Bioorganic Chemistry

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Strongly Binding Cell-Adhesive Polypeptides of Programmable Valencies**

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The control of interactions between cells and materials is a topic of fundamental interest in materials science, surface chemistry, and micropatterning technologies. Currently, cellular interactions with biomaterials are commonly controlled by using only a limited set of molecules, such as fibronectin, laminin, and peptides derived from them (RGD and YIGSR, respectively).[1–3] Although short linear peptides offer increased stability and control over surface density, they do not generally exhibit the binding strength of whole proteins. Moreover, it is difficult to systematically vary binding strengths to enable quantitative experiments for studying cellular interactions with materials and surfaces. Herein, we show that polypeptides can be engineered in such a way that the valencies of the cell-adhesion region can be precisely programmed and systematically varied (up to precisely 80 copies of a RGD repeat) to enable strong and tunable interactions between cells and materials. We also demonstrate the programmable binding strengths on the basis of a well-controlled microfluidic-flow setup for the study of cell binding.

Because the RGD sequence binds integrins with high affinity,[4–6] it is commonly used for the adhesion of cells to materials; the most popular form is a short linear peptide containing a single copy of the RGD sequence. In many previous studies, different presentations of the RGD molecule have been investigated to alter its binding properties,[7–18] including nanoclusters,[19] cyclic molecules,[1,2] and assemblies of multiple RGD molecules bound to molecular scaffolds.[20–23] Nevertheless, these methods often involve complex synthetic strategies, and the number of RGD molecules presented is limited by the size of scaffolding molecules. Additionally, it remains challenging to develop a method for the synthesis of variants of the RGD sequence that would produce monodisperse species with a large (and readily controllable) number of RGD peptides per molecule for precise quantitative studies.

In this study, we aimed to produce polypeptides with long and programmable numbers of repeats of the RGD sequence by an alternative strategy to previous synthetic approaches. Our synthetic strategy enables the construction of monodisperse polypeptides containing many repeats with commonly available reagents. The use of multivalent interactions (i.e. the presentation of multiple copies of a ligand–receptor interaction)[24,25] to mediate the binding of molecules to surfaces is beginning to be explored systematically,[26–29] for example, through the chemical coupling of peptides to a functionalized polymer.[30–32] In previous recombinant methods[33–38] for the generation of polypeptides containing repetitive cell-adhesion sequences,[39,40] limited control over the precision and total length of repeats was possible. By contrast, recursive directional ligation, a recombinant method in which interrupted palindromes are used to clone many repeats of a desired sequence, can produce monodisperse polypeptides with a high and prespecified number of repeats. However, repetitive DNA sequences are typically unstable, as they are prone to undergo spontaneous recombination, and repetitive polypeptides, especially of unstructured sequence, are often difficult to express. Hence, the feasibility of constructing long repetitive polypeptides with well-controlled numbers of cell-adhesion sequences has not yet been demonstrated.

In this initial study, we sought to repeat up to 80 times a peptide sequence containing the RGD sequence (Figure 1a). First, we cloned plasmids containing DNA sequences corresponding to precisely 20, 30, 40, and 80 repeats of an RGD-containing motif. Specifically, we repeated a peptide sequence of GSGSGSGRGDS: this sequence contains flanking residues around RGD that are important in the cell-adhesion properties of fibronectin,[41] and a GS-based linker that is flexible and hydrophilic. We adapted a cloning technique[38,42] to use BamHI and BglII, two restriction enzymes that generate compatible overhangs, to produce vectors that would contain inserts with valencies corresponding to the total valency of the two starting inserts (Figure 1b). The resultant vector can be cut by the same restriction enzymes to yield inserts with controlled and increasingly large numbers of repeats. During each round, by controlling the valency of the insert in the cut vector and the valency of the new insert, an insert containing an arbitrary valency of the RGD sequence can be precisely cloned in the vector (see legend for Figure 1).

After the cloning procedure (see the Supporting Information for details), digestion of the four vectors with BamHI and BglII yielded fragments of the four expected sizes, which corresponded to DNA coding for 20, 30, 40, and 80 repeats of

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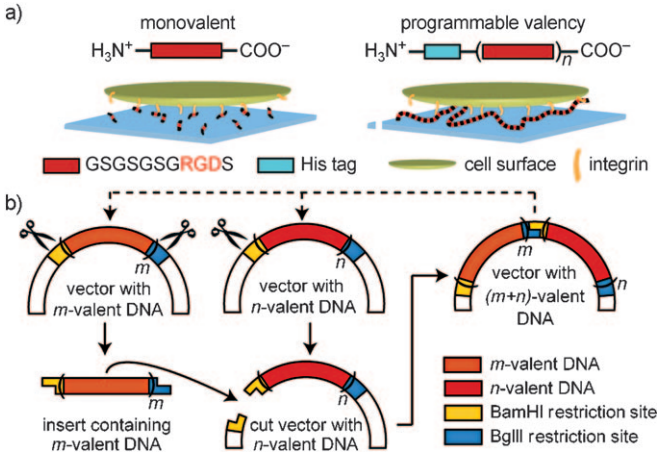

Figure 1. a) Schematic representation of polypeptides of programmable valency. b) Strategy for the synthesis of polypeptides by recursive directional ligation, whereby complementary overhangs from BamHI and BglII restriction sites are utilized to generate recombinant vectors of high valency. We ligated a 10-valent insert with a 10-valent vector to produce a 20-valent vector. We added another 10-valent insert to this 20-valent vector to create the 30-valent vector. Similarly, a 20-valent vector and 20-valent insert produced a 40-valent vector, and a 40-valent vector and 40-valent insert produced an 80-valent vector.

the RGD sequence (Figure 2a). Moreover, dideoxy sequencing of the coding region in all four multimers produced no deviation from the expected DNA sequences. (Since clones of long repetitive sequences are often unstable, as they tend to undergo spontaneous recombination, we froze the plasmids, minipreped them, and verified their size every few weeks.)

To produce the multivalent polypeptides, we expressed the plasmids in BLR-(DE3)-pLysS *Escherichia coli*. We purified the polypeptides by His-bind chromatography and ion-exchange fast protein liquid chromatography (FPLC) and obtained the products in good final yields, which ranged from 10 to 12 mg per liter of culture. SDS-PAGE of the products revealed polypeptides of approximately the right sizes (Figure 2b). Importantly, mass spectrometry (MALDI-TOF) of the four polypeptides confirmed their molecular weight to be within 144 to 682 Da (or 0.08 to 1.2%) of the expected molecular weight (Figure 2c). This margin is consistent with the accuracies of the described method for recombinant synthesis,[38,43] and in all cases, the error was well less than the length of a single RGD-containing monomer. Hence, although long, unstructured polypeptides can be difficult to express and are often easily degraded, and although the RGD sequence is also known to be susceptible to hydrolysis due to attack of the carboxylate group of the main-chain aspartic acid residue,[14,15] these data demonstrate that long repetitive cell-adhesion polypeptides can indeed be expressed and purified (at 4 mg mL^{-1} in 2% glycerol).

Further, we hypothesized that the monodispersity of the species could result in precise control over adhesion strength. Such control could enable quantitative studies of the relationship between molecular structure and binding strength: for example, previous studies have shown that increased valency can, somewhat unexpectedly, result in decreased binding specificity.[26] We conducted cell-adhesion experiments under carefully controlled flow conditions by using a microfluidic setup. First, we coupled the polypeptides to glass by using a heterobifunctional cross-linker. The N-terminal primary amine of the polypeptide reacted with the *N*-hydroxysuccinimide (NHS) moiety of the cross-linker; a sulfhydryl-containing silane on the glass reacted with the maleimide

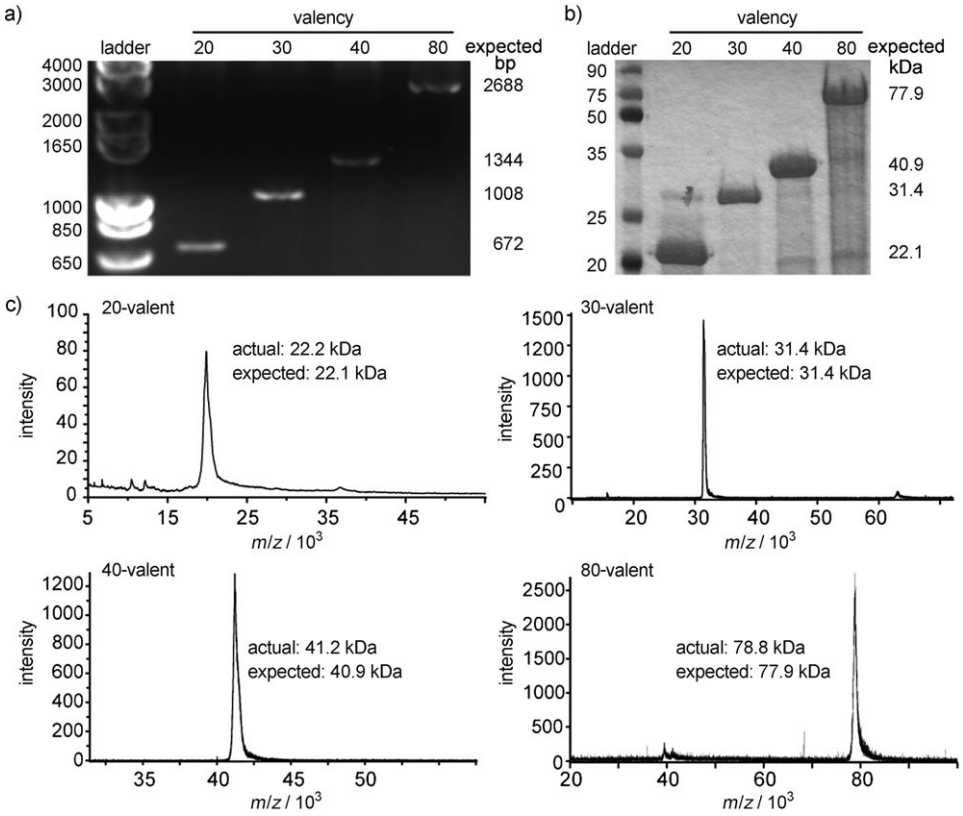

Figure 2. Confirmation of the production of cloned DNA and purified polypeptides. a) Agarose gel electrophoresis (with ethidium bromide staining) of inserts from four recombinant vectors digested with BamHI and BglII. The numbers at the top indicate the number of intended RGD repeats in the vector. b) SDS-PAGE (with Coomassie Blue staining) of the four expressed and purified polypeptides. The numbers at the top indicate the number of intended RGD repeats in the polypeptide. The slight difference in the molecular weight of the polypeptides on the SDS-PAGE gel from that expected may be due to different radii of gyration of these long polypeptides. c) MALDI-TOF mass spectra of the four synthesized polypeptides.

group of the cross-linker to form a strong covalent bond (Figure 3a). We found that, in a microfluidic system, 3T3 fibroblasts adhered only to the polypeptide-patterned area, as intended (the 40-valent polypeptide is shown in Figure 3b). Cells were well-spread after 2 h on glass derivatized with each of the multimers (as with fibronectin; Figure 3c).

We measured the level of cell coverage to test whether the multivalent polypeptides provided increased cell adhesion as compared to monovalent RGD, as well as increased resistance to cellular delamination due to fluid shear stresses. (We conducted ELISA experiments, which suggested that, for all multimers, coupling at solution concentrations of 1 mg mL^{-1} resulted in saturation of the surface; see Table S1 in the Supporting Information.) Whereas the monovalent RGD peptides did not adhere cells as readily as fibronectin (consistent with previous studies),[44] multimeric polypeptides showed binding similar to that of passively absorbed fibronectin (Figure 3d). Additionally, polypeptides of higher valencies (40- and 80-valent polypeptides) resisted fluid flow (i.e. with fewer than 20% of cells shearing off) when subjected to a 100 μL min^{-1} flow rate (approximate shear stress of 8.5 dyn cm^{-2}; comparable to values used in previous studies[45–48]). This result was similar to the level of cellular delamination observed for fibronectin (Figure 3e). The level of cellular delamination under shear was tunable and depended on the number of repeats of the cell-adhesion sequence: 40- and 80-valent polypeptides adhered cells significantly more strongly than monovalent and 20-valent polypeptides under fluid flow (Figure 3e).

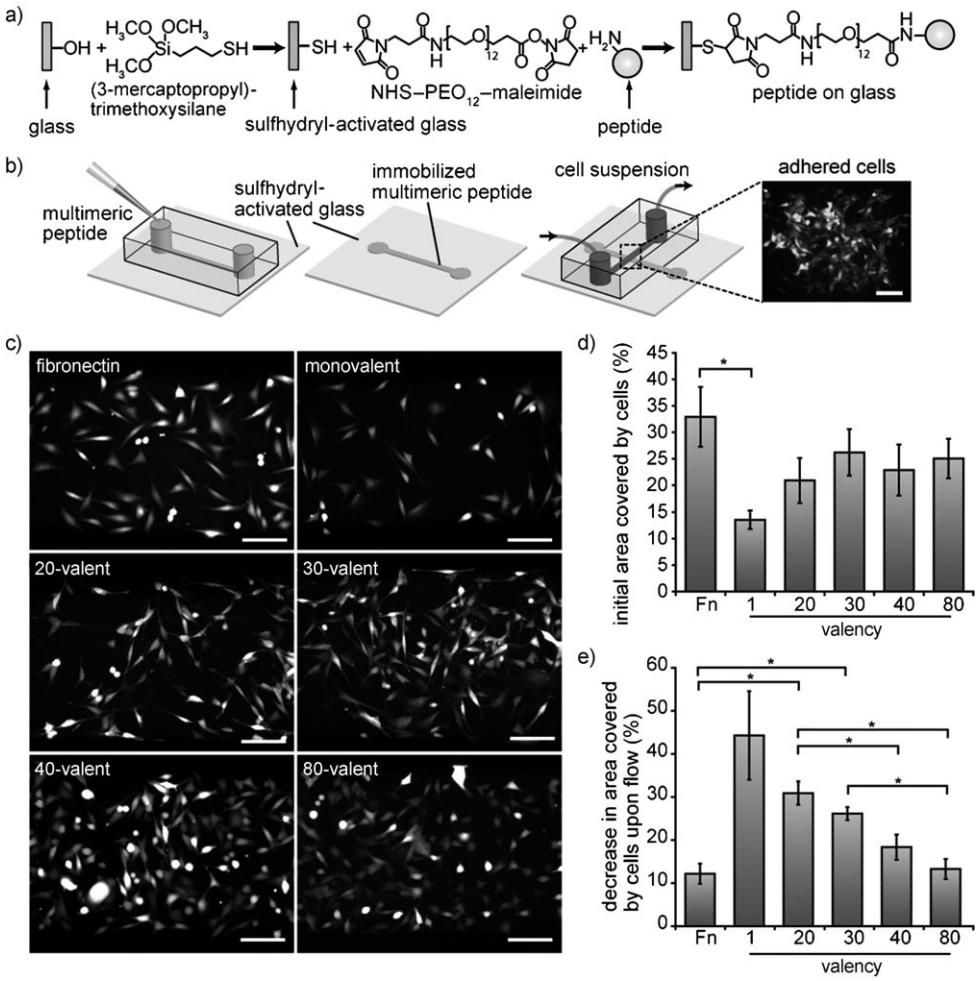

Figure 3. Adhesion of cells to surfaces derivatized by multivalent cell-adhesion polypeptides. a) Schematic representation of the cross-linking approach used to covalently attach the polypeptides to glass slides. b) Schematic diagram of the flow experiment and fluorescence micrograph of 3T3 fibroblasts adhering selectively to a region of glass derivatized by the 40-valent polypeptide. The polypeptide (1 mg mL^{-1}) reacted with the cross-linker in solution (2:1 polypeptide/cross-linker ratio) and was then introduced into a microfluidic channel containing glass silanized with 5% (3-mercaptopropyl)trimethoxysilane in toluene. After nonspecific binding had been blocked, 3T3 fibroblasts were seeded for 2.5 h and visualized under a microscope. Scale bar: 100 μm. c) Fluorescence micrographs of 3T3 fibroblasts seeded on glass derivatized with various polypeptides as compared with passively absorbed fibronectin. Scale bars: 100 μm. d) Graph showing the fraction of the total area covered by cells after incubation for 2 h. e) Graph showing the fraction of cells sheared off during the flow assay (100 μL min^{-1} for 10 min; approximate shear stress: 8.5 dyn cm^{-2}). Results are given as the mean ± standard error of the mean for $n \geq 5$. Statistical significance was determined by ANOVA ($^*p < 0.05$). Fn = fibronectin, PEO = poly(ethylene oxide).

By using image-segmentation algorithms, we analyzed the morphologies of adhered cells according to area, aspect ratio, perimeter, and other parameters (see Figure S2 in the Supporting Information). Cells adhering to RGD-containing polypeptides of all valencies showed no statistical difference for these parameters (or other cell morphological parameters; see the Supporting Information) when compared with cells adhering to fibronectin (analysis of variance (ANOVA): $n \geq 37$, $p < 0.05$). Hence, multivalent RGD polypeptides exhibit enhancement of binding (both statically and under shear) when compared to the commonly used RGD monomer. Since the level of adhesion and morphologies of adhered cells on surfaces coated with multivalent RGD polypeptides approach those of cells on surfaces coated with fibronectin, this synthetic strategy offers an alternative class of cell-adhesion molecules that exhibit high and tunable binding strengths. This simple synthetic strategy does not employ additional synergy sequences,[16] and the vectors can be readily transferred

between laboratories and expressed by using standard bacterial systems.

Despite the unstable nature of repetitive DNA and the difficulty in expressing unstructured peptides, our study demonstrates a recombinant method that enables the synthesis of monodisperse cell-adhesion polypeptides without the use of prestructured scaffolding agents. Also, since long polypeptides of this type are different in their three-dimensional conformation from highly rigid dendrimers or gold nanoparticles, our polypeptides may exhibit different properties in vivo.[32] Indeed, this study extends the capability of proteins to be used as monodisperse functionalized polymers.[36,49]

Moreover, the combination of monodispersity and the programmability of valency could enable precise and systematic studies of the adhesion of cells to materials. Thus far, studies of cell–material interactions have included variations of RGD nanoclusters[30] and multivalent hexahistidine-tagged maltose-binding proteins bound to cyclodextrin self-assembled monolayers.[50] The ability to precisely tune cell-adhesion strength may be of interest to researchers who are interested in altering the strength of interactions between cells and materials in a well-defined manner for the quantitative investigation of interactions between integrins and cell-adhesion molecules, the development of new micropatterning technologies, or the construction of biomaterials with tunable biocompatibility profiles (such as cellular delamination due to shear stress in implanted materials).[3]

In summary, we have developed a method for the synthesis of linear, repetitive, monodisperse polypeptides for cell adhesion by using readily available bacterial cloning and expression systems. Repeats of the RGD sequence restored the surface-binding strengths of cells to levels observed in whole fibronectin molecules, as tested by cellular delamination under carefully controlled flow conditions in a microfluidic setup. Moreover, the monodispersity of the species enables the systematic tuning of binding strength through the programming of different numbers of repeats of the cell-adhesion sequence. Overall, these molecules may expand the toolbox available to researchers for mediating cellular interactions with materials beyond currently used cell-adhesion molecules, such as fibronectin, laminin, and short linear RGD peptides.

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