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Promoting Extracellular Matrix Crosslinking in Synthetic Hydrogels

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Promoting Extracellular Matrix Crosslinking in Synthetic Hydrogels

A thesis Presented

by

Marcos Matias Manganare

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

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Molecular and Cellular Biology

Promoting extracellular matrix crosslinking in synthetic PEG hydrogels

A Thesis presented

by

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ABSTRACT

PROMOTING EXTRACELLULAR MATRIX CROSSLINKING IN SYNTHETIC HYDROGELS

SEPTEMBER 2015

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The extracellular matrix (ECM) provides mechanical and biochemical support to tissues and cells. It is of crucial importance for cell attachment, differentiation, and migration, as well as for ailment-associated processes such as angiogenesis, metastases and cancer development. An approach to study these phenomena is through emulation of the ECM by synthetic gels constructed of natural polymers, such as collagen and fibronectin, or simple but tunable materials such as poly(ethylene glycol) (PEG) crosslinked with short peptide sequences susceptible to the activity of metalloproteases and cell-binding domains. Our lab uses PEG gels to study cell behavior in three dimensions (3D). Although this system fosters cell attachment and crosslinking peptides susceptible to degradation by matrix metalloproteases (MMPs), the regenerative process of the ECM has not been mimicked yet in 3D synthetic gels. In an attempt to build in this functionality to PEG-based gels, I attempted to find short peptides that could be used as re-connectors of the matrix elements in order to emulate the *in vivo* reconstitution process. I performed phage display to identify short oligopeptides that bind either collagen or fibronectin to assess them as potential nucleation points for crosslinking. A phage display is a library of random oligopeptides expressed on a M13. This strategy allows identification of a phenotype and a genotype with a single screening step. In comparison with other techniques, such as antibodies or target-binding proteins, the phage display strategy could yield a short oligopeptide that would be more specific, would not depend on folding, and is inexpensive. I identified the conditions under which phage display is compatible with our targets, and I isolated and identified five peptide candidates for fibronectin binding and two for collagen. Future work include assessing whether these candidates could facilitate the formation of cell-created crosslinking in 3D synthetic hydrogels.

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CHAPTER 1

MIMICKING THE EXTRACELLULAR MATRIX AND ITS FUNCTIONS

1.1 Introduction

ECM-mimicking PEG-based hydrogels are useful to shed light upon cell behavior such as migration, metastasis and cell mechanosensing (1, 2). Their potential applications in regenerative medicine are promising based on PEG's relatively inert properties (3), and the fact that crosslinkers and proteins can be controllably added (3, 4). However, there are processes occurring *in vivo* on the ECM-cell dynamic interaction that have yet not been emulated *in vitro*, such as ECM reconstruction. Research toward finding new components that can be incorporated in the construction of these hydrogels in order to match these natural phenomena are needed.

The operational hypothesis underlying this work, as a potential strategy to imitate the ECM reconstruction process, is that generating short molecules with binding affinity for 3D PEG hydrogel components such as collagen and fibronectin will function as a crosslinking nucleation point. The objectives of this work are to 1) use a phage display to screen a peptide (7mers) library to find sequences with binding affinity for fibronectin and collagen, and 2) assess candidate efficacy in stabilizing and increasing the endurance of the PEG based hydrogels when challenged by the activity of MMPs.

1.2 Specific Aims to generate Hydrogels Crosslinkers

1.2.1 Aim 1: Use phage display to identify peptide candidates that bind to either collagen I or fibronectin.

Hypothesis: By phage display I will find peptides able to bind to fibronectin or collagen for eventual use in PEG-based hydrogels.

Method: I used a phage display Ph.D7 kit, which contains every possible 7mer peptide sequence (20^7 peptides) and screened their affinity to our interest molecules (5). I first coated plates with either collagen or fibronectin. I used *E. coli* infected with the M13-based phage library, increased the phage mass, purified the phages, and challenged them against the target molecule-coated plates. Then, by a double step panning process, I selected the high affinity phages. Phage colonies were isolated and their DNA sequenced.

1.2.2 Aim 2: Synthesize, confirm, and purify candidate peptides.

Hypothesis: I will successfully synthesize peptides identified in Aim 1.

Method: Peptides identified in Aim 1 were synthesized, as well as two additional peptides found in the literature (6, 7). I plan to compare the binding capabilities of these published peptides with those identified in Aim 1. Peptides were synthesized by solid-phase methods in collaboration with the Perry lab.

1.2.3 Aim 3: Test the potential applications of the isolated peptides as re-linkers in synthetic 3D matrices.

Hypothesis: The affinity of the peptides obtained in Aim 1 for will facilitate cell-mediated crosslinking.

Method: We will prepare PEG hydrogels including phage display-identified peptides.

Gels will otherwise contain the same components as typical in the Peyton lab (MMP-degradable peptides and integrin binding peptides). Cells will be seeded in the gels, and after 7 days gels of culture will be fixed, and ECM components detected by immunostaining. We expect to see increased fibronectin and collagen staining in the appropriate peptide-containing gels. Further, we expect to see reduced degradation in peptide-containing gels as compared to typical PEG gels.

Through this work we expect that the peptides we found in Aim 1 will bind to collagen and fibronectin, will serve as nucleation points when added to PEG hydrogels, promoting the building up of ECM elements on the gels. This strategy will improve the PEG hydrogel tool bringing it a step closer toward the ultimate goal of developing self-healing synthetic hydrogels.

CHAPTER 2

SIGNIFICANCE OF PROMOTING THE RECONSTRUCTION OF HYDROGELS NETWORKS *IN VITRO*

2.1 Tissue engineering combines artificial structures, scaffolds, and cell factors to support cell culture.

Scaffolds for tissue engineering are designed to emulate the *in vivo* cell milieu, thus they must provide some natural functions. For example, they should allow for cell attachment and migration, as well as enable diffusion of cell nutrients and secreted cell products and/or delivery and retain cells and biochemical factors (8). In order to do so, these scaffolds must reunite some requirements. They should be porous, and these pores should be of a proper size to be conducive to cell establishment and nutrient diffusion. They can also be biodegradable, and this biodegradability can facilitate cell behaviors such as migration and proliferation. Hydrogels such as polyacrylamide (PAA) and PEG (4) are some of the biomaterials suitable as scaffolds for tissue engineering due to their high water content, soft tissue-like consistency, and biocompatibility (9). Besides their eventual *ex vivo* applications, they are promising for their *in vitro* applications as model systems for studying cell proliferation, interaction, migration, adhesion, differentiation, and metastasis, within an emerging field known as mechanobiology (4).

2.2 PEG hydrogels exhibit many advantages compared to other synthetic hydrogels.

PEG hydrogels are relatively bio-inert, they have low protein adsorption properties, are nontoxic and innocuous generating minimal inflammatory response, they are water soluble, and their reagents are readily available and economical. These characteristics make them highly desirable. Moreover, they are easily amenable to chemical manipulation, molecular chain length, weight percentage and cross-linking. Furthermore, the process of gelation in PEG hydrogels can be performed under nontoxic conditions (3, 8). In comparison to matrices made of natural components such as collagen and fibronectin, PEG hydrogels exhibit the enormous advantage that bioactive functionalities such as cell anchorage sequences, cell degradable sequences, and growth factors can be added in a measurable and controllable amount to obtain desirable or favorable cell or tissue behavior (3, 10). In this way, synthetic PEG hydrogels containing components of ECM can chemically and physically mimic the ECM to some degree (10).

2.3 PEG conjugated to maleimide forms gels more efficiently than other macromolecules.

Maleimide (MAL) groups conjugated to polyethylene glycol chains are used as flexible linking molecules to bind proteins to surfaces because of their fast reaction kinetics and affinity for thiols at physiological pH (3). The double bond of the maleimide group reacts with the thiol group of a cysteine, resulting in a carbon-sulfur bond. Compared with other bio-ligand macromolecules, such as 4-arm PEG acrylate (PEG 4A), 4-arm PEG vinylsulfone (PEG-4VS) or PEG diacrylate (PEG DA), gels made of PEG 4MAL incorporate the crosslinkers and the cell-adhesive RGD sequence more efficiently, are

more efficient in gelation time, and have tighter structure. Moreover, with the low polymer weight percentage, PEG-4MAL can form gels with mechanical properties similar to gels prepared by natural extracellular matrix derived components such as collagen I (3).

2.4 Tissue engineering still faces some challenges.

In vivo, cells can move in 3D using either proteolytic (mesenchymal) or no proteolytic (amoeboid) strategies. During proteolytic migration, cells secrete proteases, which break down macromolecules of the ECM and thus create macroscopic cavities that allow their movement (1). This has been emulated *in vitro* in synthetic matrix gels by the incorporation of the mentioned MMP-sensitive cross linkers, such as GPQG-IAGQ. However, *in vivo* cells are able to dynamically reconstruct the ECM that is being digested. Emulation of this process in the PEG hydrogels is one of the major complex functionalities that tissue engineering is facing. The goal of this work is to ameliorate this issue by finding short peptide sequences that could promote the reconstruction of the hydrogels networks *in vitro* (Figure 1)

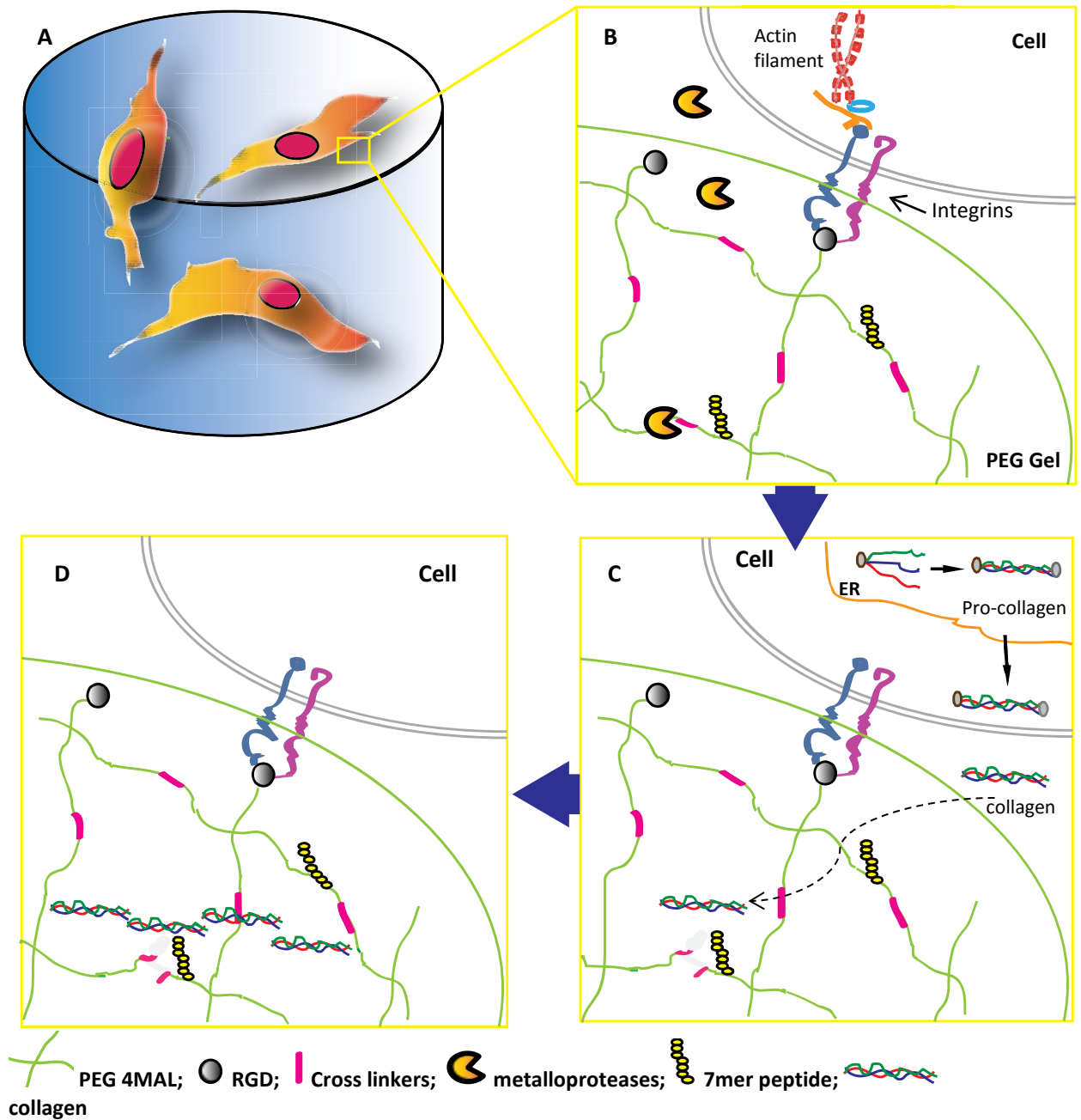


Figure 1: Representation of candidate peptides working as crosslinkers in a synthetic PEG hydrogel.

This is a representation of the main aim of this work; used here as example the potential use of 7mer peptides with collagen affinity as nucleation seeds in the construction of PEG-4MAL hydrogels. A) Cells in a 3D PEG-4MAL hydrogel. B-D) Matrix-cell dynamic interaction zone augmented. B) Cells attach to the gel by the interaction of an integrin with the cell-anchorage tripeptide RGD (grey); also, cell-secreted MMPs targeting crosslinkers (purple). C) Cell produces and secretes collagen, which is captured by a 7mer peptide nearby. D) Collagen molecules and 7mer peptides promote the formation of collagen fibrils. (Note: this is a representation; it is not at scale; the pathways and processes shown here such as the collagen synthesis are incomplete)

CHAPTER 3

INNOVATIVE GENERATION OF SELF-HEALING HYDROGELS

3.1 Innovation:

The innovative feature of this work is the generation of “self-healing” PEG hydrogels: gels that could imitate the ECM reconstruction process that occurs *in vivo*. Finding molecules that could work as re-connectors or as ECM polymer nucleation points in 3D synthetic gels could increase the use of these PEG-based hydrogels as a model system for studies in tissue engineering, regenerative medicine, and as models of pathophysiology. This new feature will allow us to study morphogenic phenomena, such as metastasis, in a scenario that recapitulates the *in vivo* scenario with the advantage of a controllable and reproducible system.

3.2 Rationale:

In vivo, weak interactions such as van der Waals, electrostatic, hydrogen bonding and hydrophobic repulsions are responsible for the assembly and architecture of supramolecular structures such as the ECM. However, these interactions are not established randomly. The exquisite and highly specific recognition ability of biomolecules is the key to weak interactions constructing the aforementioned super structures. In nature, this selective recognition ability of biomolecules is the result of ages of evolution.

In vitro, a powerful tool to seek specific recognition ability is phage display; a method to identify new peptides with selective affinity for certain molecules screened from bioengineered peptide libraries displayed on phage surfaces. Given their regular structure, these peptides might recognize superficial organization of functional groups of the target molecules, which imbue these peptides with specific affinities. Such affinities give them the potential to facilitate patterning or as catalysts for assembling target molecules into more complex structures (11). Because the phage display peptide library represents every possible peptide composed of seven amino acids (20^7 peptides), presumably it would feature all the potential physical characteristics and affinities possible, some of those will likely possess affinity to our interest molecules: collagen and fibronectin. In this way I will identify and isolate short peptides with binding properties to collagen and fibronectin to re-connect or serve as nucleation points when added to a PEG-based hydrogel containing collagen, fibronectin or both. This approach could be rationally extended to any other target protein of interest.

CHAPTER 4

AIM 1: USE PHAGE DISPLAY TO IDENTIFY PEPTIDE CANDIDATES THAT BIND TO EITHER COLLAGEN I OR FIBRONECTIN.

4.1 Hypothesis:

By phage display I will find peptides able to bind to fibronectin or collagen for eventual use in PEG-based hydrogels.

A phage display is a library of random oligopeptides expressed on an M13 phage virion with the DNA encoding each peptide on the inside (12). This approach allows identification of a phenotype and a genotype with a relatively simple screening step. This screening process is called panning, and can distinguish variants of the peptides based on their differential affinity to a target molecule. The phage display protocol consists of four crucial steps: 1) coating a plate with the target molecule; 2) challenging the target with the phage library; 3) washing the weak-binding phages away; and 4) eluting phages of interest and amplification. Then, the whole process is repeated again to increase the mass of the phage of interest and select the more specific ones to be sequenced (Figure 2).

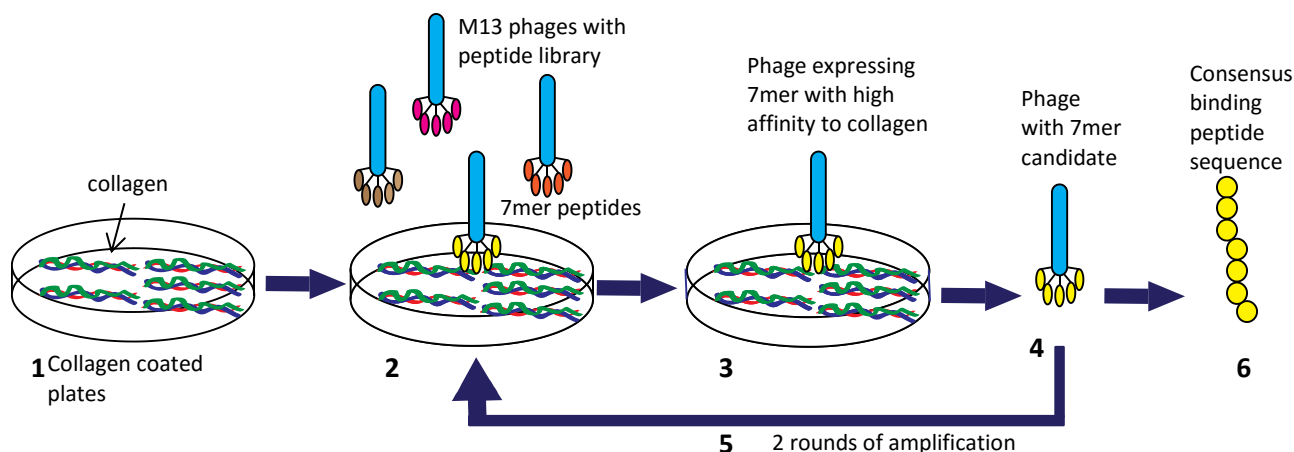


Figure 2: Phage Display Technique. 1) Coating a plate with the target molecule, in this case it is shown collagen as example; 2) Challenging the target with a phage library; 3) Washing the weak-binding phages away and selecting the phage of interest; 4) Eluting the interest phage and amplifying it; 5) Repeat two or three cycles; 6) Consensus peptide sequence, the 7mer.

4.2 Method:

4.2.1 Direct target coating:

The most straightforward way to carry out the panning process is by directly coating a plastic plate with the molecule target.

1. Following the New England BioLabs' Phage Display Ph.D 7 kit manual recommendations (NEB#E8100) a 100 µg/ml solution of Fibronectin in 0.1 M NaHCO₃, pH 8.6 and a collagen solution at the same concentration in the same solvent were prepared as coating buffers.
2. Then 1.5 ml of each was individually added by separately to two wells of two six well plates each (one for fibronectin and the other for collagen), swirled and incubated overnight at 4°C with gentle agitation in a humidified container, one for each plate.
3. Following the subsequent steps recommended by the manufacturer in the NEB#E8100 manual for the panning process, the coating solutions were poured off from each plate, replaced by Blocking Buffer (0.1 M NaHCO₃ pH 8.6, 5 mg/ml BSA), incubated by an

hour at 4°C, discarded, and each well was washed 6 times with TBST (TBS + 0.1% [v/v] Tween-20).

4. A 100-fold dilution of the library was prepared by diluting 10 µl of the peptide library with 1 ml of TBST, pipetted onto coated plate, rocked gently for 60 minutes at room temperature and discarded nonbinding phage by pouring off and slapping plate face-down onto a clean paper towel.

5. The wells were washed 10 times with TBST 0.1% (Tween 20 0.1 %) and slapped down on a clean section of paper towel each time to prevent cross-contamination.

6. The bound phage was eluted with 1 ml of 0.2 M Glycine-HCl (pH 2.2), 1 mg/ml BSA (a general buffer for nonspecific disruption of binding interactions). The elution mixture was rocked gently for 15 minutes at room temperature, then pipetted into a microcentrifuge tube, and neutralized with 150 µl of 1 M Tris-HCl, pH 9.1.

7. Finally, 1 µl of each eluate was diluted 10, 10² and 10³ folds in LB and tittered by a plaque assay method. For this, aliquots of 200 µl of *E. coli* ER2738 cultured in LB until mid-log phase (OD₆₀₀= 0.5) one for each dilution, were infected with the phage dilutions mentioned above. They were incubated for 5 minutes, introduced separately in aliquots of 3 ml of top agar at 45°C and plated on pre-warmed LB/IPTG/Xgal plates. We expected to count blue plaques (that represent the amount of phage) after incubation at 37°C for 48 hrs. Because the peptide library is constructed on a M13KE cloning vector, which carries the lacZα gene, phage plaque appear blue when plated on media containing Xgal and IPTG.

After the 48 hrs of incubation at 37°C, a single blue plaque was observed.

4.2.2. Optimized Coating conditions:

8. To find the conditions under which this system is optimized, the above process was repeated using different buffers and concentrations. In step 1, the concentration chosen for the coating protein collagen was 50 µg/ml based on the NEB #E8100 protocol (10µg-100 µg/ml) and 1µg/cm² based on standard coating protocols, which recommend this concentration in order to ensure that these proteins would not form fibers. The buffers chosen, shown in Table 1, were selected based on the NEB #E8100 Manual, standard protocols and from literature (see references 12 and 15). Based on the same literature for the conditions 3 to 8, collagen was dissolved in acetic acid 0.2M at 1 µg/µl concentration previous to dissolved again in the coating buffer. All panning processes were carried out in six well plastic culture plates. Table 1 shows the coating buffers used for collagen and Table 2 for fibronectin.

Table 1: Collagen Coating Buffers

	Collagen:
1	25 µl + 1.5 ml NaHCO ₃ 0.1M
2	9.5 µl + 1.5 ml NaHCO ₃ 0.1M
3	75 µl in AcACH 0.2M + 1.5 ml NaHCO ₃
4	9.5 µl in AcACH 0.2M + 1.5 ml NaHCO ₃
5	75 µl in AcACH + 1.5 ml 35mM NaHCO ₃ /Na ₂ CO ₃ 15mM
6	9.5 µl in AcACH + 1.5 ml 35mM NaHCO ₃ /Na ₂ CO ₃ 15mM
7	75 µl in AcACH + 1.5 ml TBS
8	9.5 µl in AcACH + 1.5 ml TBS

Table2: Fibronectin Coating Buffers

	Fibronectin:
1	9.5 ul + 1.5 ml NaHCO ₃
2	9.5 + 1.5 ml 35mM NaHCO ₃ /Na ₂ CO ₃ 15mM
3	15 ul in AcACH + 1.5 ml TBS

4.2.3 Washing curve:

9. In order to find whether the washes in step 5 of Section 1A (involving 10 washes with TBST 0.1 %) were excessive and the phages of interest were being washed away, a washing curve was performed. Six single wells of a six well plate were prepared for each one of the conditions detailed in the Tables 1 and 2 and labeled from A to F. In step 5, instead of a single step of 10 washes with TBST 0.1%, a series of different washes were carried out separately. These washing series were, those wells labeled A were washed 2 times; those labeled B were treated with 3 washes; C with 4; D were 6, E with 8 and F with 10 washes.

10. After the washing steps, the bound phage were eluted with 1 ml of 0.2 M Glycine-HCl (pH 2.2), 1 mg/ml BSA rocking them gently by 15 minutes at room temperature, then pipetted into microcentrifuge tubes, and neutralized with 150 μ l of 1 M Tris-HCl, pH 9.1.

Finally, 1 μ l of each eluate was diluted 10, 10^2 and 10^3 fold in LB and tittered by the plaque assay method as described in Section 1A step 7.

11. From the plates of collagen, binding phages eluted from coating buffer condition 4 (9.5 μ l in AcACH 0.2M + 1.5 ml NaHCO_3) washed six and eight times, were counted; 30 blue plaques in each plate at 10^3 plating dilution. From the plates of fibronectin binding phages eluted from coating buffer condition 1 (9.5 μ l + 1.5 ml NaHCO_3) were washed six and three times were counted; about 70 blue plaques in each plate at 10^3 plating dilution. The plaques count fell by half with eight washes.

12. The eluted phages from these two conditions treated with six washes were amplified by adding the eluate to 20-ml ER2738 cultures (one for each condition) at early-log ($OD_{600} \sim 0.01-0.05$) and incubating at 37°C and 250 rpm during 4.5 hours .

13. Cultures were transferred to individual centrifuge tubes and spun for 10 minutes at 12,000 g at 4°C. The supernatants were transferred to fresh tubes and re-spun.

14. The upper 80% of the supernatants (16 ml) were transferred to fresh tubes and 4 ml of 20% PEG/2.5 M NaCl was added to each tube, then left overnight to precipitate at 4°C .

15. The tubes were spun at 12,000 g for 15 minutes at 4°C, supernatants discarded, tubes re-spun briefly, and residual supernatants removed with a pipette.

16. Phage pellets were suspended in 1 ml of TBS each one, transferred to individual microcentrifuge tubes and spun at 14,000 rpm for 5 minutes to pellet residual cells.

17. The supernatant was transferred to individual microcentrifuge tubes and re-precipitated by adding 1/6 volume of 20% PEG/2.5 M NaCl. Then, they were incubated on ice for 60 minutes. They were centrifuged at 14,000 rpm for 10 minutes, the supernatants were discarded, re-spun, and any residual supernatant was removed with a micropipet.

18. Pellets were suspended in 200 µl of TBS, centrifuged for 1 minute to pellet any remaining insoluble material and the supernatant was transferred to individual tubes. These are the amplified eluates with which a second panning process was carried out.

4.2.4 Second Panning

19. A second panning process was carried out following the above procedure with few slight modifications.

In step 1 of the process described in Section 1A, a six well plate was coated with collagen using the coating conditions shown in Table 3 in triplicate. Similarly, another plate was coated with fibronectin using the coating conditions shown in Table 4 in triplicate.

**Table 3: Second Panning
Collagen Coating Buffers**

	<u>Collagen:</u>
1	75 µl in AcACH 0.2M + 1.5 ml NaHCO ₃
2	9.5 µl in AcACH + 1.5 ml 35mM NaHCO ₃ /Na ₂ CO ₃ 15mM

**Table 4: Second Panning
Fibronectin Coating Buffers**

	<u>Fibronectin:</u>
1	9.5 µl + 1.5 ml NaHCO ₃
2	9.5 + 1.5 ml 35mM NaHCO ₃ /Na ₂ CO ₃ 15mM

20. 10 µl of each eluate was diluted with 1 ml of TBST and pipetted onto a coated plate.

The phages eluted from a fibronectin coated plate in the first panning were pipetted onto a fibronectin coated plate. The process was repeated for phages eluted from collagen coated plates. These plates were rocked gently for 60 minutes at room temperature and nonbinding phage discarded by pouring off and slapping plate face-down onto a clean paper towel.

21. In order to remove any weakly bound phages, the plates were washed as described above in step 5 of Section C1, but using TBST 0.5 % (Tween20 0.5%). The wells were coated in triplicate in order to carry out three different washing series. One well of each series was washed 3 times, another 6 and the last 8 times.

22. After the elution of the phages and titting as described above in Section C, 5 blue plaques were observed on the plate inoculated with fibronectin binding phages eluted from coating condition 1 treated with six washes at plating dilution 10³; also 2 blue

plaques from collagen binding phages from coating condition 2 treated with six washes at plating dilution 10^3 (Figure 3).

Figure 3

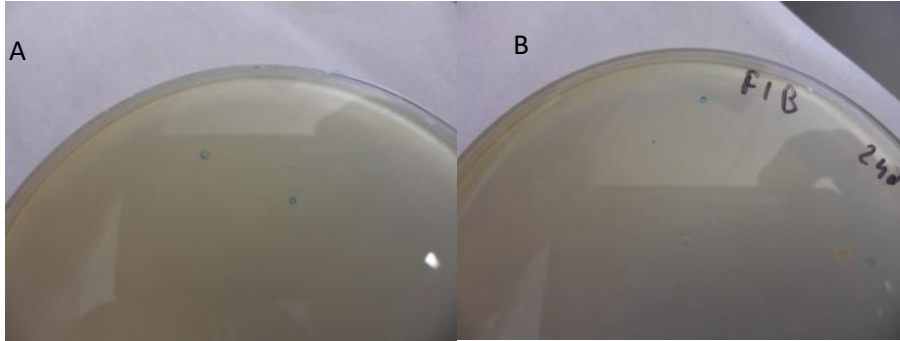


Figure 3: Collagen and fibronectin phage titrating. A) Xgal/IPTG LB plate showing two blue plaques form a collagen binding phage titrating diluted 10^3 . B) Xgal/IPTG plate from fibronectin binding phage at same dilution

4.2.5 Phage amplification / phage stock preparation:

Because of dilution, each single isolated blue plaque obtained should correspond to a single phage clone. These were amplified by inoculation of *E. coli* ER2738 culture.

1. An overnight culture of ER2738 was diluted 1:100 in LB and aliquot into eight culture tubes by 1 ml each.
2. Using a sterile wooden stick each single blue plaque from the titering plates was stabbed and transfer to a single tube containing the diluted culture. Having well separated, isolated plaques increases the chances that each plaque contains a single DNA sequence.
3. The tubes were spun at 37°C and 250 rpm for 4.5 minutes.
4. The cultures were transferred to microcentrifuge tubes, and spun at 14,000 rpm for 30 seconds. The supernatants were transferred to fresh tubes and re-spun. Then the upper 80% of the supernatants (800 µl each) were transferred to fresh tubes. These are the amplified phage stock.

4.2.6 Phage DNA purification and sequencing:

The DNA of each phage clone was purified using the E.Z.N.A M13 DNA Mini Kit from Omega Bio-tek (D6900-01) following the manufacturer's recommendations. This kit is designed to purify single-stranded DNA from phage supernatant.

The isolated DNA samples were sequenced by GENEWIZ sequencing service by Sanger sequencing using the sequencing primer -96 gIII : 5'- HOCCC TCA TAG TTA GCG TAA CG -3'

Resulting in the following consensus sequences:

Fibronectin:

[illegible]

Collagen:

[illegible]

A bioinformatics analysis of the obtained DNA sequences by using NCBI BLAST tool

(16) confirmed the position of the indel mismatch (peptide engineered library) at the N

terminal of the pIII gene of the phage M13 (See reference 14) where it is expected to be (Figures 4 and 6). Finally, by using the blastx tool of NCBI website(17), the sequences peptide sequences were obtained (Figure 5 and 7).

Enterobacteria phage M13 isolate WT variety Rutgers, complete genome
Sequence ID: [gb|JX412914.1](#) Length: 6407 Number of Matches: 1

Range 1: 700 to 1680 [GenBank](#) [Graphics](#) [Next Match](#) [Prev](#)

Score	Expect	Identities	Gaps	Strand
1492 bits(1654)	0.0	937/1021(92%)	41/1021(4%)	Plus/Minus
Query 38	TGAATTTTCTGTATGGGATTTTGCTAAACAACCTTTCAACAGTTTCGGCCGAACCTCCACC	97		
Sbjct 1680	TGAATTTTCTGTATGGGATTTTGCTAAACAACCTTTCAACAGTTTCAGCGGA-----	1630		
Query 98	AAAAGACCACTTCGACAACTAGAGTGAGAATAGAAAGGTACCACTAAAGGAATTGCGAA	157		
Sbjct 1629	-----GTGAGAATAGAAAGGAACAACTAAAGGAATTGCGAA	1594		
Query 158	TAATAATTTTTTTCACGTTGAAATCTCCaaaaaaa-GGCTCCAAAAGGAGCCTTTAATT	216		
Sbjct 1593	TAATAATTTTTTTCACGTTGAAATCTCCAAAAAAAAGGCTCCAAAAGGAGCCTTTAATT	1534		

Figure 4: Fibronectin binding phage BLAST. Alignment of a section of the sequence obtained from the isolated fibronectin binding phages (query) and a section of the phage M13 genome corresponding to the pIII gene

gene III
Sequence ID: [prf|1814345A](#) Length: 424 Number of Matches: 1

Range 1: 6 to 34 [GenPept](#) [Graphics](#)

Score	Expect	Method	Identities
53.9 bits(128)	6e-07	Composition-based stats.	29/40(73%)
Query 120	FAIPLVVPFYSHSLSKWSFGGGSAETVESCLAKSHTENS	1	
Sbjct 6	FAIPLVVPFYSHS-----AETVESCLAKSHTENS	34	

Figure 5: Fibronectin Blastx. Peptide sequence obtained by blastx for fibronectin binding 7mer.

Enterobacteria phage M13 isolate WT variety Rutgers, complete genome
Sequence ID: [gb|JX412914.1](#) Length: 6407 Number of Matches: 1

Range 1: 833 to 1692 [GenBank](#) [Graphics](#) [Next Match](#) [Pri](#)

Score	Expect	Identities	Gaps	Strand
1342 bits(1488)	0.0	835/900(93%)	41/900(4%)	Plus/Minus
Query 31	GACGTTAGTNNMTGAATTTTNCNGNATGGGATTTTGCTAAACAACCTTTCAACAGTTTCGGN	90		
Sbjct 1692	GACGTTAGTAAATGAATTTTCTGTATGGGATTTTGCTAAACAACCTTTCAACAGTTTC---	1636		
Query 91	CGNNNTNCACCATGAAGCTCCGACAGCAGCAAGAGTGAGAATAGAAAGGTACCACTAA	150		
Sbjct 1635	-----AGCG-----GAGTGAGAATAGAAAGGAACAACTAA	1606		
Query 151	AGGAATTGCGAATAATAATTTTTTTCACGTTGAAATCTCCaaaaaaa-GGCTCCAAAAG	209		
Sbjct 1605	AGGAATTGCGAATAATAATTTTTTTCACGTTGAAATCTCCAAAAAAAAGGCTCCAAAAG	1546		

Figure 6: Collagen binding phage BLAST. Alignment of a section of the sequence obtained from the isolated collagen binding phages (query) and a section of the phage M13 genome corresponding to the pIII gene

gene III
Sequence ID: [prf|1814345A](#) Length: 424 Number of Matches: 1

Range 1: 10 to 38 [GenPept](#) [Graphics](#)

Score	Expect	Method	Identities
47.0 bits(110)	2e-04	Composition-based stats.	25/40(63%)
Query 120	LVVPFYSHSWLSLHGXHXETVESCLAKSHXNSXTNV	1	
Sbjct 10	LVVPFYSHS-----AETVESCLAKSHTENSFTNV	38	

Figure 7: Collagen blastx. Peptide sequence obtained by blastx for collagen binding 7mer.

4.2.7 Conclusions of Aim 1:

The sequence of the 7mer isolated from the collagen binding phages is WSLSELH and the sequence of the 7mer isolated from fibronectin binding phages is SLSKWSF.

CHAPTER 5

AIM 2: SYNTHETIZE, CONFIRM, AND PURIFY CANDIDATE PEPTIDES.

5.1 Hypothesis:

We will successfully synthesize peptides identified in Aim 1 (Chapter 4)

5.2 Method:

We synthesized the peptides identified in Aim 1 as well as two peptides found in literature, one reported to bind to fibronectin, GLAGQRGIVGLPGQRGER (6) and the other reported to bind to collagen, AAHEEICTTNEGVM (7).

Peptides were synthesized by solid-phase method by using a 9-fluorenylmethyloxycarbonyl (Fmoc) strategy. The peptide chains were assembled on a Rink amide MBHA resin, which results in amino termini on both ends of the peptide. For coupling, N,N-diisopropylethylamine (DIEA) was used, as well as oxyma for coupling, and piperidine for Fmoc removal. All of the reactions were performed using dimethylformamide (DMF) as a solvent. To cleave the peptide from the resin and remove the side chain protecting groups, the peptide resin was treated with trifluoroacetic acid (TFA), triisopropylsilane and water. The peptides were identified satisfactorily by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDITOF-MS).

CHAPTER 6

AIM 3: TEST THE POTENTIAL APPLICATIONS OF THE ISOLATED PEPTIDES AS RE-LINKERS IN SYNTHETIC 3D MATRICES.

6.1 Hypothesis:

The affinity of the peptides obtained in Aim 1 (Chapter 4) and synthesized in Aim 2 (Chapter 5) for collagen and fibronectin are requirement enough for these peptides function as re-linkers or nucleation points.

6.2 Method:

As mentioned in the significance section, PEG-4MAL is a very useful tool in mechanobiology and tissue engineering because of its efficiency on incorporating the metalloproteases degradable cross linkers and RGD, its gelation time and tight structure. Moreover, with PEG-4MAL gels can be prepared with mechanical properties that mimic those of ECM derived components. Thus, we are interested to try whether we could improve PEG-4MAL properties by adding the peptides synthesized in Aim 2. We expect, these peptides could function as “nucleation seeds” promoting the building up of ECM elements on the hydrogels. In this way, this *de novo* building up of ECM elements could restore the gel’s stiffness, which is weakened by the protein digestion occurring during cell migration. We will prepare PEG-4MAL hydrogels following standard protocols with a slight modification. The peptides will be diluted separately in DMSO at 1mM concentration and added to a 2 mM RGD solution. Gels will then be prepared as described in the attached protocol and the peptide-RGD solution will be mixed with

PEG-4MAL at a ratio of 1:100 RGD-peptide:PEG-4MAL. Cells will be seeded on the gels and after 7 days will be fixed with 4% formaldehyde and ECM components detected by immunostaining and fluorescent images taken using imageJ.

Thomas McCarthy, a Peyton Lab member, had carried out a first attempt to this experiment using a modified version of the peptides sequenced in Aim1 and a mesenchymal stem cell h-Tert cell line (no further specifications on the cell line used). These peptides, also synthesized in Aim2, were CWSLSELHC for collagen and CSLSKWSFC for fibronectin. Cells were labeled with mouse anti-collagen type 1 (MAB3391 Millipore) and TRITC conjugated anti-mouse secondary antibody (RED) (non specified); DAPI to stain the nuclei (blue) and alexa fluor 488 conjugated mouse anti-fibronectin (563100, BD Pharmingen, green). On this experiment, it has not been observed building up of ECM elements on the matrix gels (Figure 8), suggesting that these peptides did not serve as nucleation point. However, this experiment should be repeated and optimized to fully explore the potential of *de novo* nucleation points.

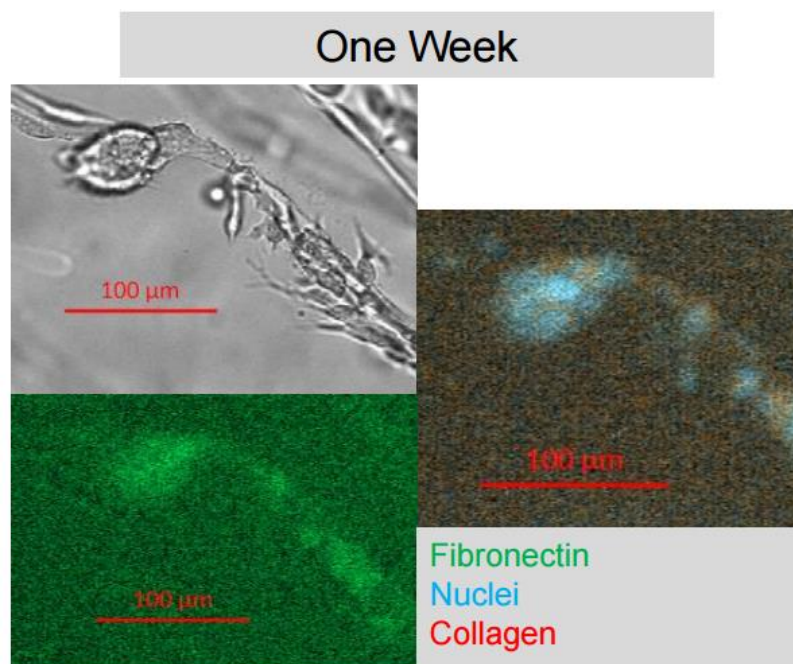


Figure 8:
Immunofluorescent assay. Fluorescent immunostaining to analyze ECM elements building up in PEG-4MAL gel prepared with short peptides. Pictures have been taken with imageJ. No ECM elements observed on gel. Courtesy of Thomas McCarthy.

CHAPTER 7

PROJECTIONS ON SHORT PEPTIDES AS ECM ELEMENTS' NUCLEATION POINTS/CROSSLINKERS

7.1 Potential Pitfalls:

The short peptides might not bind to the target molecules with the same strength that the respective phages they are derived from do or may not bind to the target molecules when they are free from the phage structure. It has been demonstrated that the interaction of the free peptides with the target molecules is significantly weaker than their phage counterparts (13). This could be the result of a synergy of the affinity of the five peptide copy on the phage coat results in an enhanced affinity when they are attached to it.

Another reason could be that the conformation and orientation of the peptides are crucial to their affinity, or when they are free from the phage, their affinity to other copies of the same peptide is higher than the affinity to the target protein.

7.2 Potential solutions:

A practical way to check whether the peptides are capable of binding to their target molecules is by ELISA assay. Cover slips should be coated with the peptides, and then they should be challenged by the target molecules and finally using antibodies against the target molecules to verify their interaction.

In case the peptides do not bind to the target molecules, a structure could be constructed that mimics the phage structure. In this way the peptides would conserve their tridimensional orientation (13).

7.3 Future Directions:

As a short term future direction the ELISA assays proposed above should be carried out.

Also, the testing of the peptides on the hydrogels described in aim 3 should be completed.

Once the function of the aforementioned peptides is confirmed, their applications in different hydrogel systems such as PEG-PC (phosphorylcholine) should be explored.

Also, their potential applications in drug testing may be assessed; for example to detect an inhibitor of ECM building up or metalloproteases inhibitors. Another potential application is tissue engineering, to explore how these peptides could contribute to the rebuilding or healing of tissues using synthetic scaffolds. In case these peptides do not function as proposed, a future direction would be the construction of structures that mimic the phage structure as proposed on the previous section.

APPENDIX A:

PEG-MAL HYDROGEL PROTOCOL

Materials:

4-arm Polyethylene glycol-maleimide (PEG- 4MAL), MW 20 kDa, >95% purity (JenKem) cross-linking polymer

- Pan-MMP (degradable) sequence: GCRDQGW~~I~~LGQPGDRCG (GenScript).
 - Note: Other degradable sequences can be made for specific MMPs.
 - J, Patterson, JA Hubbell, Biomaterials 31 (2010) p.7836-7845
 - Generally, these peptides are purchased in aliquots of 0.5 mg to avoid weighing errors.
- Linear PEG dithiol, MW 1.5kDa, >95% purity (JenKem or Sigma)

Adhesive ligand

- RGD sequence: RGDC, >95% purity (GenScript)
 - Note: other adhesive ligands can be added to the gel.

PBS, pH 7.4

200 mM Triethanolamine (TEOA) in PBS, pH 7.4

Gel making protocol:

1. Gels are calculated by weight percent. Crosslinking is determined by matching moles of crosslinker to moles of PEG-MAL.
2. Warm polymer solutions to room temperature and weigh out appropriate mass of polymers needed.
3. Make 200 mM TEA in PBS, pH 7.4 (maleimide-thiol reaction only occurs at pH 7-7.5).
4. Dissolve crosslinking polymers in sterile TEOA solution and PEG-MAL in PBS.
5. Functionalize hydrogel.
 - a. Mix PEG-MAL and RGD at 100:1 ratio.
 - b. React at room temperature for 30 min.
6. If encapsulating cells, begin to split the cells and spin down the desired cell amount into a pellet.
7. Re-suspend cell pellet in the PEG-RGD solution. Minimize the time cells spend in uncrosslinked PEG solution.
8. Cast hydrogels by mixing PEG-RGD-cells with crosslinking solution at 10:1 ratio.
 - a. Note: Gels polymerize quickly.
9. Incubate gel at 37°C ~15minutes or until fully gelled.
10. Add media (or PBS if no cells) to swell gel.

Notes:

TEOA

- Higher TEOA concentrations may be used to decrease reaction time and increase reaction rate.
- Maleimide-thiol reaction occurs at pH range in between 7 and 7.5. Bring the pH to 7.4 when encapsulating cells.

PEG-MAL

- Should be stored with nitrogen gas at -20°C in a sealed container.

Peptides

- Should be stored at -20°C.
- Each adhesive ligand will affect the gelation process differently and must be experimented with. Check the pH of the solution if you have gelation problems.

APPENDIX B:

NOTES:

Thomas McCarthy, a Peyton Lab member, had carried out the experiment explained in chapter 6 using a modified version of the peptides sequenced in Aim1 (Chapter 4) and a mesenchymal stem cell h-Tert cell line (no further specifications on the cell line used).

Thomas McCarthy has also provided the figure 8 corresponding to mentioned experiment.

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