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A photoclickable peptide microarray platform for facile and rapid screening of 3-D tissue microenvironments

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ABSTRACT

Microarrays are powerful experimental tools for high-throughput screening of cellular behavior in multivariate microenvironments. Here, we present a new, facile and rapid screening method for probing cellular behavior in 3D tissue microenvironments. This method utilizes a photoclickable peptide microarray platform developed using electrospun fibrous poly(ethylene glycol) hydrogels and microarray contact printing. We investigated the utility of this platform with five different peptide motifs and ten cell types including stem, terminally differentiated, cancer or immune cells that were from either primary origin or cell lines and from different species. We validated the capabilities of this platform to screen arrays consisting of multiple peptide motifs and concentrations for selectivity to cellular adhesion and morphology. Moreover, this platform is amenable to controlled spatial presentation of peptides. We show that by leveraging the differential attachment affinities for two cell types to two different peptides, this platform can also be used to investigate cell-cell interactions through miniature co-culture peptide arrays. Our fibrous peptide microarray platform enables high-throughput screening of 3D tissue microenvironments in a facile and rapid manner to investigate cell-matrix interactions and cell-cell signaling and to identify optimal tissue microenvironments for cell-based therapies.

1. Introduction

Regenerative medicine holds great promise for treating patients with tissue deterioration and organ failure due to disease, aging or trauma. Development of effective regenerative therapies, therefore, requires precisely-engineered smart and intelligent biomaterials capable of recapitulating the biochemical and biophysical complexity of the native microenvironments. Composition, structure and rigidity of the native extracellular matrix (ECM) is highly specific to the form, age, and function of each tissue [1–4]. Moreover, during development the ECM transforms as it transitions from an immature to a mature tissue [5,6]. Diseases, such as tumorigenesis and fibrosis, can also alter the ECM structure and rigidity [7,8], whose influence on cells is further confounded by variations in ECM composition, density and stiffness [9–12]. Mechanistic insights into these coordinated events involving various ECM parameters in the regulation of cell fate are, however, still lacking and, in particular, in the context of 3D tissue architectures. The significance of 3D micro/nano fibrous architectures of ECM in modulating cellular adhesion, viability, proliferation, self-renewal and differentiation has been established [13–16]. Yet, engineering of 3D tissue microenvironments that mimic the native ECM environment where parameters such as composition, rigidity and architecture provide vital cues, remains technically challenging. The development of robust 3D tissue-like fibrous biomaterial platforms that can recapitulate key aspects of the *in vivo* environment, but in an

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in vitro setting, is critically needed. Such a platform would enable probing of complex cellular behavior in multivariate microenvironments.

A myriad of signals arise from the complexity and compounding effects of different 3D extracellular matrix parameters. Conventional screening methods are inefficient, laborious, and expensive for designing multivariate microenvironments that combine physical, chemical, and biological cues. Currently available microarrays of ECM proteins [15,17–23] are insufficient to address this critical need due to the complications that arise as a result of variations in protein source [24] and protein structure [25], and are further confounded by tethering approaches that can alter protein activity [26]. Alternatively, peptides can be incorporated into a biomaterial with greater precision and without affecting bioactivity. While several strategies have been proposed to incorporate synthetic peptides into tissue-relevant microenvironments [27–29] the challenge remains to develop 3D peptide microarray platforms that are facile, inexpensive, and amenable to automation, while boasting a powerful portfolio of spatially and compositionally complex physiological designs. To this end, we have developed a new peptide microarray platform using fibrous hydrogels and sequential photoclick reactions that enable decoupling and systematic integration of peptides to emulate tissue microenvironments for high-throughput parametric studies. To demonstrate the wide applicability of our platform, we used five different peptide motifs and ten cell types including stem, terminally differentiated, cancer or immune cells that were from either primary origin or cell lines and from different species. We validated the capabilities of this platform to screen arrays consisting of multiple peptide motifs and concentrations for selectivity to cellular adhesion and morphology.

Our 3D photoclickable peptide microarray platform integrates innovations in thiol-ene "photoclick" chemistry, 3D fibrous manufacturing, and high-precision microcontact printing. Our system takes advantage of a photo "clickable" step-growth polymerization between norbornene and thiol containing monomers, which represents a simple, orthogonal and highly efficient reaction [30], to produce crosslinked fibrous scaffolds via electrospinning methods. By reacting off-stoichiometry, free norbornenes are preserved along the fibers, enabling a second-step photo "click" reaction that introduces peptides into the fibrous matrix. The bioorthogonal reaction ensures the free norbornenes react exclusively with thiols such that any biological or chemical molecule containing thiols can be introduced into the engineered architecture with high fidelity. In addition, cysteine-containing peptides are deposited in a microarray format using a robotically controlled commercially available contact printer. Collectively, these technologies enable our multivariate peptide platform that can be used for probing cellular behavior of different cell types in a 3D fibrous tissue-like environment in a high throughput manner. The significance of our approach is that each of the enabling technologies, thiol-ene photoclick chemistry, electrospinning and microcontact printing, are relatively inexpensive, amenable to scale up, automation, and manufacturing, and our peptide microarray platform is adaptable to integration with current high throughput screening technologies. To our knowledge, none of the existing microarray platforms combines these technological advances [31].

2. Materials and methods

2.1. Poly(ethylene glycol) norbornene synthesis

Four-arm poly(ethylene glycol) norbornene (PEGNB) was synthesized following methods described in detail elsewhere [32]. Briefly, first 5-norbornene-2-carboxylic acid (Sigma) (8x molar excess nobornene to amine-terminated PEG arms) in dimethylformamide (DMF) was pre-reacted for 5 min under argon with 2-(1H-7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate methanaminium (HATU, 4x molar excess, Chem Impex INT'L, Inc) and N,N-diisopropylethylamine (DIEA, 4x xcess, Sigma) at room temperature. The pre-reacted mixture was added to 4-arm PEG amine (JenKem Technology USA, Inc.) in DMF, and allowed to react for 24–48 h under argon at room temperature. The reactant, PEGNB, was precipitated in ice-cold ethyl ether, purified by dialyzing against deionized (DI) water for 2–3 days, sterile filtered (0.2 μm) and then lyophilized. Using ^{1}H NMR spectroscopy, norbornene conjugation (carbon-carbon double bond peaks, δ = 5.9–6.3 ppm) per 4-arm PEG molecule (methylene peaks, δ = 3.4–3.9 ppm) was determined to be 95%.

2.2. Peptide synthesis

All the peptides used in this study (CRGDS, CRGES, CIKVAV, CYIGSR, CDGEA, CVAPG) were synthesized via standard Fmoc solid-phase methodology, rink-amide resin, and HBTU/HATU activation using PS3 peptide synthesizer (Protein Technologies, Inc). After completion of peptide synthesis, simultaneous cleavage from resin and side chain deprotection was achieved by treatment with a trifluoroacetic acid (TFA) cocktail (92.5% TFA, 2.5% Ethanedithiol (EDT), 2.5% water and 2.5% triisopropylsilane (TIS)) for 5 h. Crude peptide was precipitated and washed (4 times) with ice-cold diethyl ether, and then allowed to dry overnight. The dry peptide powder so obtained was then dissolved in Milli Q water, filtered using 0.2 μm filter, and then lyophilized. This lyophilized powder was used for the experiments without further purification. The molecular mass of all the peptides was confirmed by matrix-assisted laser desorption, ionization time-of-light (MALDI-TOF) mass spectrometry (Applied Biosystems DE Voyager) using α-cyano-4-hydroxycinnamic acid matrix (Sigma). MALDI-TOF-MS: [M + 1H]+: CRGDS: Calculated (536.58), Observed (536.34); CRGES: Calculated (550.60), Observed (551.10); CIKVAV: Calculated (631.85), Observed (632.01); CYIGSR: Calculated (697.82), Observed (697.72); CDGEA: Calculated (493.51), Observed (493.91); CVAPG: Calculated (445.55), Observed (446.03).

2.3. Fluorescent peptide synthesis

CDGEAK peptide was synthesized via standard Fmoc solid-phase methodology, rink-amide resin, and HBTU/HATU activation. Further, N-terminal of the peptide was capped with acetic anhydride (Ac-CDGEAK), cleaved from the resin and processed as described earlier. MALDI-TOF-MS: [M + 1H]+: Calculated (663.72), Observed (663.49). Ac-CDGEAK peptide (12.5 mg) was dissolved in DMF (2.45 mL) containing Alexa Fluor® 488 carboxylic acid, 2,3,5,6-tetrafluorophenyl ester (1 mg) or Alexa Fluor® 568 carboxylic acid, succinimidyl ester (1 mg) with N,N-Diisopropylethylamine (DIEA, 50 μL) and reacted overnight protected from light [33]. The samples were concentrated using rotovap, dissolved in Milli Q water, and lyophilized. These fluorescently labeled peptides (Ac-CDGEAK-A488 and Ac-CDGEAK-A568) were used without further purification.

2.4. Electrospinning

Fibrous hydrogels were prepared by electrospinning a solution of 4-arm PEGNB (5 kDa, 10 wt%), poly(ethylene glycol) dithiol (Sigma, 1 kDa, thiol: ene = 0.9), poly(ethylene oxide) (5 wt%, MW: 400 kDa), and photoinitiator Irgacure 2959 (0.1%) in DI water using a custom set up equipped with a 18G needle, 14-mm syringe and a high voltage power supply (Gamma High Voltage, Inc).