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Epitope topography controls bioactivity in supramolecular nanofibers

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Abstract

Incorporating bioactivity into artificial scaffolds using peptide epitopes present in the extracellular matrix (ECM) is a well-known approach. A common strategy has involved epitopes that provide cells with attachment points and external cues through interaction with integrin receptors. Although a variety of bioactive sequences have been identified so far, less is known about their optimal display in a scaffold. We report here on the use of self-assembled peptide amphiphile (PA) nanofiber matrices to investigate the impact of spatial presentation of the fibronectin derived epitope RGDS on cell response. Using one, three, or five glycine residues, RGDS epitopes were systematically spaced out from the surface of the rigid nanofibers. We found that cell morphology was strongly affected by the separation of the epitope from the nanofiber surface, with the longest distance yielding the most cell-spreading, bundling of actin filaments, and a round-to-polygonal transformation of cell shape. Cell response to this type of epitope display was also accompanied with activated integrin-mediated signaling and formation of stronger adhesions between cells and substrate. Interestingly, unlike length, changing the molecular flexibility of the linker had minimal influence on cell behavior on the substrate for reasons that remain poorly understood. The use in this study of high persistence length nanofibers rather than common flexible polymers allows us to conclude that epitope topography at the nanoscale structure of a scaffold influences its bioactive properties independent of epitope density and mechanical properties.

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Keywords

Supramolecular Nanofibers; Epitope Presentation; RGDS Epitope; Focal adhesion

Introduction

Artificial scaffolds for tissue engineering or regenerative medicine aim to recreate a cellular microenvironment that provides cells with physical support as well as cues for desired cell functions such as proliferation, differentiation, migration and morphogenesis.^{1, 2} The cell-material interface is perceived as a critical component in the design of scaffolds in order to engineer biochemical and biophysical signals.^{3, 4} While these signals are provided by a set of ECM proteins in the native tissue, modifying the surfaces of artificial scaffolds with entire protein molecules faces several practical challenges, such as the high cost associated with protein synthesis and purification, decline of the scaffold mechanical properties, and the risk of pathogen transfer and immune reactivity.^{5, 6} Instead, using ECM-derived short peptide sequences that bind to the cell-surface integrin receptors is synthetically less demanding, and sequences of interest can be conjugated to chemically diverse scaffold materials.⁷ Substantial improvement in bioactivity of artificial matrices has been reported following modification with peptide ligands, as observed by enhanced cell adhesion, spreading and proliferation.^{5, 8, 9} This bio-functionalization strategy also allows for eventually rendering more complex scaffold functions such as dynamic regulation of epitope activity.^{10–12}

Peptide based self-assembled biomaterials have recently drawn attention for their ability to present epitopes at a very high density as well as facile control over epitope density.¹³ The peptide amphiphile (PA) construct, which conjugates a hydrophobic alkyl tail to one terminus of a peptide sequence, offers a number of advantages from a supramolecular design point of view.¹⁴ Specifically, peptide sequences can be used not only to incorporate biological signals, but also to control the morphology of the biomaterial's nanoscale structure and its mechanical properties.^{15, 16} All three factors controllable with peptide sequences can impact on biological response.^{17–19} Additionally, the ability to present bioactive peptide epitopes derived from ECM proteins or soluble growth factors has made PAs a common platform for a wide variety of tissue engineering applications ranging from angiogenesis²⁰ to bone,²¹ cartilage,²² enamel²³ and nerve tissue regeneration.²⁴ For example, a PA molecule displaying the laminin-derived IKVAV sequence improved functional recovery from acute spinal cord injury,²⁴ while PAs displaying the fibronectin-derived RGDS sequence promoted enamel regeneration²³ and increased the efficacy of therapeutic cell delivery.²⁵ Interestingly, when RGDS sequence was presented on a branched peptide backbone or co-assembled with filler PA molecules lacking epitopes, the cell adhesion and spreading response to the RGDS sequence was substantially enhanced;^{26,25} both observation suggest that the spatial arrangement of the epitopes on PA nanofibers could further play a prominent role in determining their bioactive efficacy.

Several strategies have been investigated in an attempt to enhance the epitope efficacy presented on a scaffold; a majority of these studies used the RGD sequence as a model

epitope as its interaction with integrin receptors is well understood.²⁷ Cell response to RGD functionalized substrates is found to be highly dependent on a number of factors including epitope density, peptide conformation and the presence of a supporting epitope.^{28–30} A cyclic peptide conformation offers higher affinity binding to $\alpha_v\beta_3$ integrin compared with a linear sequence, resulting in an enhanced cell attachment and focal adhesion formation.^{29, 31} Incorporation of the fibronectin derived synergy sequence PHSRN has been shown to greatly improve RGD mediated cell adhesion and spreading.^{30, 32} Enhanced cell adhesion and spreading is also observed with an increase in epitope density,^{33, 34} but a very high epitope density can reduce the response.^{26, 35} Further studies carried out using nanopatterned surfaces reveal that epitope distribution, in addition to their density, is critical for the cell adhesion, spreading and motility.^{36–38} However, the estimated critical RGD spacing for cell spreading varied substantially between experimental set ups (60–440 nm).^{28, 37, 39} While the intrinsic properties of the cell types used in these studies could underlie the variability, a difference in spatial presentation of epitopes on different substrates, modifying epitope accessibility to integrin receptors can also contribute.

A spacer moiety has been used previously in order to improve RGD epitope accessibility and enhance their interaction with integrin receptors.^{40–42} RGD epitopes presented with a spacer of four glycine residues or higher on an alginate scaffold have been shown to enhance the spreading and proliferation of fibroblasts.⁴¹ The spacer chemistry also influenced RGD-mediated cell response on gold nanopatterned surface, where a polyproline spacer elicited a better cell adhesion kinetics and focal adhesion formation over a spacer containing polyethylene glycol (PEG) or aminohexanoic acid.⁴² Interestingly, in this study, the integrin binding affinity of RGD was found to decrease on increasing the PEG or aliphatic spacer length. The effects of epitope spacing on cell response varied substantially in the studies mentioned above as well as in other similar studies that used polymeric scaffolds.^{43, 44} Since cells are highly responsive to extrinsic biophysical cues,^{45, 46} variables such as polymer cross-linking density and flexibility of the backbone could influence the epitope-specific response of cells in these polymer systems. In contrast non-covalent networks of PA nanofibers emulate the fibrous architecture found in the natural ECM. Given the growing interest on the use of biologically-inspired self-assembling materials as scaffolds for regeneration, investigating how spacers influence epitope bioactivity in these systems is crucial in order to optimize their molecular design. Furthermore, the use of high persistence length PA nanofibers in such studies avoids the confounding effects of backbone flexibility on the effect of spacers and the knowledge obtained could be applied to control bioactivity in a variety of supramolecular polymers beyond PAs.

The present work investigates how the spatial presentation of epitopes relative to the nanofiber surface (we use the term epitope topography to designate this relationship) affects PA bioactivity. A series of RGDS epitope-presenting PAs were designed where the epitope was connected to the base peptide backbone by a linker moiety; the length and the molecular flexibility of the linker were varied to modify the epitope presentation on the nanofiber surface. The bioactivity of nanofibers due to such change in the details of epitope display was evaluated by observing fibroblast cell response, focusing on their morphology changes, focal adhesion formation and cytoskeletal organization. Finally, the mechanism contributing

to the change in the nanofiber bioactivity was investigated by looking at integrin activation, strength of cell adhesion and cell spreading dynamics.

Experimental

PA Synthesis

PAs were synthesized using a standard fluorenylmethyloxycarbonyl (Fmoc) solid phase peptide synthesis (SPPS) on Rink Amide MBHA resin as described previously.⁴⁷ Amino acid couplings were performed either manually or on a CEMLiberty microwave-assisted peptide synthesizer. Rink Amide MBHA resin, Fmoc-protected amino acids and 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were purchased from Novabiochem; Fmoc-NH-PEG₄-CH₂COOH was purchased from ChemPep Inc.; palmitic acid was purchased from Acros Organics; Fmoc-(4-amino)benzoic acid and Fmoc-(4-aminomethyl)benzoic acid were purchased from VWR and Chem-Impex International Inc., respectively. All other reagents and solvents were purchased from Sigma-Aldrich and used as received. Fmoc deprotection was performed using 30% piperidine in *N,N*-dimethylformamide (DMF) and amino acid and palmitic acid couplings were performed with 4 molar equivalent (eq) protected amino acid or palmitic acid, 3.95 eq HBTU, and 6 eq of *N,N*-diisopropylethylamine (DIEA) in DMF alone or in a solvent mixture of 1:1:1 DMF:dichloromethane (DCM):*N*-methyl-2-pyrrolidone (NMP). The coupling reaction for the PEGylated amino acid was performed similarly to other standard Fmoc-protected amino acids, using Fmoc-PEGylated amino acid (3 eq), HBTU (2.95 eq), and DIEA (4.5 eq) in DMF. For the coupling of Fmoc-(4-amino)benzoic acid and Fmoc-(4-aminomethyl)benzoic acid, both were converted into acid chloride first (procedure described below) to increase the coupling yields. The coupling reaction was performed by using 4 eq of Fmoc-(4-amino)benzoyl chloride or Fmoc-(4-aminomethyl)benzoyl chloride and 6 eq of DIEA in NMP. Synthesized PA molecules were cleaved from the resin using a mixture of 95% trifluoroacetic acid (TFA), 2.5% water, and 2.5% triisopropylsilane (TIPS). After removing TFA by rotary evaporation, the product was precipitated with cold diethyl ether, dried, and purified using preparative scale reverse phase high performance liquid chromatography on a Varian Prostar Model 210 system equipped with a Phenomenex Jupiter Proteo column (C12 stationary phase, 10 mm, 4μm particle size and 90 Å pore size, 150 × 30 mm). A linear gradient of acetonitrile (2% to 100%) and water with 0.1% TFA (added to aid PA solubility) was used as the mobile phase for purification. ESI mass spectrometry (Agilent 6510 Q-TOF LC/MS) was used to identify the pure fractions, which were then combined together and lyophilized after removing excess acetonitrile by rotary evaporation. To prepare co-assembled PA nanofibers consisting of more than one PA species, PA molecules were first dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), followed by mixing of epitope-presenting PAs and the diluent PAs (*e.g.* base PA) at 1:9 weight ratio (so that epitope presenting PAs consists of 10 wt% in the mixture). The mixed solution was lyophilized, and finally dissolved in water prior to use. This procedure ensures intimate mixing of two PA molecules in the co-assembled nanofibers as reported previously.^{47, 48} PA fibers consisting of a single PA species were also subjected to similar HFIP treatment and lyophilization for consistency.

Fmoc-4-(aminomethyl)benzoyl chloride—To a suspension of 4-(aminomethyl)benzoic acid (612 mg) in 5 mL dry CH₂Cl₂ was added oxalyl chloride (4.0 mL). 10 μ L of DMF was added to this mixture and the reaction mixture was stirred at room temperature (RT) for 2 h as the reaction mixture became homogeneous. The pale yellow solution was concentrated *in vacuo*, taken up in CH₂Cl₂, and concentrated *in vacuo* again. The product was then dried under high vacuum to afford the product as a white powder, which was used in SPPS without further purification. ¹H NMR (CDCl₃): δ 8.08 (d, *J* = 8.0 Hz, 2H), 7.78 (d, *J* = 7.5 Hz, 2H), 7.60 (d, *J* = 7.5 Hz, 2H), 7.32–7.45 (m, 6H), 5.17 (s, 1H), 4.53 (d, *J* = 7.0 Hz, 2H), 4.45 (d, *J* = 6.5 Hz, 2H). ¹³C NMR (CDCl₃): δ 168.25, 156.67, 146.84, 143.92, 141.58, 132.07, 127.96, 127.79, 127.28, 125.12, 120.25, 66.88, 47.52, 44.69.

Fmoc-4-aminobenzoyl chloride—To a suspension of 4-aminobenzoic acid (950 mg) in 10 mL dry CH₂Cl₂ was added 40 μ L of DMF, followed by dropwise addition of oxalyl chloride (800 μ L). The reaction mixture was stirred at RT for 48 h as the reaction mixture became homogeneous. The solution was concentrated *in vacuo*, then taken up in CH₂Cl₂ and concentrated *in vacuo* again. The product was then dried under high vacuum to afford the product as a white powder, which was used in SPPS without further purification. ¹H NMR (CDCl₃): δ 8.06 (d, *J* = 6.5 Hz, 2H), 7.80 (d, *J* = 7.5 Hz, 2H), 7.62 (d, *J* = 7.0 Hz, 2H), 7.50 (s, 2H), 7.44 (t, *J* = 7.5 Hz, 2H), 7.35 (t, *J* = 7.5 Hz, 2H), 6.97 (s, 1H), 4.64 (d, *J* = 6.0 Hz, 2H), 4.29 (t, *J* = 6.0 Hz, 1H). ¹³C NMR (CDCl₃): δ 167.45, 152.87, 144.44, 143.56, 141.60, 133.40, 128.16, 127.78, 127.42, 124.97, 120.36, 117.94, 67.46, 47.15.

Measurements of the linker length were performed using Hyperchem modelling software. The PA structures were geometry-optimized *in vacuo* using an MM+ model. After completion of energy minimization was obtained, the distance between the carbonyl carbon of the outermost lysine residue in the peptide backbone and the amide nitrogen of the Arginine residue was measured.

Cryogenic Transmission Electron Microscopy (CryoTEM)

CryoTEM images were acquired on a JEOL 1230 microscope operating at an accelerating voltage of 100 kV. PA samples were prepared on 300 mesh copper grids with a lacey carbon support (Electron Microscopy Sciences), treated with air plasma (Harrick Plasma) for 30 s prior to use. 6 μ L of 0.1–0.2% (w/v) PA solution in water was deposited on a grid, blotted using a Vitrobot Mark IV (FEI) vitrification robot at 90–100% humidity, and vitrified by plunging the grid into a liquid ethane reservoir. The samples were placed into a Gatan 626 cryo-holder under liquid nitrogen, and a Gatan 831 bottom-mounted CCD camera was used to acquire the image.

Small Angle X-ray Scattering (SAXS)

SAXS measurements were performed at the Synchrotron Research Center at the Advanced Photon Source, Argonne National Laboratory using the beamline 5ID-D DuPont-Northwestern-Dow Collaborative Access Team (DND-CAT). PA solution at 1% (w/v) in water or a basal media was placed in 1.5 mm quartz capillaries (Charles Supper, 0.01 mm wall thickness). An incident photon energy of 15 keV corresponding to a wavelength λ =

0.83 Å was selected using a double-crystal monochromator, samples were exposed for 5 s, and the scattering data was collected using a CCD camera (MAR) positioned 245 cm behind the sample. Two-dimensional scattering data was reduced to one dimensional intensity profile by radial integration using the program FIT2D, and plotted on a relative scale (I) as a function of the wave vector $q = (4\pi/\lambda)\sin(\theta/2)$, where θ is the scattering angle. Background subtracted scattering profiles were obtained by subtraction of the 1D profile from a capillary containing only solvent from the 1D profile obtained for each sample. No attempt was made to convert the units to an absolute scale. Fitting of SAXS spectra was done using software provided by NIST (NCNR SANS package on IGOR); a polydisperse core-shell cylinder model was used to fit the spectra as described previously.⁴⁷

Circular Dichroism (CD)

CD measurements were performed on a Jasco J-715 spectropolarimeter. PA solution at a concentration of 0.04% (w/v) in water was added to a quartz cuvette of 1.0 mm path length. Spectra were collected at RT over a wavelength range of 190 - 300 nm with a step size of 1 nm. The data were averaged over three spectra for each sample.

Rheology

Rheological measurements were performed on a Paar Physica MCR 300 oscillating plate rheometer equipped with a 25mm-diameter cone-plate geometry and a gap of 0.05 mm. PA solutions at a concentration of 1% (w/v) in water were pipetted (175 µL) onto the rheometer plate and gelled by exposure to ammonia vapor for 10 min. All measurements were done at 25 °C, and the gels were allowed to equilibrate for 5 min at 0.1% strain prior to measurement. Data were collected at 0.1% strain over a frequency range of 1 to 100 s⁻¹ and traces from three separate runs were averaged for each condition.

PA Coating

PA coatings were prepared on sterile glass coverslips (12mm diameter) or tissue culture plates following a previously described method.⁴⁷ Briefly, the sample surface was first coated with 0.01% (w/v) poly-D-lysine, followed by application of a thin layer of 0.25% (w/v) sodium alginate (HF120RBS, FMC BioPolymer) in milliQ water. The alginate layer was gelled with 10 mM aqueous CaCl₂ solution. After a removal of CaCl₂ solution, 0.05% (w/v) PA solution in milliQ water was added onto the surfaces, and they were incubated overnight at 37 °C. Before the addition of cells, the PA solution was removed from the sample surface and gently rinsed with DMEM twice.

Cell Culture

NIH 3T3 mouse embryonic fibroblasts were maintained in growth medium consisted of Dulbecco's Modified Eagle's Medium (DMEM) with high glucose, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (P/S). The cells were grown in 75-mm² flasks (BD Falcon) and passaged every three days. All culture reagents were purchased from Gibco. For cell morphology experiments on PA coated substrates, fibroblasts were seeded at a low density (26 cells/mm²) in order to minimize cell-cell contacts, and incubated (at 37 °C, 5% CO₂) under serum free condition (DMEM + 1% P/S) following a published

protocol.⁴⁷ The serum free media was used to eliminate any confounding effects from serum adsorption to the nanofibers. Within the time-period (5 h) of experiment, no adverse cellular responses were observed from serum deprivation or serum shock after a transfer from serum containing growth media. For cell proliferation experiments, 4,000 fibroblasts were first plated in each well of a 96-well plate (Corning®) and incubated in growth medium. After 4 h of incubation, the media was exchanged with fresh growth medium to remove any unattached cells. Cells were incubated for additional 48 h prior to quantification of cell number. For experiments involving pharmacological inhibition of actin and microtubule polymerization, cells were allowed to spread on PA substrate for 2 h in serum-free media before the addition of inhibitors. Microtubule polymerization was inhibited by 5 μ M nocodazole (Sigma-Aldrich) and actin polymerization was inhibited by 0.5 μ M latrunculin A (Calbiochem). Following the addition of inhibitor in the culture media, cell morphology was monitored by live cell imaging or fixed after 2 h for fluorescence imaging or SEM.

Fluorescence Staining

Cells were fixed with 4% paraformaldehyde in PBS and 1 mM CaCl_2 for 30 min at RT. 1 mM CaCl_2 was added to the fixing solution and all other solutions subsequently used in staining and washing steps in order to prevent the disruption of the Ca^{++} cross-linked alginate layer that holds the PA layer on top. For immunostaining, fixed samples were first permeabilized with 0.1% Triton X-100 in PBS (5 min, RT), followed by blocking with 10% normal goat serum and 2% BSA in PBS (1 h, RT). Blocked samples were incubated with primary antibody in blocking solution overnight at 4 °C. The following primary antibodies were used in this work: Mouse monoclonal anti-vinculin, Sigma-Aldrich, 1:200 dilution; rat monoclonal anti-integrin β 1, Millipore, 1:250 dilution; mouse monoclonal anti- α -tubulin, Millipore, 1:1000 dilution. Primary antibodies were detected with Alexa Fluor conjugated secondary antibodies (Life Technologies, 1:1000 dilution, 1 h at RT). Actin filaments were fluorescently labeled with rhodamine or AlexaFluor-488-conjugated phalloidin (Life Technologies; 1:200 dilution, 1 h at RT) for visualization. Cell nuclei were counterstained with DAPI (Life Technologies).

Image Acquisition and Analysis

Images of fluorescently stained samples were obtained using an inverted confocal laser scanning microscope (Nikon A1R) or TissueGnostics cell imaging and analysis system mounted to an upright microscope (Zeiss). Cell morphology was quantified from phalloidin stained fluorescent images acquired by a 20X objective from randomly selected regions on the coverslip. Acquired grayscale images were background subtracted and thresholded to convert into binary images using ImageJ software (NIH). Cell morphology parameters were measured from these binary images using build-in functions in Matlab (Mathworks). The cell 'circularity' was defined as $4\pi \cdot (\text{area}/\text{perimeter}^2)$.⁴⁹ More than 300 cells from three independent experiments were analyzed for each substrate, unless otherwise mentioned.

Cell Proliferation Assay

Cell number was quantified after 4 h and 52 h of incubation using CyQUANT® Direct Cell Proliferation Assay (Life Technology) following the manufacturer's protocol. Prior to the quantification, the growth medium was exchanged once with a phenol-free Hank's Balanced

Salt Solution (HBSS, GIBCO®) to remove any unattached cells and phenol present in the growth medium. Fluorescence reading was performed on a Cytation 3 cell imaging multi-mode reader (BioTek Instruments, Inc.). Cell proliferation was estimated by taking the ratio of fluorescence intensities at 52 h and 4 h.

Live Cell Imaging

PAs were coated as described above on a 4-chambered Hi-Q4 culture dish (Nikon) or an 8-well chambered cover glass (Lab-Tek). Cells were seeded under serum-free conditions and imaged using a Nikon Biostation IM at 37 °C, 5% CO₂.

Scanning Electron Microscopy (SEM)

Cells on PA-coated glass coverslip were fixed with 2.5% glutaraldehyde in PBS (containing 1 mM CaCl₂) for 1 h at RT. Fixed samples were dehydrated by exposure a graded series of water-ethanol mixture. Once in 100% ethanol, samples were dried at the critical point of CO₂ using a critical point dryer (Tousimis Samdri-795) to preserve structural details. Dried samples were then coated with 14 nm of osmium using an osmium plasma coater (Filgen, OPC-60A), and imaged using a Hitachi S-4800 Field Emission Scanning Electron Microscope working at an accelerating voltage of 5 kV.

Western Blot

Cells were serum-starved for 12 h before plating onto PA-coated tissue culture plates, and incubated at 37 °C in a serum-free environment. After a predetermined duration, cells were washed once with PBS and lysed on the culture plate using M-PER protein extraction reagent with the addition of protease and phosphatase inhibitors (Thermo Scientific). The lysed product was further sonicated on ice using a digital cell disruptor (Branson) and centrifuged at 14 rcf for 15 min. The supernatant was mixed with SDS sample buffer, boiled for 10 min, and loaded in a Bis-Tris 4–12% gradient polyacrylamide gel (NuPAGE, Life Technologies). After running electrophoresis using MOPS SDS buffer (200 V, 50 min, 4 °C), proteins were transferred onto polyvinylidene difluoride (PVDF) membrane (350 mA, 120 min, 4 °C). The membrane was blocked with 5% bovine serum albumin (BSA, Sigma-Aldrich) in Tris buffered saline containing 0.1% Tween-20 (TBST) for 1 h at RT before incubating with primary antibody diluted in 1% BSA in TBST for overnight at 4°C. Primary antibodies used in this study are anti-phospho-FAK (rabbit polyclonal, Cell Signaling 3283, 1:500 dilution) and anti-GAPDH (mouse monoclonal, Millipore MAB374, 1:2500 dilution). The primary antibodies were detected by using horseradish peroxidase (HRP) conjugated secondary antibodies (goat anti-rabbit IgG, Cell Signaling, 1:2000 dilution and goat anti-mouse IgG, Bio-Rad, 1.5:10000 dilution) and enhanced chemiluminescence substrate (Thermo Scientific). All substrate conditions and time-points were included in a single experiment and proteins were run in a single block of gel. Data from 3 sets of experiments were used for the quantification by densitometric analysis of band intensity (using ImageJ software).

Statistical Analysis

Statistical analysis was done using nonparametric Kruskal-Wallis test with Dunn's post-hoc comparison between selected pairs, unless indicated otherwise. The data is presented in a box-and-whisker plot where the box displays the median value with upper and lower quartile, and the whiskers represents the value at 10th and 90th percentile.

Results and discussion

Design and characterization of RGDS epitope-presenting PA nanofibers

We selected RGDS as a model peptide epitope to study the influence of epitope presentation on bioactivity of nanofibers because it is the most well characterized ECM-derived epitope⁵⁰ and its interaction with integrin receptors has been extensively studied. RGD binds to a variety of integrin subtypes including $\alpha v \beta 3$ and $\alpha 5 \beta 1$,⁵¹ as shown by crystal structure examinations of the integrin binding site in the extracellular domain.²⁷ RGDS epitope-presenting PA molecules were synthesized by attaching the epitope to a peptide backbone having a consensus sequence of three valine, three alanine and three lysine residues (VVVAAAKKK; Figure 1a) that has been previously shown to form rigid cylindrical nanofibers.¹⁸ The valine residues placed next to the alkyl tail help stabilize the supramolecular nanofibers by promoting β -sheet hydrogen bonding along the long axis of the nanofiber.^{16, 52} Charged lysine residues on the distal end of the peptide sequence were included to enhance water solubility and facilitate formation of a nanofiber network in the presence of physiological salt solution. Here a positive charge (lysine) was chosen over a negative charge (*e.g.* glutamic acid) because the positively charged PAs allow a uniform and reproducible coating on the glass or plastic surfaces for cell assays.^{18, 47} Furthermore, to regulate the distance between the epitope and the peptide backbone, we used linkers consisting of glycine residues. The absence of a side chain makes glycine amino acids particularly suitable as linkers in order to eliminate steric or charge effects. In this study, we considered three different linker lengths consisting of one, three or five glycine residues (the PAs thus referred to as G1 PA, G3 PA and G5 PA). In a geometry-optimized PA conformation, these linkers would separate the RGDS epitope from the peptide backbone by approximate distances of 4.82 Å, 12.2 Å and 19.3 Å.

The epitope-presenting PAs were further co-assembled with PA containing the peptide backbone only (base PA, Figure 1b) at a ratio of 1:9 by weight (*i.e.* epitope-presenting PA content in the co-assembled nanofibers is 10 wt%). Our laboratory has previously shown that this co-assembly improves the epitope bioactivity, presumably by reducing epitope crowding on the nanofiber surface.²⁶ In addition, the combination would allow the nanofiber morphology and mechanical properties to be better conserved across various epitope-presenting PAs tested, because the properties would be determined by the dominant PA component of the nanofibers—the base PA. To ensure intimate mixing, two PAs were first mixed in HFIP to disassemble any preformed structures, lyophilized and finally dissolved in water, as previously described.⁴⁷ A molecular graphics representation comparing the location of RGDS epitopes relative to the surface of co-assembled nanofibers for PAs with three different linkers is shown in Figure 1c.

The nanostructures formed by the self-assembled PA molecules were characterized using cryogenic transmission electron microscopy (cryoTEM) and small angle X-ray scattering (SAXS). CryoTEM images revealed that the base PA formed long cylindrical one-dimensional nanostructures in aqueous solution (Figure 2a); a similar morphology was observed for the G1, G3 and G5 PAs (Figure 2b–d). The cylindrical morphology of the nanofibers was further confirmed by SAXS analysis. Scattering traces from the PA samples demonstrated a power law dependence of q^{-1} in the low q regions, characteristic of a cylindrical shape (Figure 2e).⁵³ As expected, fitting the traces to a core-shell cylinder model showed an increase in the average diameter with increasing linker length (*c.a.* 8.6 nm in G1 PA vs. 9.0 nm in G3 PA and G5 PA; base PA diameter 8.1 nm). However, we observed a less-than-perfect fit for the co-assembled fibers (Figure S1) and attribute it to the surface features created by the epitope and linker structures on otherwise uniform cylindrical nanofibers. Based on circular dichroism (CD) spectroscopy, all nanofibers were found to contain a dominant β -sheet secondary structure with minima and maxima at 219 nm and 202 nm, respectively, although there were some variations in the peak intensity values (Figure 2f). We attribute this variation in intensity without any shift of the peak to a difference in aggregation of nanofibers in the solution. The SAXS and CD experiments confirmed that the overall morphology and the internal order of the nanostructure assemblies were conserved across the PA compositions investigated here.

After confirming that the co-assembled RGDS-presenting nanofibers with various linkers have effectively the same morphology, we compared their mechanical properties using rheological measurements of gels formed by the nanofibers. Previous work from our group has shown that a measurement of such bulk gel property correlates well with the cell response observed as a function of nanofiber stiffness.¹⁸ Measurement of the storage modulus (G') and the loss modulus (G'') of 1% (w/v) gels over an angular frequency range of 1–100 s^{-1} revealed that even an addition of 10 wt% epitope presenting PA to base PA caused a moderate reduction in gel stiffness (Figure S2). The storage modulus (a measure of the elastic modulus or stiffness) of gels formed by base PA and G1 PA were found to be 5.7 ± 0.5 kPa and 2.8 ± 0.4 kPa, respectively at an angular frequency of 10 s^{-1} and 0.1% strain. The change in the linker length, however, produced much less variation in stiffness with the storage modulus remained within the 2–3 kPa range for G1 PA, G3 PA and G5 PA.

Epitope presentation and cell response

We investigated whether spatial separation of the epitopes on the nanofibers had any effect on cell response using fibroblasts as a model cell type. The response of fibroblasts can be assessed by evaluating the cell morphology, cytoskeletal organization and focal adhesion formation.^{26, 28, 29, 47} PAs were coated on glass coverslips using a method we developed previously that involves sequential coating of poly-D-lysine, alginate and PA;⁴⁷ this procedure enables uniform dense networks of PA nanofiber coating (Figure S3). 3T3 fibroblasts were incubated on these coatings for 5 h, stained for actin and analyzed. A serum free culture medium was used to minimize any contribution from the serum proteins adsorbed to the nanofiber surface. On the base PA, cells were poorly spread and mostly round in shape (Figure 3a). Qualitatively, cells on G1 PA had a similar morphology except dendritic extensions were observed more often (Figure 3a, phalloidin stained images). On

G3 PA coated substrate, however, cells appeared more spread, and the shape assumed a more polygonal form; cell spreading was further enhanced on G5 PA while maintaining the polygonal shape. This striking difference in cell morphology was absent, and cell shape was similar to that found on the base PA when the RGDS sequence on G5 PA was replaced by a mutated non-bioactive RGE sequence (RGE PA). This observation suggests that the cell response was specific to the RGDS epitope and not due to a secondary effect of the linker.

The change in cell morphology was further assessed quantitatively by measuring the projected cell area, convex hull and circularity. The projected area was not significantly different between cells cultured on base PA and G1 PA ($1371 \pm 34 \mu\text{m}^2$ and $1488 \pm 35 \mu\text{m}^2$ respectively), whereas a 1.5 and 1.8 fold increase was observed on G3 PA ($2124 \pm 42 \mu\text{m}^2$) and G5 PA ($2467 \pm 43 \mu\text{m}^2$), respectively (Figure 3b). Cell area on the RGE PA ($1492 \pm 33 \mu\text{m}^2$), which also bears the five-glycine-residue linker, was similar to that of base PA, confirming the RGDS epitope-specific response. The convex hull, which is defined by the convex area enclosing the distal ends of the cell processes, showed a trend similar to that of the projected area (Figure 3c), except the hull area was slightly but significantly higher on the G1 PA compared with the base PA ($2023 \pm 47 \mu\text{m}^2$ vs. $1709 \pm 43 \mu\text{m}^2$). This difference is attributed to the small increase in dendritic processes extension observed on G1 PA, which has a minimal effect on projected area but contributes to a greater increase in convex hull. The difference in morphology between cells on G1 PA and base PA was also reflected by a significant reduction in the circularity (defined as $4\pi \times \text{area} / \text{perimeter}^2$ where circularity of a perfect circle is 1; see Figure 3d) on G1 PA (0.42 ± 0.01 on base PA vs. 0.34 ± 0.01 on G1 PA). A polygonal cell shape on G3 and G5 PA caused a further reduction of the circularity index to ~ 0.18 .

Apart from the changes in cell morphology, linker length also caused striking differences in the cellular distribution of actin filaments and focal adhesion formation. On G5 PA substrate, cells exhibited a highly organized network of actin filaments (visualized by phalloidin staining), which frequently bundled to form stress fibers (Figure 3a). The termini of these stress fibers were anchored to the PA substrate through focal adhesions as revealed by staining against vinculin. Moreover, the presence of $\beta 1$ -integrin receptors in these adhesion structures suggests their possible involvement in focal adhesion formation. In contrast to G5 PA, on the G1 PA nanofiber substrate (as well as on base PA and RGE PA) the arrangement of actin filaments in the cell soma was less ordered; actin filament bundles were observed only around the cell circumference, and focal adhesion formation was not observed. Cells on G3 PA substrate exhibited an intermediate level of actin filament organization and focal adhesion formation. Our results suggest that the linker length influences the interaction at the cell-PA interface, resulting in a characteristic change in cytoskeletal organization and focal adhesion.

A prominent difference in cell morphology at 5 h motivated us to explore whether the linker length also influences the cell response at a longer time-scale. For this purpose, we measured fibroblast proliferation on PA substrates after 52 h of culture (Figure S4). Cells incubated on the base, G1 and RGE PA showed a modest 1.5–1.9 fold increase in cell number during this period (differences between substrates were not significant). However, an increase in cell number was significantly higher on G3 PA (2.5 ± 0.3 fold increase) and

further increased on G5 PA (3.3 ± 0.4 fold increase). Thus the cell proliferation result indicates that the influence of the linker length on bioactivity of PA nanofibers displaying RGDS epitopes is maintained at a longer time-scale.

An enhanced fibroblast response to RGDS on co-assembled PA nanofibers containing longer linker suggests more efficient binding of epitopes to the integrin receptors located on the cell membrane. This improvement of integrin binding could result from, (a) the separation of the epitope region from the charged and bulky amino acids of the peptide backbone in an individual PA molecule, and/or (b) spacing out the epitope from the surface of the nanofiber, that is by changing the topographical presentation of epitope. To investigate how epitope topography on nanofibers contributes to the bioactivity, we co-assembled G5 PA with RGEs PA instead of Base PA. Nanofibers consisting of 10% G5 PA in RGEs PA have a comparable RGDS epitope density to the G5 PA co-assembled with base PA (as used before). However, the RGDS sequence in this system is no longer projected out of the nanofiber surface (Figure 4a and b). The average projected area of fibroblasts on G5/RGEs PA substrate was found to be 35% less than on G5/Base PA substrate (Figure 4d and S5), indicating a strong reduction in bioactivity. In a conventional polymeric scaffold such difference is not expected since it would be difficult to define topography of epitope display in flexible chain of the polymer. Our results suggest that the spatial presentation of epitopes on the co-assembled nanofibers could be critical for cellular interaction even the density of bioactive molecular species remains similar. Interestingly, the cell area was reduced by only 10% when pure G5 PA nanofibers (consisting of 100% G5 PA molecules) were used instead of co-assembled G5/RGEs PA nanofibers. Although the display of RGDS epitopes from the nanofiber surface is expected to diminish similarly for both G5/RGEs PA and pure G5 PA nanofibers (Figure 4c), we attribute the partial rescue of cell spreading in the latter system to an excess of available epitopes on the nanofiber surface. At the same time, our results demonstrate that linker-bearing PAs achieve a favorable spatial presentation of epitopes in co-assembled nanofibers, which can elicit even higher cell response at a much lower epitope density than pure (undiluted) linker-bearing PAs.

Dynamics of cell spreading on PA substrates

The actin network inside the cell not only helps to maintain its shape, but also regulates dynamic cell functions such as extension and retraction processes (lamellipodia and filopodia) and migration. As discussed earlier, the intracellular organization of actin changed strikingly on glycine-linker-series of PA substrates, along with a change in the cell morphology. We therefore explored whether the linker length also has an influence on cell spreading dynamics. We acquired time-lapse images of fibroblasts on G1 PA and G5 PA coated substrates, as the maximum difference in the cell morphology was observed between these two PAs. Fibroblasts showed frequent extensions of filopodial processes on both PA surfaces (Figure 5a, Movie S1 and S2). On most occasions, these processes were quickly retracted on G1 PA coated surfaces, so there was effectively no change in cell spreading. However, the filopodial processes were more stable on G5 PA coated substrates and frequently followed by an outward expansion of the leading edge of the cell membrane. As the filopodial movements were different on these two PA surfaces, we examined their

morphology and distribution on the cell surface by using scanning electron microscopy (SEM). After 5 h of culture, filopodial extensions were observed along the cell edge on both nanofiber substrates (Figure 5b and S6). In addition, many filopodia-like processes were distributed over the surface of fibroblasts on G1 PA substrate. We speculate that these short processes—resembling filopodia in morphology, but not attached to the substrate—represent the remnants of retracted filopodia, observed during live imaging. Furthermore, these processes were far fewer on the surface of cells on the G5 PA substrate, where filopodial retraction events were less frequent. Taken together, these findings indicate that reduced filopodial retraction afforded by the G5 PA substrate favors higher spreading of the fibroblasts.

Integrin Activation and Focal Adhesion

Filopodial and lamellipodial processes are formed from a deformation in the cell membrane caused by the actin polymerization near the distal edge of the membrane, while retraction of these processes is prevented by an anchorage of the actin filaments to the ECM bound integrin complexes. In our experiments, PA nanofibers were presented with RGDS epitopes to provide specific binding to the integrin receptors. Therefore frequent retractions of filopodia on G1 PA and restrained retractions on G5 PA would suggest a weaker and a stronger RGDS-integrin coupling on these two PAs, respectively. To verify whether the linker length in PA molecules could influence the epitope-integrin interactions, we assessed integrin activation on PA substrates. Ligand binding to the extracellular domain of the integrins brings about specific conformational changes in their intracellular domain, which in turn initiates local recruitment and phosphorylation of a set of specific proteins including focal adhesion kinase (FAK). By Western blot analysis of the phosphorylated form of FAK (pFAK, phosphorylation of tyrosine 397 residue), we observed that cells on G5 PA substrate have an increased level of pFAK compared with those on the G1 PA substrate, even within only 30 min of incubation (Figure 6a). This was confirmed by densitometric analysis of blot intensity (GAPDH expression was used as an internal control), where a three-fold higher level of pFAK was observed in the cells on G5 PA substrate compared with the cells on G1 PA substrate (Figure S7). Moreover, the mutated control RGEs PA, which also had a five glycine residue linker, failed to show any enhancement of pFAK expression. Thus, both the RGDS epitope and the long linker are necessary for the integrin activation.

In addition to phosphorylation of FAK, RGDS epitopes in G5 PA also promoted integrin clustering manifested by the formation of β 1-integrin rich mature focal adhesions. According to the tensegrity model of the cell, these focal adhesions play an important role in maintaining cell shape. The final geometry of the cell is determined by the contractile force generated by the actomyosin network counterbalanced by the compressive force from microtubules (which form a meshwork of rigid filaments within the cytoplasm) and external support from ECM through focal attachment.⁵⁴ Therefore, to better understand how the cell-PA interface contributes to maintaining cell shape, we perturbed the force equilibrium by inhibiting microtubule polymerization. Fibroblasts were allowed to spread on PA coated substrates for 2 h, and then treated with 5 μ M nocodazole, a pharmacological inhibitor of microtubule polymerization. Time-lapse imaging showed that cells on G1 PA collapsed into a spherical blob within minutes after addition of the drug, indicating that microtubules play a

critical role in maintaining the cell spreading on this substrate (Movie S3). The cells, however, did not collapse when nocodazole treatment was accompanied by latrunculin (500 nM), an inhibitor of actin polymerization (Figure 6b). This observation confirms a direct involvement of actomyosin generated contractile force in inducing the collapse. A resistance to somatic collapse was observed when fibroblasts were plated on G5 PA coated surfaces and similarly treated with nocodazole (Movie S4). The partially contracted cells often had a cogwheel appearance where multiple long cytoplasmic processes radially extended out from the cell circumference. These radially-directed cytoplasmic processes appeared to be anchored to the underlying substrate at their distal end, and a complete cell collapse was observed when these anchorage points were lost. Phalloidin staining further revealed that these radial processes contained bundles of actin, which were connected to the actin network within the cell soma (Figure 6b). We hypothesize that the contractile forces generated by the somatic actin network are transmitted by these radial bundles of actin to the substrate anchorage points. In turn, the binding of integrins to G5 PA nanofibers at the anchorage points is sufficiently strong to counterbalance the actin generated tension, even in absence of microtubules. In comparison, the weak attachment of cells to G1 PA nanofibers was unable to bear the actin-generated tensional force after nocodazole treatment, leading to immediate somatic collapse. A direct measurement of the binding affinity between integrin and RGDS epitope on PA nanofibers could have provided additional information on how the length of the linker influences the cell-nanofiber interaction; however, this is experimentally challenging for cylindrical supramolecular systems, especially those that tend to form highly entangled networks of fibers, and therefore was not investigated in the present study.⁵⁵

Linker Flexibility and Cell Response

The experiments involving fibroblast spreading, integrin activation and cell-substrate adhesion strength support the conclusion that the presence of a longer linker in PA molecules facilitates the interaction between RGDS epitopes and integrins. Our findings also suggest that a favorable topographical presentation of epitopes on PA nanofibers afforded by the long glycine-linker contributes to this effect. We speculate that a long linker spaces out the epitopes from the nanofiber surface, helping to overcome steric barriers that might otherwise interfere with signaling to cell surface integrin receptors by epitopes in the crowded environment of the supramolecular nanofibers. A recent study using gold nanoparticle patterned surfaces has reported that the cell response to cyclic RGD sequence depends on the chemistry of the spacer used; a stiff polyproline spacer elicited faster cell spreading and higher focal adhesion formation compared with a flexible PEG or aminohexanoic acid spacer of similar length.⁴² Therefore, to investigate how the flexibility of linker influences the cell response to PA nanofibers, we designed two additional linkers having the length roughly equal to five-glycine residues (extended length 19.3 Å) but with different molecular flexibility (Figure 7a). Four repeating units of polyethylene glycol (PEG) were employed to construct a flexible linker (extended length 17.9 Å). Similarly a rigid linker was synthesized using three tandem aryl amide residues (extended length 18.6 Å), where the rigid aromatic chain is expected to hinder chain deformation.⁵⁶ RGDS epitopes were presented on the PA nanofibers using both flexible and rigid linkers, and fibroblasts were cultured on these PA substrates. Examined after 5 h of incubation, cells on both substrates appeared to be well spread with a polygonal shape and exhibited fibrillar focal

adhesions and actin stress fibers (Figure 7b). Analysis of morphological parameters showed that cell morphology on both flexible and rigid linker PAs were not significantly different than on G5 PA (Figure 7c). This result suggests that the length of the linker rather than its flexibility is the major determining factor for the epitope bioactivity on the supramolecular PA nanofibers.

Ligand density and stiffness are known to be two key parameters in a materials substrate that independently control the response of cells such as spreading, focal adhesion formation and actin fiber bundling.^{33, 57} We demonstrated here a differential cell response to nanofiber substrates with comparable rigidity and epitope density, but with varied epitope topography. Topography of the RGD sequence in fibronectin is thought to play an important role in efficient integrin binding. The RGD epitope in the native protein is presented at the apex of a hairpin loop that projects out from the β -sandwich rich flat fibronectin module surface, making it accessible to integrins.⁵⁸ Forced unfolding of the protein in molecular dynamics simulations reveals a gradual shortening of the distance between RGD epitope and module surface and predicts an associated reduction of epitope accessibility to the integrins.⁵⁹ An incremental cell response to PA nanofibers with increasing linker-length suggests a gradual enhancement of epitope accessibility to integrin receptors. Although we have not investigated here the cell response to linkers longer than five-glycine residues, previous reports on glycine spacers indicate that the increasing strength of RGD-integrin binding interactions quickly saturate beyond this spacing distance.^{40, 41} Our findings confirm that the cell response on bioactive nanofiber substrates is critically regulated by epitope topography at the cell-material interface.

Conclusion

We have shown here that the spatial presentation of RGDS epitopes on PA nanofibers strongly influences the bioactivity of the nanofibers. Increasing the length of the linker moiety that spaces out epitopes from the nanofiber surface was associated with augmentation of RGDS specific cell response, manifested by increased cell spreading, formation of focal adhesions and actin fiber bundling. The cellular effects are mediated by integrin receptor activation and stronger attachment of cell processes to nanofiber substrates, presumably from a favorable RGDS interaction to the ligand binding sites on integrins afforded by a longer linker. We have further demonstrated that the flexibility of the linker, unlike length, has minimal influence on the cell response. In this work we have demonstrated strategies to precisely control supramolecular topography of bioactive epitopes in high persistence length fibers, which constitute a key feature of biomimetic scaffolds for regenerative medicine.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

PA	peptide amphiphile
ECM	extracellular matrix
cryoTEM	cryogenic transmission electron microscopy
SAXS	small angle X-ray scattering
CD	circular dichroism

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Figure 1.

(a) Chemical design of RGDS epitope-presenting PAs, where a glycine linker of variable length presents the epitope. (b) A PA containing the peptide sequence lacking the RGDS signal (base PA) was used to co-assemble with epitope-presenting PAs and this way vary the average epitope density. (c) Molecular graphics representation of RGDS epitope display on the co-assembled nanofiber surface, where the linker consists of one, three, or five glycine residues (named as G1 PA, G3 PA and G5 PA, respectively).

Figure 2.

(a–d) CryoTEM images of base PA (a), G1 PA (b), G3 PA (c) and G5 PA (d) in H_2O (G1, G3 and G5 PAs were co-assembled with base PA at a 1:9 weight ratio); long cylindrical nanofibers are observed for all the compositions investigated. Scale bar 100 nm. (e) SAXS and (f) CD spectra of the same set of PAs. In the SAXS plot, a line with -1 slope is drawn at the low q region (characteristic of cylindrical morphology) to compare the slopes of PA traces.

Figure 3.

Fibroblast morphology on PAs presenting the RGDS epitope using linkers of different length. (a) Representative images of 3T3 fibroblasts cultured on PA-coated substrates for 5 h and stained for actin (phalloidin staining, top row), vinculin (middle row), and β1-integrin (bottom row). Scale bar 20 μm. (b–d) Cell morphology on PA substrates is compared by measuring the projected cell area (b), convex hull (c) and circularity (d). In the plots, the box represents the median value with upper and lower quartile, while the whiskers represent the value at 10th and 90th percentile. (** $p < 0.01$, *** $p < 0.001$; calculated against the base PA, unless indicated, using Kruskal-Wallis test with Dunn's post-hoc comparison; $n > 300$ cells for each condition from three independent experiments).

Figure 4.

(a–c) Molecular graphics illustration of RGDS epitope presentation on the surface of PA nanofibers consisting of 10% G5 PA in base PA (a), 10% G5 PA in RGEs PA (b; RGEs PA is shown in yellow, arrowheads point to RGDS sequences), and 100% G5 PA (c). (d) Comparison of fibroblast projected area on these PA substrates (** $p < 0.01$, *** $p < 0.001$; calculated against the G5/base PA using Kruskal-Wallis test with Dunn's post-hoc comparison; $n > 350$ cells for each condition from two independent experiments).

Figure 5.

Dynamics of fibroblast spreading on PA nanofiber substrates. (a) Phase contrast images showing fibroblast morphology over time (with time noted on image) on G1 PA (top) and G5 PA (bottom) coated surfaces. The background texture and lines in these images appear from a minor inhomogeneity of thickness in the alginate layer. (b) Scanning electron micrographs of fibroblasts cultured for 5 h on PA nanofiber substrates; many filopodia-like extensions can be observed on the top surface of the cells on G1 PA, but cells on G5 PA exhibit a smooth surface. Scale bars: a, 10 μm , b, 5 μm .

Figure 6.

Integrin activation and stability of focal adhesions on PA substrates. (a) Western blot analysis of pFAK expression in fibroblasts measured after incubating for 15 or 30 min on RGDS epitope-presenting PAs with short (G1) and long (G5) linker, or on mutated epitope (ES) control. (b) Fibroblasts were allowed to spread on PA substrates for 2 h and then treated with nocodazole (5 μ M) or nocodazole and latrunculin (500 nM) for another 2 h. Representative micrographs of cells stained with phalloidin, (green), anti α -tubulin antibody (red) and DAPI (blue) show the cell morphology and the distribution of actin and microtubules after drug treatment. Arrowheads indicate cell boundary. Scale bar 10 μ m.

Figure 7.

Effects of linker rigidity on cell response. (a) Chemical structures of RGDS presenting PAs, where the epitopes are presented by a flexible PEG or a rigid aromatic linker with a length equivalent to five glycine residues. (b) Fibroblast morphology after 5 h of culture on flexible and rigid linker PA substrates (actin, red; vinculin, green). Scale bar 20 μm. (c) Cell morphology parameters on flexible and rigid linker PAs are compared with G5 PA. (*n.s.* $p > 0.05$; Kruskal-Wallis test with Dunn's post-hoc comparison; $n > 300$ cells for each condition from three independent experiments).