estimated in terms of hydrated viscoelasticity (Mf) using quartz crystal microbalance-dissipation (QCM-D) measurements. Mf was calculated as

$$\mathrm{Mf} = \frac{D_{\mathrm{sample,wet}} - D_{\mathrm{gold,wet}}}{f_{\mathrm{gold,dry}} - f_{\mathrm{sample,dry}}} \qquad \text{(eq. 1)}$$

where f denotes the resonance frequency at 35 MHz and D is the energy dissipation factor (see the SI). The value of Mf indicates the normalized viscoelastic nature of a polymer surface in its hydrated state. Therefore, a highly dynamic segment such as a weakly cross-linked hydrogel or polymer brush on the surface would have a large D.[9] In a previous study, we reported that PRX block-copolymer surfaces showed much higher Mf values than the corresponding random-copolymer surfaces, even though their chemical compositions were highly similar.[10] In this study, the same trend in Mf was observed for the prepared RGD-presenting surfaces (Figure 1a,b). The Mf value for the RGD100-PRX surface was ∼3 times higher than that for the RGD100-Random surface, even though the two polymer lineups showed similar levels of hydrophilicity and RGD contents. The interactions between integrins and surface RGD peptides were analyzed using surface plasmon resonance (SPR) measurements. Although several integrins such as $\alpha_5\beta_1$ and $\alpha_{11b}\beta_3$ require specific synergy domains in addition to the RGD sequence for strong binding,[11] the integrin β_1 subunit is a good indicator for investigating RGD−integrin interactions.[12] A solution of the β_1 subunit was flowed over the sample surfaces, and an increase in the SPR signal was observed for both the RGD-PRX and RGD-Random surfaces. No such change was evident when a solution of control protein (fibronectin) was used. This indicates that the specific interaction of RGD with integrin β_1 was responsible for the change in the SPR sensorgrams. Of particular interest is the rapid association and slow dissociation observed for the RGD100-PRX surface (Figure 1c). This indicates fast and strong specific binding of the β_1 subunit to the surface RGD. In contrast, integrin binding to the RGD100-Random surface was much slower and reached only approximately half the level of the PRX surface in the same contact time (Figure 1d−f). As a result, the binding constant for the β_1 subunit and the RGD group was much higher on the

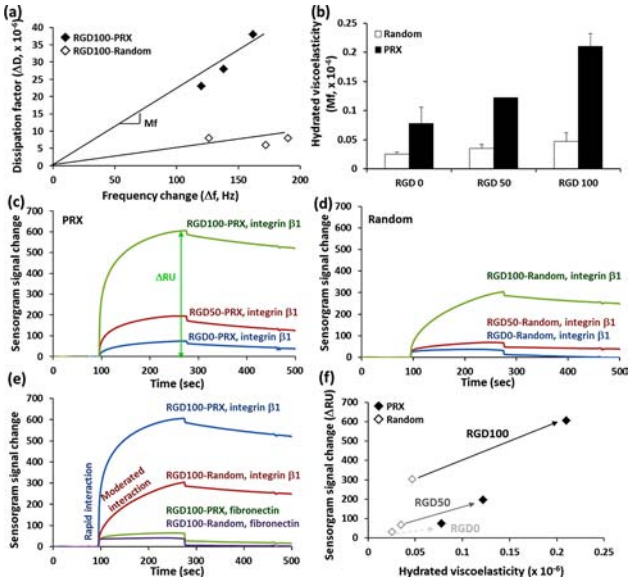


Figure 1. (a) Plots of dissipation factor change (ΔD) vs mass-dependent frequency change (Δf) for RGD100-PRX and RGD100-Random surfaces. (b) Calculated hydrated viscoelasticities. (c, d) SPR sensorgram changes for (c) RGD*x*-PRX and (d) RGD*x*-Random surfaces monitored during the flow of integrin $\beta 1$ solution (flow rate = 30 μL/min, contact time = 180 s, concentration = 10 μg/mL in PBS). (e) Comparison of RGD100-PRX and RGD100-Random under flowing integrin $\beta 1$ or fibronectin solution (10 μg/mL in PBS). (f) Plots of maximum changes in sensorgram signal vs hydrated viscoelasticity.

RGD-PRX surface (6.67×10^{11} M^{-1}) than on the RGD-Random surface (2.77×10^{10} M^{-1}).

Initial integrin−RGD binding and subsequent cell adhesion to the polymer surfaces were investigated using human umbilical vein endothelial cells (HUVECs) by means of real-time QCM-D measurements. The f and D values obtained from real-time QCM-D monitoring are effective means by which to estimate molecular interactions on surfaces.[13] A change in f is induced by molecular deposition such as protein adsorption or cell adhesion on the sensor surface, whereas D represents the ability of the surface molecules to dissipate the vibrational energy of the system.[14] Therefore, if units are tethered on the sensor surface through specific molecular interactions such as integrin−ligand binding, a rapid increase in D should be observed.[15] Figure 2a shows how D changed during the initial stage of HUVEC interaction with the RGD*x*-PRX block-copolymer surfaces. Because f remained constant throughout the measurement period (90 min), the changes in D are likely due to the initial binding of the membrane integrin with the surface RGD ligands prior to the adhesion stage. Furthermore, the stable f value also indicates that there was no significant nonspecific molecular deposition, such as protein adsorption (Figure S11). The D value for the RGD100-PRX surface began to increase as soon as the HUVECs were injected. When the RGD density was reduced to 50%, the slope decreased, and at 0% RGD the D value remained almost constant. This result indicates that a significant molecular interaction occurred immediately after HUVEC contact with the PRX surfaces containing RGD peptide. On the RGD*x*-Random copolymer surfaces, a time lag of ∼20 min was required before an increase in D was observed, which indicates the time necessary for the initial molecular interaction with the RGD (Figure 2b). Such an

dx.doi.org/10.1021/ja400817q | *J. Am. Chem. Soc.* 2013, 135, 5513−5516

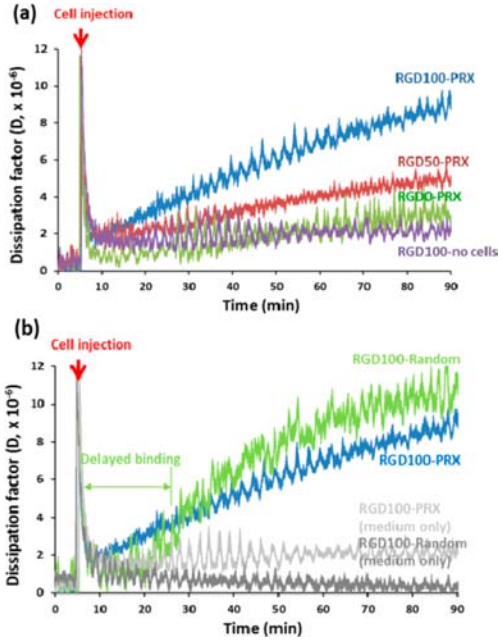


Figure 2. (a) Real-time monitoring of the dissipation factor on RGD-PRX surfaces during HUVEC adhesion. (b) Comparison of RGD100-PRX and RGD100-Random surfaces during HUVEC adhesion.

initial time lag (generally >5 min) is commonly observed when cells are seeded on RGD-immobilized surfaces.[15]

Living cells are known to interact dynamically with their environment via their surface molecules. Plasma membrane molecules such as integrins, immunoglobulins, lectins, and syndecans dynamically sense specific peptide or carbohydrate ligands in order to communicate with other cells or surfaces.[16] The binding efficiency of these specific molecular interactions is thought to be greatly enhanced when the two molecules are able to move and be positioned closer to each other.[5] The time lag that occurred on the random-copolymer surfaces is possibly the minimum time required for the induction of specific recognition between the integrin and the RGD peptide immobilized on the hydrophilic surface. In contrast, on the PRX block copolymer, the highly dynamic (hydrated viscoelastic) nature of the RGD-containing CDs is thought to enhance the frequency with which an integrin on the plasma membrane comes into contact with an RGD ligand.

Another interesting feature is the higher *D* value for the RGD100-Random surface after the initial time lag, which contrasts the initial fast interaction that was observed for the RGD100-PRX surface as seen by SPR and QCM-D measurements (Figure 2b). This is presumably due to a difference in adhesion to the RGD100-PRX surface in comparison with the RGD100-Random surface. Cell adhesion can be considered to consist of two independent steps: initial ligand recognition and the metabolic adhesion that follows. Although dynamic ligand surfaces have the advantage of a fast initial recognition process, this does not ensure the occurrence of metabolic adhesion. Conversely, stable focal adhesion that is accompanied by actin polymerization could be suppressed because both the ligand surface and the cell membrane are dynamic systems. To confirm this hypothesis, a similar QCM-D experiment was performed with cell suspensions containing cytochalasin D, which is a well-known inhibitor of actin polymerization (a major metabolic process).[17] An increase in *D* was still observed on the RGD100-PRX surface. However, the RGD100-Random surface showed a drastic decrease in *D*, which was almost the same as that of the no-cells state (Figure S12). The QCM-D result obtained with cytochalasin D is well-consistent with the SPR result (Figure 1). This indicates that most of the changes in the signal on the Random surface are induced by metabolic cell motion such as elongation and contraction. In contrast, the PRX surface can still maintain the binding between the RGD and the plasma membrane because of the dynamic nature of the threaded macromolecules, as depicted in Scheme 1. The adhesion of the HUVECs to the different surfaces was assessed by observing the morphologies of adhering cells after 3 h of incubation using a confocal laser microscope (Figure 3). On

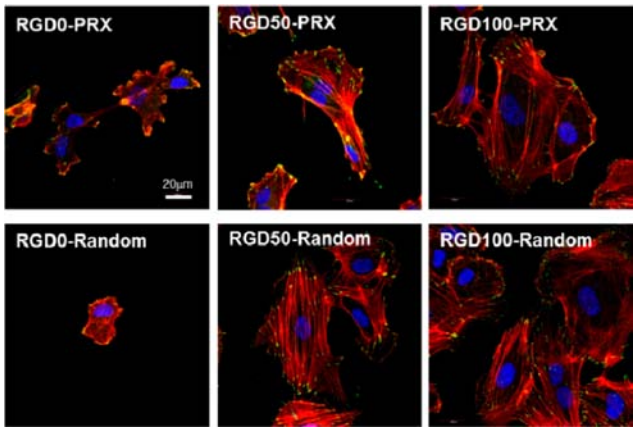


Figure 3. Confocal laser microscope images of adhering HUVECs after 3 h of incubation (blue, nucleus; red, F-actin; green, phospho-focal adhesion kinase).

both the RGD*x*-PRX and RGD*x*-Random surfaces, the projected cell area gradually increased with increasing amount of surface RGD, indicating that the adhesion of the HUVECs was induced by specific interactions between the RGD and the integrins. The larger spreading area on the random surfaces is presumably induced by the above-mentioned metabolic process, which can easily take place on the nondynamic surface.

Various factors that regulate cellular behavior on artificial materials have been investigated in order to further the development of useful biomedical devices. However, there have been no reports on techniques that can be used to regulate the time required for cells to respond to a specific ligand-presenting surface. The possibility to regulate freely the response time of cells to various surfaces provides enormous potential for novel biomedical devices such as high-performance cell-sorting surfaces or biochips. This report has demonstrated the feasibility of such an approach.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental procedures and analytical data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

dx.doi.org/10.1021/ja400817q | *J. Am. Chem. Soc.* 2013, 135, 5513−5516

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