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Biological Signaling

Dynamic Display of Bioactivity through Host–Guest Chemistry**

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The extracellular matrix (ECM) not only forms the supporting structure around cells but also offers them essential mechanical and chemical cues. Such cues can instruct cells to adhere, migrate, proliferate or differentiate and, therefore, mediate tissue development, tissue regeneration and wound healing.[1] These biological processes require a molecularly dynamic ECM to allow spatiotemporal control over the display of signals.[2] A number of bioactive short peptide sequences have been identified over the past few decades which mimic these signals. The peptide sequence RGDS, found in the extracellular protein fibronectin, has been extensively investigated to develop artificial matrices. This sequence has been found to bind transmembrane integrin proteins to initiate the recruitment of multiple proteins giving rise to attachment domains to the cell's cytoskeleton, known as focal adhesions.[3] The formation of focal adhesions allows cells to spread and activate various pathways which results in gene expression. Studies have shown that the presence of the RGDS sequence conjugated to artificial matrices resolves in the formation of focal adhesions.[4] For example, attachment to such artificial matrices has been shown to induce adhesion and spreading of fibroblasts,[5] human mesenchymal stem cells,[6] and human endothelial cells,[7] among others.[8] Artificial matrices in which cues could be displayed dynamically would help to understand biological processes and enable the development of therapies for tissue regeneration.[9]

We recently reported the development of a matrix in which the RGDS sequence is conjugated to a photo-cleavable nitrobenzyl ester and, therefore, initial spreading of fibroblasts could be reversed by irradiation.[10] Conversely, in other work, a cyclic RGD cue inactivated by a photolabile ester was covalently attached to silica surfaces to induce spreading upon irradiation.[11] There is also an example in the literature describing dynamic signaling through the use of electrochemical stimuli.[12] However, it remains a challenge to control the bioactive cues displayed on an ECM mimic in a dynamic and minimally invasive fashion.

We demonstrate here that a molecular host covalently attached to a matrix enables dynamic control of cue display through exchange of guest molecules. Specifically, we engraft β-cyclodextrin (CD) to alginate which is well known as a non-spreading surface for 3T3 fibroblasts. We found that addition of soluble RGDS-containing guest molecules to the culture media induced focal adhesion formation and cell spreading. The observed spreading of cells on the substrate can be reversed by simply adding competitive guest molecules bearing the mutated non-bioactive sequence RGES. This shows that the expressed cue on this artificial ECM can be controlled dynamically.

β-Cyclodextrin (CD) is a seven-membered sugar ring with a hydrophobic cavity that can host small hydrophobic molecules such as adamantane carboxylic acid and naphthoic acid with high affinities.[13] Although both complexes have relatively high association constants, they remain dynamic, and the presence of a guest with a higher association binding constant can replace them.[14] This property is an essential requirement for the exchange of the bioactive cues displayed on the matrix. We attached the RGDS sequence to two hydrophobic guest molecules, 1-adamantanecarboxylic acid and 1-naphthoic acid, through a pentaglycine (G_5) linker. The G_5 linker was introduced in the molecular design to ensure that the RGDS was not sterically crowded by the CD and, therefore, available for display to the integrin transmembrane proteins.[14] Both 1-adamantanecarboxylic acid and 1-naphthoic acid were coupled to the N-terminus of a NH_2-GGGGGRGDS peptide sequence on a solid-phase synthesis resin to give 1-adamantane-amide-G_5-RGDS (ada-RGDS) and 1-naphthalene-amide-G_5-RGDS (naphthyl-RGDS), respectively. As a control, the N-terminus of G_5-RGDS was also capped with acetic acid (acetyl-RGDS) which is expected not to be hosted by β-cyclodextrin. Finally, the biologically inactive control RGES (an RGDS analog mutated by the replacement of an aspartic acid by a glutamic acid) was coupled to 1-adamantane-carboxylic acid to give 1-adamantane-amide-G_5-RGES (ada-RGES) (Figure 1a and Table S1 in the Supporting Information).

The formation of supramolecular complexes between CD and the synthesized guests was confirmed using electrospray ionization mass spectroscopy (ESI-MS). The chromatograms of solutions of CD and guest-G_5-RGDS showed the presence of free guest and CD as well as the CD–guest complex (Figure S3). By means of isothermal titration calorimetry (ITC), the complexation of the newly designed guests with CD was further examined. It was confirmed that ada-RGDS, ada-RGES, and naphthyl-RGDS all bind to CD in a 1:1 ratio.

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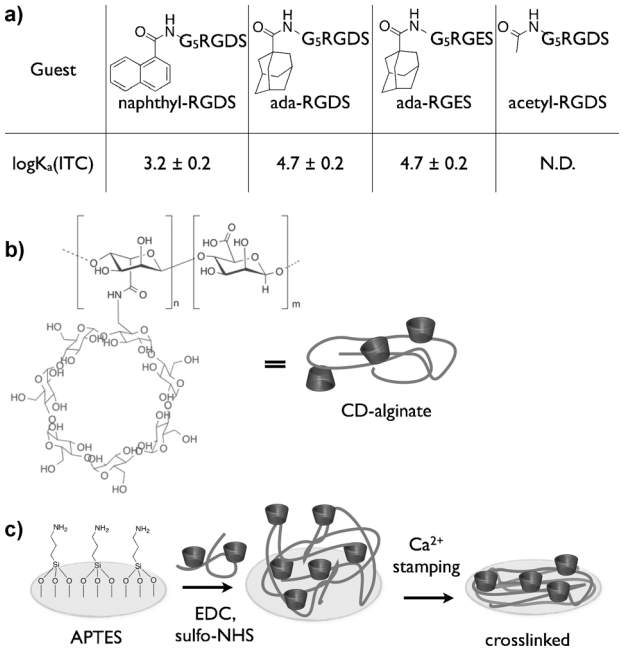

Figure 1. a) Chemical structures of molecular guests. Complex formation with β-cyclodextrin was studied by means of ITC. b) Structure of β-cyclodextrin functionalized alginate, CD-alginate. c) Reaction scheme of covalent attachment of cyclodextrin-functionalized alginate to glass.

Both the ada-RGDS and ada-RGES form complexes with CD with association constants of $\log K_a = 4.7$, whereas the CD naphthyl–RGDS complex was found to have an association constant of $\log K_a = 3.2$. All these binding constants were in the same range as values found in the literature.[13] The association constant for acetyl-RGDS with CD could not be determined by means of ITC, most likely because of its poor complexation with CD.

As a dynamic artificial extracellular matrix we chose to functionalize sodium alginate, a non-adhesive biocompatible polymer, currently explored for its use in regenerative medicine.[16] Alginate was functionalized by covalently engrafting mono-6-amino-6-deoxy-β-cyclodextrin[17] to 6% of the carboxylates using amide coupling chemistry yielding CD-alginate (Figure 1b). Subsequently, a fraction of the remaining carboxylates of the CD-alginate was converted to the activated *N*-hydroxysuccinimide ester and reacted with (3-aminopropyl)triethoxysilane (APTES) treated glass. To homogenize and crosslink the CD-alginate layer on the glass, cover slips were stamped with a $CaCl_2$ soaked agar gel.[18] Upon coating plasma treated glass with APTES, the contact angle increased from $4.7 \pm 1.1°$ to $56 \pm 2.6°$ corresponding to a decrease in hydrophilicity.[19] Subsequent attachment of the CD-alginate increased the hydrophilicity to a contact angle of $14 \pm 2.2°$. Fluorescence microscopy on fluorescein-labeled alginate confirmed the presence and confluency of the alginate layer (Figure S5). Finally, the presence of the CD on the surface was confirmed by incubating the coverslips in a solution of fluorescent probe Rhodamine B, which is known to have an affinity for the hydrophobic interior of CD.[20] Indeed, after 30 min of incubation the surface of the coverslips was more fluorescent than coverslips with non-functionalized alginate (Figure S3).

The ability of cells to adhere to the cyclodextrin-conjugated surfaces was investigated by incubating mouse 3T3 fibroblasts,[21] a cell line extensively studied on RGDS-displaying surfaces.[22] First, the guests, at two concentrations (10 and 100 μM), were added to different wells containing the CD-alginate-functionalized coverslips and incubated for 15 min. Then, 10^4 cells were added to each well and monitored for cell spreading. After 3 h of incubation, the cells were fixed and stained with DAPI (nuclei) and alexaFluor-488-conjugated phalloidin (actin filaments). In the absence of the dynamic RGDS cues, the cells attached to but did not spread on the surfaces and remained relatively round (Figure 2a and d). This attachment is most likely due to non-specific interactions between the substrate and the cell. In the presence of the naphthyl-RGDS and ada-RGDS, on the other hand, a drastic increase in cell spreading was observed and the cells showed a polygonal shape as compared to the round cells in the absence of the bioactive sequence. Moreover, the organization of the actin network was more distinctly observed for cells in the presence of naphthyl-RGDS and ada-RGDS. This actin organization into stress fibers is a typical feature observed for focal adhesion induced attachment as a result of signaling by the extracellular matrix.

To quantify the observed difference in morphology, the projected surface area, convex hull, and circularity were

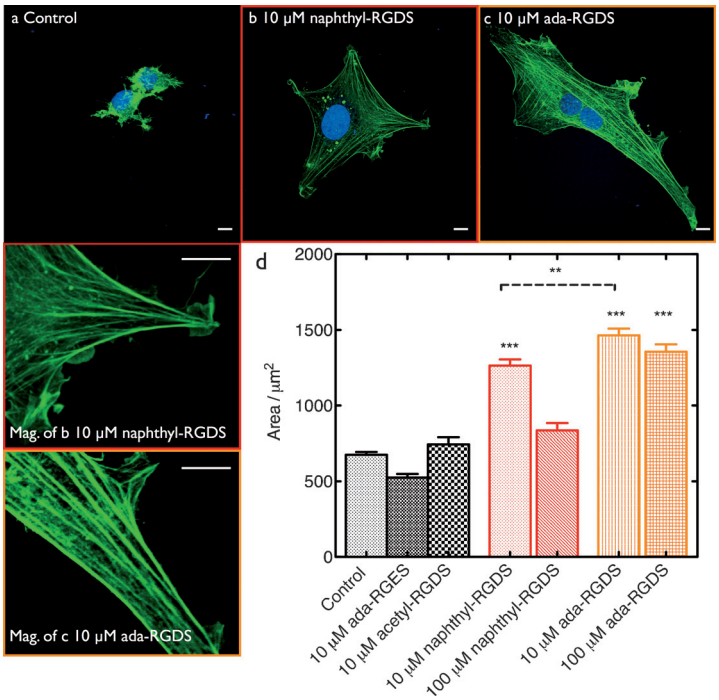

Figure 2. Selected confocal micrographs of 3T3 fibroblasts on CD-alginate, a) without guest, b) with 10 μM naphthyl-RGDS, and c) 10 μM ada-RGDS. Scale bars correspond to 10 μm. d) Quantification of the projected surface area of the cells in the presence of various guests. Statistical significance given compares samples to the control. Error bars correspond to standard error. ***: Statistical significance with $P < 0.001$, **: $P < 0.01$.

Table 1: Summary of fibroblast morphology on alginate-CD surface in the presence of various guests.[a]

Guest	CD-alginate with controls			CD alginate with naphthyl-RGDS		CD-alginate with ada-RGDS		**Dynamics:**
	without guest	10 μM acetyl-RGDS	10 μM ada-RGES	10 μM	100 μM	10 μM	100 μM	CD alginate with 10 μM naphthyl-RGDS. After 3 h addition of 10 μM ada-RGES
Projected area [μm^2]	795 ± 33	745 ± 46 (n.s.)	732 ± 34 (n.s.)	1260 ± 37 (***)	835 ± 49 (n.s.)	1587 ± 51 (***)	1444 ± 53 (***)	709 ± 28 (n.s.)
Convex hull [μm^2]	1293 ± 57	1276 ± 81 (n.s.)	1436 ± 63 (n.s.)	2001 ± 69 (***)	1429 ± 92 (n.s.)	2565 ± 85 (***)	2402 ± 90 (***)	1123 ± 56 (n.s.)
Circularity	0.35 ± 0.01	0.32 ± 0.01 (n.s.)	0.34 ± 0.01 (n.s.)	0.30 ± 0.01 (*)	0.28 ± 0.01 (***)	0.28 ± 0.01 (***)	0.25 ± 0.01 (***)	0.35 ± 0.01 (n.s.)
Stress fibers observed [%]	8.4	4.8	12	33	6.2	48	43	12

[a] Statistical significance given compares samples to the "without guest" column. Noted errors correspond to standard errors. ***: statistical significance with $P < 0.001$, **: $P < 0.01$, *: $P < 0.05$, n.s.: $P > 0.05$.

measured using image analysis (Figure 2d, Table 1 and Figure S6. See Supporting Information for definitions). In the absence of any RGDS or guest, the projected surface area was found to be $795 \pm 33\ \mu m^2$, the convex area was found to be $1293 \pm 57\ \mu m^2$, and the circularity 0.35 ± 0.1. In the presence of 10 μM of the non-binding acetyl-RGDS or the mutated ada-RGES, no significant change in projected cell surface area, convex hull or circularity was observed when compared to the untreated control. In the presence of 10 μM naphthyl-RGDS, however, both the projected surface area and the convex hull increased by a 1.5-fold, whereas the circularity of the cell decreased. Surprisingly, addition of 100 μM naphthyl-RGDS did not significantly alter the surface area, convex area or circularity. These surprising results will be discussed later. In the presence of both 10 and 100 μM ada-RGDS the convex hull and projected surface area increased by a factor 2.0 as compared to the controls while the circularity decreased. Finally, the fraction of cells that showed actin stress fibers was counted. In the absence of guest, 8.4% of the cells displayed actin stress fibers and this number increased to 12% in the presence of ada-RGES. However, in the presence of 10 μM naphthyl-RGDS or 10 μM ada-RGDS this number increased significantly to 33% or 48%, respectively.

From these first observations, we conclude that 3T3 fibroblasts respond to RGDS that is non-covalently displayed on surfaces. Upon recognition of RGDS by the transmembrane integrins, a biochemical cascade is initiated resulting in the formation of focal adhesion points, stress fiber formation and cell spreading.[23] The observed formation of actin stress fibers, cell spreading and focal adhesions points to a RGDS-integrin mediated adhesion mechanism. This conclusion is corroborated by the observation that ada-RGES, mutated by one amino acid, did not induce spreading. Interestingly, statistically significant differences were observed between the spreading induced by naphthyl-RGDS and ada-RGDS. We hypothesize that this difference is the result of either less cues displayed on the surface because of the lower binding constant of naphthyl-RGDS compared to ada-RGDS or a lower average lifetime of the host–guest complex for naphthyl-RGDS compared to ada-RGDS.

We attribute the difference between 10 μM and 100 μM naphthyl-RGDS to the antagonist effect of free RGDS molecules, thus inhibiting integrin-mediated signaling. In agreement with our findings, previous work has shown that inhibition of integrins occurs at concentrations in the range of 100 μM soluble RGDS.[24] This difference is not observed for ada-RGDS, and we attribute this effect to the self-assembly of ada-RGDS molecules into aggregates unable to inhibit integrin receptors as a result of their supramolecular configurations. Evidence for self-assembly of ada-RGDS is demonstrated by a Nile Red assay[25] (Figure S7) and the appearance of turbidity in the cell media. The use of solvatochromic probe Nile Red assay revealed that both ada-RGDS and ada-RGES indeed assemble into aggregates bearing a hydrophobic domain at a critical aggregation concentration around 50 μM. This assembly driven by hydrophobic collapse was not observed for naphthyl-RGDS or acetyl-RGDS at concentrations up to 1 mM. We assume that the supramolecular aggregate formed in this case does not display RGDS sufficiently to induce cell signaling. The excess of ada-RGDS is therefore not free in solution and cannot inhibit the cell signaling. This observation corroborates our earlier report that the organization of supramolecular aggregates is critical to bioactivity.[26]

Encouraged by the results described above, we examined the system's dynamics. As ada-RGES has a higher association constant compared to naphthyl-RGDS, it is expected to displace bound naphthyl-RGDS. Indeed, ESI-MS confirmed that the main complex formed in a solution of 0.5 mM CD, naphthyl-RGDS and ada-RGES is the CD–ada-RGES complex (Figure S3). In other words, a biologically active CD-alginate surface hosting naphthyl-RGDS can be rendered biologically inactive by the addition of ada-RGES. To test the dynamics, fibroblasts were incubated on the CD-alginate surfaces and incubated with 10 μM naphthyl-RGDS for 3 h resulting in attachment, focal adhesion formation, and a 1.5-fold increase in spreading as compared to an untreated

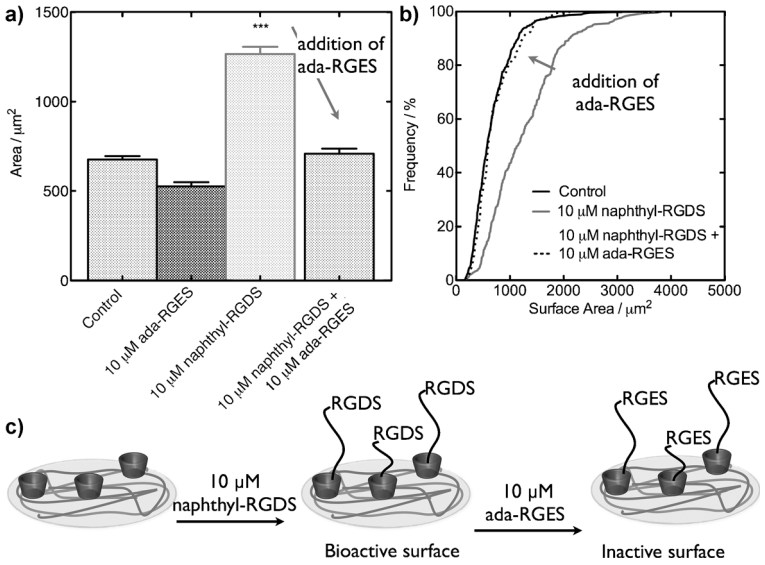

Figure 3. Projected surface area of the cells in the presence of various guests, significance is compared to an untreated control. Addition of ada-RGES to bioactive surfaces incubated with naphthyl-RGDS for 3 h renders them inactive. Error bars represent standard error. b) Cumulative distribution plot of projected cell surface area, where each point represents the total percentage of cells smaller than the given area. The data shows a decrease in cell spreading upon addition of ada-RGES. c) Schematic representation of the dynamic bioactive surfaces. Error bars correspond to standard error. ***: statistical significance with $P < 0.001$.

control (Figure 3a). After 3 h of incubation and spreading, 10 μM ada-RGES was added to displace the RGDS bioactive cues on the alginate surface with biologically inactive RGES. Addition of the ada-RGES and incubation for another 3 h indeed resulted in a drastic decrease in cell projected surface area to $709 \pm 28\ \mu m^2$, similar to the control experiment. Moreover, the number of cells displaying stress fibers decreased from 33% to 12%. This significant response demonstrates that indeed upon exchanging RGDS for RGES the cells collapse and reorganize their actin network. More generally, this result demonstrates that the display of biological signals in a dynamic fashion can be designed through the use of host–guest chemistry.

We have demonstrated dynamic control over bioactivity on surfaces by simple addition of guest molecules to cell culture media. The possibility of gaining spatiotemporal control over the display of signals on artificial ECMs opens new opportunities for the field of regenerative medicine. For example, we envision a dynamic 3D scaffold that can trigger stem cells to first attach, subsequently proliferate and finally differentiate into the desired cell types. The system described here using host–guest chemistry offers some of the requirements for such a process using a minimally invasive molecular strategy.

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