I, Jangwook Philip Jung, hereby submit this original work as part of the requirements for the degree of:

Doctor of Philosophy

in Biomedical Engineering

It is entitled:

Engineering Modular Self-Assembling Biomaterials for Multifunctionality

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This work and its defense approved by:

Committee Chair: Daria Narmonova, PhD

Abstract

The objective of this thesis was to design self-assembling biomaterials whose physical and biological properties can be systematically adjusted to modulate cell growth and differentiation. The intended applications of these biomaterials include defined 3D cell culture scaffolds as well as coatings for existing prosthetics.

The complex and dynamic nature of extracellular matrices necessitates the precise integration and adjustment of multiple physical, chemical, and biological features within engineered biomaterials, but this has been challenging for previous scaffolds owing to the fact that these features tend not to be adjustable independently. Instead, these properties tend to be conflated and entangled, limiting the ability to systematically engineer scaffolds with multiple components. As a step towards addressing this issue, this thesis describes the development of a modular self-assembling biomaterial system capable of incorporating multiple physical or biological functions into precisely defined biomaterials without affecting other material properties. Three families of different biological and physical functionalities were designed, synthesized, and investigated: those that modulate matrix mechanics, those that mediate cell-matrix binding, and those that can release soluble effector molecules.
All materials were based on a short, synthetic, self-assembling peptide sequence, Q11, which formed self-supporting hydrogels in physiological conditions. To independently modulate matrix mechanics, Q11 derivatives were developed possessing chemoselective functional groups that could be polymerized via native chemical ligation. This method produced significantly stiffened gels, which also significantly enhanced endothelial cell proliferation in an independent manner.

To develop modular self-assembling ligand-bearing peptides, endothelial cell-interactive ligands, RGDS, REDV, IKVAV, and YIGSR amino acid sequences were added to the N-terminus of Q11 (X-Q11). The incorporation of X-Q11 into hydrogels was quantitative and did not significantly alter stiffness when different ligands were included in the hydrogels. The ligands were physically presented on the surface of fibrils, retained their biological activities, and interacted with cell surface receptors to modulate endothelial cell behaviors.

To develop Q11 derivatives capable of releasing soluble effectors, Q11 peptides were synthesized containing a nitric oxide (NO) donor compound. The conjugation efficiency was about 88%, and fibril morphologies were not significantly altered by the NO donor compound, allowing quantitative incorporation of this peptide into Q11 hydrogels.
The last stage of the project employed a statistical method, design of experiments, to capitalize upon the modularity of the developed co-assembling matrices, with the purpose of maximizing the growth of endothelial cells on the materials. Through several rounds of multifactorial experimentation, an optimal formulation of multiple peptides was determined, resulting in endothelial cell attachment and proliferation comparable to the native matrix protein, fibronectin. Such a calculation of an optimal formulation would be prohibitively costly, both in terms of time and materials, for conventional biomaterials not constructed in a modular fashion. These results suggested that modular Q11-based self-assembling systems enable facile manipulation of multiple factors, allowing the efficient targeting of a desired response, in this case endothelial cell growth. This approach should allow for the systematic design of biomaterials for a wide range of applications, without relying on ad hoc strategies.
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Organization of Dissertation

This dissertation is organized as a collection of several manuscripts prepared for publications. Each chapter starts with an introduction, materials and methods, results and discussion. This organization was intended to combine each chapter as a coherent dissertation with defined objectives and clearly stated conclusions.

Chapter 1 presents an overview of the dissertations and each chapter’s specific aims and hypotheses.

Chapter 2 presents the backgrounds relevant to the experiments conducted in Chapters 3 to 6. A brief review of self-assembly and β-sheet fibrillizing materials, followed by the strategies to achieve multifunctionality in cell-matrix binding, the impact of matrix mechanics to cell behaviors, a variety of nitric oxide (NO)-donor molecules considered for NO-release, and the design of experiment (DOE) approaches.

Chapter 3 describes the covalent crosslinking via native chemical ligation to increase the stiffness of self-assembled biomaterials. Chapter 4 describes the functionalization of the self-assembling Q11 peptide with extracellular matrix (ECM)-derived ligands so as to modulate the attachment and growth of endothelial cells. In Chapter 5, formulations of multiple ECM-derived ligand-bearing peptides were systematically optimized through DOE approaches in order
to produce biomaterials with improved endothelial cell attachment and growth. Chapter 6 described the conjugation of NO to self-assembling Q11 and the release pattern of NO with respect to varying degrees of incorporation in Q11 hydrogels.

Finally, Chapter 7 presents future research directions which can be sought to attain the goals in our laboratory.
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List of Abbreviations

ANOVA: analysis of variance
BM: basement membrane
CCD: central composite design
CD: circular dichroism
cGMP: cyclic guanosine monophosphate
DAPI: 4',6-diamidino-2-phenylindole
DOE: design of experiment
DTNB: 5,5'-dithiobis-(2-nitrobenzoic acid)
EC: endothelial cells
ECM: extracellular matrix
EGM: endothelial growth medium
ELISA: enzyme-linked immunosorbent assay
FTIR: Fourier transform infrared spectroscopy
HPLC: high-performance liquid chromatography
HSD: honestly significant difference
HUVEC: human umbilical vein endothelial cell
MALDI: matrix-assisted lased desorption/ionization
MPAA: 4-mercaptophenylacetic acid
MS: mass spectrometry
MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NCL: native chemical ligation
NO: nitric oxide
NOS: nitric oxide synthase
NONOate: diazeniumdiolate
PBS: phosphate buffered saline
PECAM: platelet-endothelial cell adhesion molecule
PEG: poly(ethylene glycol)
PMT: photomultiplier tube
RSM: response surface methodology
RSNO: S-nitrosothiol

TCEP: tris(2-carboxyethyl)phosphine
TCPS: tissue culture polystyrene
TEM: transmission electron microscopy
TFA: trifluoroacetic acid
TIS: triisopropylsilane

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SMC: smooth muscle cells

vdW: van der Waals
Chapter 1

Overview of the dissertation and specific aims

1.1 Motivation of the project

In 2006, 448,000 coronary artery bypass surgeries were performed on 253,000 patients in the US, estimated by the National Center for Health Statistics. This procedure utilizes autologous saphenous veins or mammary arteries to treat peripheral arterial occlusive disease and coronary heart disease. Although such autografts are currently the gold standard for bypass procedures, there are several disadvantages, including the limited availability, size mismatch, and insufficient long-term patency of autologous tissues. In contrast, synthetic grafts theoretically have limitless availability, and their design is flexible enough to match any demand for surgical operations. One of the most significant limitations of these prostheses, however, is that they generally do not support the regeneration of functional endothelial cells (ECs) on their luminal surfaces. This contributes to the high-failure rate of small- (<4 mm) to medium-diameter (<7 mm) devices, which additionally require chronic anticoagulation therapy in most cases [1-4]. The primary patency rate of PTFE (polytetrafluoroethylene) prosthetic bypasses drops to only 39% after 5 years [5]. One strategy for endothelializing such devices is to mimic interactions that would naturally exist between the
endothelium and the vascular basement membrane (BM) using synthetic polymers, peptides, and tissue engineering approaches [6]. However, strategies employing polymers with a mixture of surface-bound or released factors are difficult to optimize, particularly if they present multiple factors together. Because of this, their development has previously been undertaken with a somewhat *ad hoc* approach [7]. In this way, such materials are unlikely to replicate the complexity and functionality of native BMs, requiring the development of new approaches where multiple factors can be reliably integrated, manipulated, and optimized.

The key to tackling the limitations of current vascular biomaterials is to design cell-material interfaces where multiple factors can be manipulated to initiate endothelialization and sustain normal functionality. The candidates of such factors would ideally be sequences derived from BM proteins or soluble factors known to modulate the functions of ECs. However, it is not only proteins that can directly contribute to the functions of a BM. Properties that can modulate the functions of the microenvironment around the ECs can be considered possible candidates as well, factors such as the mechanical properties of BMs.

In this dissertation, the goal is not to produce specific coating materials for currently existing prosthetics nor application of such coatings for *in vitro* or *in vivo* models, per se. Instead, with the motivation described above, I designed and characterized a novel method of producing *modular* hydrogel-based materials,
where multiple distinct molecular features can be independently adjusted and systematically optimized in a multifactorial manner. To accomplish this, I designed, synthesized, and developed a series of self-assembling peptides that can be assembled together to provide materials capable of supporting the rapid growth of endothelial cells.

Self-assembly is a process in which components spontaneously organize themselves from a less ordered state into a final state that is more ordered [8]. The process can occur on almost any length scale, from molecular to macroscopic, and it is driven by weak interactions such as van der Waals (vdW) forces, hydrogen bonding, electrostatic interactions, or hydrophobic interactions. The reversibility and adjustability exhibited by self-assembling systems is a key advantage in constructing materials. Self-assembling materials can be constructed in a modular way, enabling independent addition or deletion of intended functionalities and facilitating systematic exploration of multiple factors. In the materials developed in this thesis, modularity arises from the non-covalent construction of the materials, which enables the precise combination of multiple independently synthesized components simply by mixing them and inducing co-assembly into integrated matrices. In contrast, for whole proteins or cross-linked synthetic polymers, systematic tuning of multiple functionalities would be considerably, if not prohibitively, more challenging. In most cases, the
physicochemical and biological properties of biopolymers (biologically derived materials) are complexly interrelated [7]. Chemically defined and self-assembling materials, however, can allow more systematic investigation of multiple functionalities.

The strategy developed in this thesis for incorporating modular functionality into self-assembling hydrogels includes a) identifying an easily synthesized self-assembling component whose self-organization can proceed even when it is attached to multiple pendant functional components, b) adding functionality to this basic self-assembling peptide by synthesizing a family of co-assembling variants, each with its own independent function, c) mixing multiple functional peptides and assembling them into one hydrogel, and d) systematically adjusting multiple functionalized peptides towards a specific quantifiable output. Specifically, in this thesis three properties were modulated and will be discussed in the following chapters; 1) matrix mechanics, 2) cell-matrix binding, and 3) release of soluble factors.

1.2 Organization of the dissertation

This section overviews the main organization of this thesis. It is primarily organized as a series of manuscripts, two of which have been previously published (Chapters 3 and 4), and one of which is currently submitted (Chapter 5).
Background material for the thesis is provided in Chapter 2, auxiliary experiments not planned for journal publication are discussed in Chapter 6, and recommendations for future work are discussed in Chapter 7. At the beginning of each chapter, a brief introduction will explain the main idea, focus, and rationale for the experiments conducted.

In Chapter 3, the self-assembling peptide used throughout this dissertation, Q11, was functionalized with crosslinking functional groups in order to utilize a chemoselective ligation approach for modulating mechanics. This ligation chemistry increased the stiffness of non-covalently assembled hydrogels via covalent crosslinking. This strategy is advantageous, compared to conventional crosslinking chemistry, in that the crosslinking reaction proceeds in aqueous conditions, is compatible with hydrogel environments, is chemically specific, and does not undergo side reactions. I found that it also did not interfere with the structures of the self-assembling peptide. This exemplifies a modular strategy to increase stiffness of the self-assembled hydrogels.

Chapter 4 describes the design, synthesis, and evaluation of self-assembling Q11 peptides functionalized with extracellular matrix (ECM)-derived sequences to provide for cell-matrix binding. By incorporating such sequences at one terminus of the self-assembling peptide, the peptide can present this ligand sequence on the assembled hydrogels. Multiple functionalized peptides can be
mixed and presented on a single hydrogel in order to mimic some of the key multifunctional features of the BM. The individual peptide mixing behavior, ligand presentation, and mechanical properties were all investigated. Since this chapter deals with characterization of each functionality, the first logical step was to test binary mixtures of the ligand-bearing and the basic modular self-assembling peptide. If fibrillization was unaffected, ligand presentation was reliable, and mechanical properties were not affected by incorporation of the ECM-derived ligands, that peptide was considered as a modular factor for subsequent experimentation. This step was necessary for constructing hydrogels with multiple peptides and their subsequent systematic, discussed in Chapter 5.

In Chapter 5, multiple ECM-derived sequence-bearing peptides were designed and synthesized, and mixtures of them were systematically optimized through multifactorial experimentation. Starting from concentration ranges of peptides identified singly in the experiments described in Chapter 4, the concentrations of several of these functionalized peptides were systematically varied together in order to approach optimal formulations for maximizing endothelial growth. In addition, these experiments aimed to uncover any synergisms or antagonisms that may have existed between the different peptides. This was accomplished through a series of multifactorial experiments and “Design of Experiments (DOE)” approaches. The modular self-assembling
properties of these peptides were essentially required for this type of experimental design, as integrated matrices of nearly limitless different combinations of the peptides could be easily produced by mixing them in different ratios and inducing co-assembly. Despite the modularity of the system, it was nevertheless impractical to test all possible combinations and concentrations of functionalized peptides, so DOE approaches were necessary. These experiments are described in Chapter 5.

Beyond factors that mediate cell attachment to biomaterials, the release of soluble signaling factors can significantly drive cellular responses to biomaterials coatings as well. For endothelial cells, one such factor is nitric oxide (NO), a well-known regulator of the physiology of the vascular wall. In Chapter 6, preliminary data is presented that outlines the synthesis of a modular co-assembling peptide that releases nitric oxide (NO). Although the biological effectiveness of this drug-eluting peptide has not been determined within this thesis, it should be easily incorporated as an additional co-assembling factor in the construction of modular Q11-based matrices.

In summary, this dissertation characterizes and optimizes 1) matrix mechanics, 2) cell-matrix binding, and 3) release of soluble factors from a self-assembling system by combining multiple functionalities with systematic methods to achieve directed responses from co-assembled hydrogel materials. The
schematic description of three different functionalities discussed in Chapters 3 through 6 and their potential combinations is shown in Figure 1.

### 1.3 Specific aims and hypotheses for the experimental chapters

The specific aims and hypotheses that have driven the work described in Chapters 3, 4, 5, and 6 are described below. The goal of the entire dissertation is the development of a modular self-assembling biomaterial system enabling the incorporation of multiple physical or biological functions into precisely defined biomaterials via their systematic optimization without affecting other material properties.

**Chapter 3**

**Specific aims:** Integrate chemical functional groups into self-assembling peptides to enable polymerization post-assembly via chemoselective ligation. Investigate the effect of chemoselective ligation on hydrogel stiffness by using oscillating rheometry. Test endothelial cell growth responses on hydrogels with altered stiffness.

**Hypotheses:** Self-assembling peptides functionalized with thioester reactive groups and allowed to polymerize covalently after self-assembly will produce stiffened hydrogels. The chemoselective ligation chemistry will not alter
the assembly of the self-assembling peptide. The stiffened hydrogels will support
more rapid growth of endothelial cells compared to softer, un-crosslinked
hydrogels.

Chapter 4

Specific aims: Functionalize the self-assembling peptide with ECM-
derived sequences. Quantify the gelation and incorporation of the ECM-derived
functional peptides into backgrounds of the basic self-assembling peptide.
Determine the extent to which the ligands are presented on the surface of the self-
assembled fibrils. Determine the effect of ligand-bearing peptide incorporation
on hydrogel stiffness. Evaluate endothelial cell attachment, spreading, and
growth on hydrogels containing ECM-derived sequence-bearing peptides.

Hypotheses: Fusion peptides containing self-assembling domains and
ECM-derived ligand domains will assemble predictably into fibrils and hydrogels.
Ligand incorporation will not compromise the mechanical properties of the self-
assembled matrix. Incorporation of the ligand-bearing peptides within Q11
matrices will confer the functionality of the ECM-derived sequence (integrin
binding, in most cases). Ligand-bearing peptides will improve endothelial cell
attachment, spreading, and growth.
Chapter 5

Specific aims: Utilizing a statistical method for optimization, search for one or more formulations of multiple ECM-derived sequence-bearing peptides which would maximize the growth of ECs in an *in vitro* context. Compare the identified formulation(s) against conventional positive controls, such as fibronectin.

Hypothesis: The formulation(s) identified from the statistical method will enhance the growth of ECs significantly better than ECs cultured on hydrogels whose formulations are not adjusted from the statistical method.

Chapter 6

Specific aims: Conjugate a nitric oxide (NO) donor compound to the self-assembling peptide. Characterize the release profile of NO in EC culture medium.

Hypotheses: The release of NO will be controlled by incorporating a specific amount of the NO-conjugated peptide in the context of the modular self-assembling peptide.
Figure 1.1 Schematic for co-assembly of multiple peptides into integrated matrices. A self-assembling peptide sequence (purple) is modified with chemical groups enabling cross-linking (Chapter 3), ligands for cell attachment (Chapters 4 and 5) or the release of soluble factors (Chapter 6). Simple mixing in solution and induction of self-assembly provides scaffolds where multiple different factors are predictably and precisely integrated (right).
Chapter 2

Background and literature review

2.1 Introduction

This chapter is provided as a background for the experimental work that follows in Chapters 3-6. The background, technologies, techniques, phenomena, concepts, and paradigms that are relevant to the thesis’ research will be discussed roughly in the order that these topics arise in Chapters 3-6.

First, self-assembly itself will be described, including the origins of the self-assembling peptide Q11 and previous fibrillizing structures created from β-sheet forming peptides. Before the applications of self-assembling materials are discussed, significance of promoting endothelialization on synthetic surfaces will be discussed. Multifunctionality will be discussed through three sub-sections, cell-matrix binding (Chapters 4 and 5), matrix-mechanics (Chapter 3), and release of nitric oxide (Chapter 6). First, the design of self-assembling molecules each possessing an independent function will be discussed, followed by a brief review of EC-interactive ligands utilized in Chapter 4 and 5. Subsequently, chemoselective ligation and mechanical properties of self-assembled biomaterials will be discussed, along with recent findings highlighting the importance of
specifying mechanical properties in cell scaffolds. The chemoselective ligation chemistry discussed in Chapter 3 will also be compared with other more conventional crosslinking strategies. Next, a brief review will be provided for the rationale that underlies the use of NO-releasing peptides in Chapter 6. Lastly, the rationale for employing Design of Experiments (DOE) approaches will be provided, along with a general description of the statistical methods used.

2.2 Self-assembly

Self-assembly is a process whereby disorganized molecules, components, or structures spontaneously arrange into a final state with significantly more structural order and the scale of this process can range from molecular length scales up into the macroscopic regime [8]. Biology is in essence a product of self-assembly, and self-assembled structures are ubiquitous in biological systems. Self-assembled structures include molecular motors, ECM components, lipid bilayers, the cytoskeleton, chromatin, and a long list of other biological materials created through specific organization of smaller subunits. In chemistry and materials science, examples include the formation of micelles from amphiphilic molecules such as detergents, molecular electronics, and nanoparticles for zeolite templates, to name a few [9]. Self-assembly processes are energetically favorable, occur spontaneously, and tend to have low thermodynamic barriers. The driving
forces for many self-assembly processes, including those exploited in this thesis, are weak interactions such as van der Waals forces, Coulombic interactions, hydrogen bonds, and entropic forces such as solvation effects. Such weak interactions are inherent properties of many molecules in solution, especially proteins, and the formation of ordered structures by self-assembly tends not to rely upon significant amounts of externally supplied energy or mass. Practically, inducing self-assembly typically entails making only a small change in the pH, temperature, or ionic conditions of a solution, creating an environment whereby the intermolecular forces listed above lead to self-organization. This is in stark contrast to more conventional construction techniques for synthetic matrix materials such as covalent polymerization, cross-linking, or adding catalysis, initiators, or reagents to modify entire materials’ composition by covalent chemical reactions. From a materials design perspective, therefore, self-assembly is convenient because it utilizes non-covalent interactions, which are rapid, reversible, and triggerable by adjustment of solution conditions.

This dissertation project exploited and developed the use of modular multifunctionality by using self-assembling peptides. The bigger goal of the project consisted in systematically adjusting the multiple functionalities towards a targeted response, that of rapid endothelial cell growth. To accomplish this, peptides were developed that consisted of a common assembly domain and a
variable region containing one of several functional domains. Using this system, materials aimed at different biological responses can in principal be produced through combination and optimization of the peptides developed. This is the design rationale for the self-assembling peptide called Q11, for which a brief description will follow in the section below (for more extensive details, see ref. [10]).

2.3 β-sheet fibrillizing structures

The mode of self-assembly that has been exploited in this thesis is β-sheet fibrillization. In the past 10-15 years, this particular process of self-assembly has received significant attention for producing self-assembling biomaterials, culture matrices, and hydrogels [7, 11-20]. The following section deals with the potential of β-sheet structures, fibrils formed from β-sheets, and materials constructed from β-sheet fibrillization, especially in the context of synthetic scaffolds. Portions of this section have been adapted from Jing et al., *Nanostructured materials constructed from polypeptides*, in Nanoscience and its Applications in Biomedicine, D. Shi, Editor, Springer-Verlag, (2009) [21].

β-sheets are one of the fundamental secondary structures in proteins, consisting of two or more β-strands laterally connected by hydrogen bonds between the N-H and C=O groups from one peptide bond and the C=O and N-H
Figure 2.1 Parallel (a) and anti-parallel (b) β-sheet folding. β-sheet fibrils often possess “cross-β” structure, in which the backbone of the peptide is perpendicular to the fibrillar axis; (c) schematic of the self-assembly of a designed β-hairpin peptide, reprinted from Kretsinger, et al. Biomaterials 26, 5177-5186 (2005).
groups of a second peptide bond, respectively (Figure 2.1). This conformation generates β-pleated-sheet structures, causing the side chain residues to project alternatively above and below the sheet. β-sheet structure is also supported by weak interactions such as hydrophobic effects, vdW forces, and Coulombic interactions between side chains and the backbone. Although it is challenging, but continuing to rise understand to predict the secondary structures that will form from a particular amino acid sequence [22], it is known that peptides with alternating hydrophobic and hydrophilic residues in their primary structures have a high propensity for forming β-sheet structures in water [23]. This arises from the fact that alternating hydrophilic and hydrophobic residues tend to place all hydrophobic residues on one side of the sheet and all hydrophilic residues on the other. The resulting planar amphiphiles then have a strong driving force to assemble in water into tertiary structures such as stacked β-sheets or fibrils. Such fibrils consist of β-sheet strands running across the width of the fibrils, perpendicular to the fibrillar axis, in a conformation known as “cross-β” [24]. The fibrils tend to be relatively unbranched, laterally associated into bundles and tangles, and generally consistent in this morphology.

Despite the tendency of alternating hydrophilic/hydrophobic peptides to form β-sheet fibrils, such alternation is not required for assembly. For example, several proteins and peptides with varying sequences, lengths, and patterns of
hydrophilic and hydrophobic residues are able to form β-sheet fibrils. For example, transthyretin, the SH3 domain, and lysozyme are three distinctly different proteins, yet they are all able to form similar β-sheet fibrillar structures, and none of them possess alternating hydrophilic/hydrophobic primary structures [25]. In fact, the ability to form β-sheet fibrils is shared by several types of molecules that do not even possess primary L-α-amino acid structures, as shown in Figure 2.2. This suggests that β-sheet fibrillization is an intrinsic property of polyamides, proteins, or peptides, where the fibrillar structures are likely stabilized by hydrogen bonds between the amide backbones rather than specific interactions occurring between the side chains of residues, as occurs in globular protein folding [26, 27]. As a consequence, this inherent tendency of a wide range of different polyamide structures to form β-sheet fibrils indicates that β-sheet fibrillization may be a self-assembly mechanism that can still proceed even when amino acid structures are significantly altered or engineered. In the work described in this thesis, this property has been exploited.

Fibrillization or growth of a β-sheet is believed to start with a seed of aggregated proteins or peptides. From a recent study with bovine pancreatic insulin in bulk solution, the kinetics of β-sheet growth were shown to evolve in two stages [28]. The first stage was related to the formation of nonfibrillar aggregates, and the second stage was found to be associated with the growth of
Figure 2.2 Negative-stained transmission electron micrographs of fibrils created from β-sheet folding. (a) fibrils formed from the peptide GGRGDSGGQKFKFFQEQ; (b) fibers formed from by a peptide amphiphile terminated by a cyclic RGD sequence reported by Stupp and coworkers, reprinted with permission from Guler et al. Biomacromolecules 7, 1855-1863 (2006); (c) fibrils formed by a β-hairpin peptide reported by Schneider, Pochan, and coworkers, reprinted with permission from Ozbas et al., Macromolecules 37, 7331-7337 (2004).
fibrils to form protofibrils, which then laterally associated to produce mature fibrils with widths of approximately 10-15 nm [29]. These fibrils can then form extended tangles to produce physically networked hydrogels. This entire process of nucleation and growth is affected by the local solution conditions such as pH, salt concentrations, temperature, and the presence of other seeds for possible nucleation events.

The ability to induce β-sheet fibril assembly using minor changes in solution conditions is an attractive aspect. In particular, the sensitivity of self-assembly to the addition of salts provides a facile trigger for induction of gelation. The phenomenon of salt-sensitivity of self-assembling peptides has been investigated and described by Caplan et al. In this work, (FKFE)₃ or KFE12 peptide fibrillization was dependent on the valency of the added salts. When KCl, K₂SO₄, or K₃Fe(CN)₆ were used, higher valances required less salt to stimulate self-assembly of the KFE12 peptide [30]. The theoretical basis of this observation originates from the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory. In accordance with DLVO theory, when electrostatic repulsive forces are decreased by charge-shielding and reduction of the Debye length in solutions, attractive vdW forces can become dominant and cause self-assembly.

Salt sensitivity in polypeptides with alternating hydrophilic and hydrophobic residues was first observed by Brack and Orgel [23], but the
development of such peptides as biomaterials was pioneered in the 1990s by Zhang and colleagues [31-33]. In this early work, peptides with alternating patterns such as (RADA)$_4$, (FEFEFKFK)$_2$, or (AEAEAKAK)$_2$ were initially insoluble and partially assembled in water. However, when salt-containing buffers, i.e. phosphate buffered saline (PBS), were added to these solutions, transparent hydrogels were formed nearly instantaneously [34, 35]. The solution behavior of Q11, the peptide primarily used in the research reported in this thesis, is similar to these β-sheet fibrillizing peptides.

2.4 The self-assembling peptide Q11

This section outlines the previous development of the self-assembling peptide used as the basis for the work described in this thesis, Q11 [10, 30-33, 35-41]. One of the primary motivations for the development of Q11 was the general mechanical inferiority at the time of matrices constructed from self-assembling peptides, which tended to possess low storage moduli on the order of ~1kPa. Further development of such peptides as cell culture scaffolds was limited by these poor mechanical properties [10]. In an attempt to address this issue, Q11 was developed in 2003 as a self-assembling peptide sequence that could be cross-linked by an enzyme, transglutaminase [44]. To achieve this, a reliably fibrillizing peptide previously designed by Aggeli et al. [45-47], Ac-
QQRFWQFQEQQ-NH₂, was modified through two substitutions to provide a peptide that could both self-assemble and possess transglutaminase activity. First an Arg→Lys modification was made to enable the formation of glutamine-lysine cross-links by transglutaminases. Next, a Trp→Phe substitution was made to mimic residues 61-65 of microfibril-associated glycoprotein 1, MAGP-1, which form a native β-sheet structure [48]. Additional considerations included the fact that amyloidogenic peptides such as polyglutamines are well known β-sheet forming peptides [49], but longer polyglutamine sequences possessing 30-100 glutamine residues were known to be cytotoxic. However, short polyglutamine repeats were not toxic [50], so the length of Q11 was kept as short as possible, only 11 residues. Consequently, the specific sequence of Q11 peptide (AcQQKFQFQFEQQ-NH₂) was finalized through these design considerations.

In pure water, Q11 is soluble up to at least 60 mM [10, 44, 51]. Upon addition of salt containing buffers such as PBS, it forms fibrils or translucent hydrogels within 0.5-1 h, depending on concentration. At concentrations below about 2 mM, soluble fibrils are formed, whereas at concentrations above about 2 mM, hydrogels are formed. In previous work, polyethylene glycol (PEG)-containing and oligoethylene glycol-containing derivatives of Q11 were synthesized and characterized [52], and it was found that Q11 could self-assemble even when functionalized with such structure-disrupting chemical groups as PEG.
compounds. Although Q11 in the end was found to be only a mediocre substrate for transglutaminases [44], its unintentional characteristic of being able to robustly self-assemble even when appended to other chemical groups has proven to be extraordinarily useful in the context of the work described in this thesis, as described below.

Despite the convenient self-assembling properties of Q11, its hydrogels are biologically non-functional, as are other β-sheet fibrillar materials for synthetic scaffolds [21]. In order to endow these materials with the ability to engage specific receptors or physiological processes, they must be functionalized with or conjugated to other biologically active components to render them bioactive. The following section will discuss functionalization of Q11 and other fibrillar materials for synthetic scaffolds.

2.5 Promoting endothelialization on synthetic surfaces

This section provides a brief background and significance of biological process of endothelialization. Graft thrombosis is the result of neointimal hyperplasia and is a particular problem for small- to medium-diameter vascular conduits [1-4]. Indeed, graft thrombosis is the cause of 80% of vascular access dysfunction, resulting in an associated health-care cost of more than $1 billion per year in the US [54]. After two years, the primary patency rate of PTFE
(polytetrafluoroethylene) bypasses was 69% and after 5 years it was 39% [5]. In native vessels, thrombosis and intimal hyperplasia are naturally controlled by the endothelium. A crucial limitation to most vascular prostheses is that they do not support the regeneration of this important cell monolayer on their surfaces, diminishing such devices’ safety and long-term patency. Owing to this, the endothelialization of vascular prostheses has been intensively sought, with many strategies seeking to recapitulate molecular features of endothelial cells’ natural substrate, the basement membrane (BM). However, significant improvements in clinical efficacy have yet to be realized. Critically, most strategies that seek to retain ECs on graft surfaces do not actively ensure that they retain their anti-thrombotic, anti-inflammatory properties. On device surfaces, long-term patency would be improved with successful endothelialization and the maintenance of EC homeostasis for the lifetime of the patient [55-59]. One way to address the current limitation of synthetic vascular prostheses is to develop biomaterials that promote appropriate EC attachment, growth and their native functions.

Designing biomaterials that can achieve such a goal is very challenging but it is also possible to develop reliable ways to integrate precise combinations of these factors into a defined, optimized biomaterial, which is a main objective of this dissertation. The use of synthetic biomaterials such as expanded polytetrafluoroethylene (ePTFE) or polyethylene terephthalate (Dacron) for
vascular grafts has increased tremendously over the last three decades [1, 60, 61]. Despite their prevalence, the existing synthetic materials are not suitable for coronary bypass or for small- to medium-diameter caliber arteries owing to high occlusion rates [2, 62]. It is recognized that in order to overcome this limitation it is important to establish an endothelial lining. Various approaches have been used to achieve this goal, including the seeding of autologous ECs ex vivo [63, 64], or the treatment of the materials with extracellular matrix (ECM) proteins [65-71], and blood plasma proteins [72, 73]. Even surfaces with improved endothelialization still tend to demonstrate incomplete coverage of the monolayer and initiation of EC pathology, ultimately resulting in damaging the endothelium. Therefore, the focus of the following sections will discuss the design of biomaterials so as to precisely incorporate multiple functionalities and their systematic optimization.

2.6 Multifunctionality from β-sheet fibrillar hydrogels

This section discusses the functionalization of self-assembling β-sheet fibrillizing peptides specifically for their use as synthetic scaffolds. The motivation of the project is to recapitulate the function of natural ECMs by providing self-assembling fibrillar materials with multiple functionalities that can be systematically adjusted with respect to each other. This section contains three
subsections, dealing with modifications that can mediate cell-matrix binding (2.6.1), modifications that can modulate matrix mechanics (2.6.2), and modifications that can achieve the controlled release of soluble effectors (2.6.3). Portions of this section have been adapted with permission from J. P. Jung et al., *Biopolymers, Peptide Science* 94, 49-59 (2010) [14].

### 2.6.1 Cell-matrix binding mediated by ECM-derived sequences

Synthetic scaffolds in biomedical applications are designed to recapitulate the functions of naturally existing ECMs. Fibrillar morphology mimics the structure of ECMs, and the attachment of bioactive sequences can endow functionality to synthetic scaffolds. However, ECMs are extremely multifunctional, dynamic, and incompletely characterized. ECMs communicate adjacent cellular microenvironments through cell-matrix interactions, mechanical signaling, and the controlled release of soluble factors. In addition, they are continuously remodeled. It is the summation and integration of all of these signals together that drives specific cell behaviors [74]. It is highly challenging to engineer synthetic scaffolds that present and mimic these functionalities since precise control and independent adjustment of biochemical and biophysical properties are required [7, 75].
To address this challenge, strategies for constructing synthetic scaffolds from self-assembling components are beginning to emerge [12, 17, 76-79]. Generally, these strategies are characterized by the design of a basic peptide-based building block that can self-assemble into fibrillar hydrogel networks, which can then be modified to produce functional variants. The advantages of these peptide-based systems are that once a robust self-assembling base material is designed, many different modifications of the base material (e.g. those that contain cell surface receptor ligands, crosslinking domains, degradable sequences, or drug-releasing components to name a few) can then be co-assembled into integrated multi-functional scaffolds. Because these materials are constructed non-covalently via self-assembly, the different factors can, in principle, be explored as combinations much more efficiently than within covalently assembled or polymerized biomaterials (Figure 1.1). Again, this design skips the steps of reinventing chemistry of materials or ad hoc approaches - one material for one application.

One goal of the work reported in this thesis is to develop a co-assembling set of molecules that can be co-assembled without altering global properties of the materials. In an ideal system, different cell adhesion ligands would be able to be swapped with other bioactive amino acid sequences or biochemical functionalities without significantly altering the supramolecular framework of the material, such
as its fibrillar morphology, mechanical properties, or gelation kinetics [7]. An ability to achieve this would enable the independent tuning of each factor. This approach is also supported technologically by previous research in which functionalized self-assemblies have been reported. For example, strategies have been taken recently to add functional domains to self-assembling peptide biomaterials, incorporating cell-binding ligands, mechanical factors, enzymatically cleavable domains, or controllably released soluble factors [80-82]. A range of directed responses have been achieved to date from such functionalized matrices, including cell attachment, growth, and cell type-specific differentiation, but importantly, this previous work has almost exclusively investigated only one factor, or in rare cases two factors. The materials discussed in this thesis provide routes for incorporating many different factors into co-assembled matrices.

Examples of previous materials include the strictly alternating polar/non-polar peptides (RAD16 family) first described by Zhang and coworkers [33, 83, 84], Q11 peptides [44, 85, 86], and peptides from amyloidogenic proteins such as transthyretin [87]. In order to confer specific biofunctionality to these otherwise purely structural assemblies, functional amino acid sequences have recently been appended to either their N-termini or C-termini [37]. In many cases the functional sequences are presented on the surface of the self-assembled fibrils in
configurations that allow them to influence the behaviors of cells in contact with them. For instance, the RAD16 family of peptides is well known to form stable β-sheet fibrillar hydrogels in salt-containing buffers with peptide concentrations as low as 1-10 mg/mL [40, 79]. Several different functional sequences have been attached either at the N-terminus, including the laminin-derived YIGSR and RYVVLPR sequences and the type IV collagen TAGSCLRKFSTM sequence [36] for attachment of cells and the osteopontin-derived DGRGDSVAYG cell adhesion sequence [37]. In cultures of human aortic endothelial cells, gelled RAD16-I peptides bearing YIGSR and TAGSCLRKFSTM ligands modulated growth and nitric oxide production compared to unmodified RAD16-I peptides, indicating that a sufficient number of ligands were presented by the self-assembled fibrils to induce these changes in cell behavior [36]. Gelled RAD16 peptides bearing bone marrow homing sequences at their C-termini were also shown to promote neural stem cell adhesion and differentiation in the absence of soluble neurotrophic factors [88].

Regarding the Q11 peptide, its functionalization and characterization is detailed in Chapter 4 and the method for its systematic adjustment is described in Chapter 5. In those two chapters, the cell-binding ligands selected for functionalization were RGDS, REDV, IKVAV, and YIGSR. These peptides were selected based on their previously documented functions to enhance EC
attachment and growth. The most extensively investigated of these functional peptides is the integrin-binding sequence, RGDS. This sequence has been shown to support cell attachment and is located close to the center of the fibronectin cell binding domain [89, 90]. The RGDS sequence found in fibronectin, laminin, and other ECM proteins is well known to enable cell attachment for many cell types by binding multiple integrins including $\alpha_5\beta_1$, $\alpha_i\beta_3$, $\alpha_{IIb}\beta_3$ and several others [91-93]. From a biomaterials perspective, this sequence provides a simple and defined means to enhance EC attachment and spreading when conjugated to synthetic surfaces [91, 94-96].

The REDV peptide, similar to the RGD sequence [97], was originally reported to enhance attachment of murine melanoma cells [98], but it also supported attachment and spreading of ECs [99, 100]. This peptide is located near the C-terminus in the alternatively spliced type III connecting segment (IIICS) domain of the plasma fibronectins [101], and integrin $\alpha_4\beta_1$ is the receptor for REDV-mediated adhesion of human umbilical vein endothelial cells (HUVECs). This sequence, when incorporated into artificially constructed ECMs [102, 103] or porcine small intestinal submucosa-derived ECMs [104], has been shown to enhance attachment of ECs on such biomaterials.

The peptide IKVAV, from a cryptic sequence near the carboxy-terminal end of the $\alpha_1$ chain of laminin-111, is known in the field of biomaterials as a
modulator of neuronal cell attachment and neurite outgrowth [105-107]. This sequence is also found to influence attachment, migration, morphology, and matrix remodeling for ECs as well [108, 109], though the mechanisms responsible for these cellular responses are not fully understood. Previous studies have suggested that the IKVAV sequence binds $\alpha_3\beta_1$ and $\alpha_6\beta_1$ integrins in cultures of adenoid cystic carcinoma cells [110] while others have identified a 110 kDa non-integrin cell surface laminin-binding protein that recognizes an IKVAV-containing peptide [111]. In cultures of ECs, surface-bound IKVAV peptides have been found to promote weak cell adhesion [108, 109, 112], and soluble IKVAV has been found to promote cell migration and the formation of tube-like cellular morphologies [113], illustrating that its influence on EC behavior is subtle, multifaceted, and most likely context-dependent.

The major cell-binding site from the $\beta_1$ chain of laminin is identified as CDPGYIGSR whose amide form of the peptide (YIGSR-NH$_2$) was known to reduce the formation of lung melanoma colonies when it was added in soluble form [114]. For cell binding, the spatial arrangement of tyrosine and arginine residues are critical for maintaining hydrogen bonding [115], which should be separated by glycine to allow a turn structure in the peptide backbone [116]. The role of isoleucine is not essential, as shown by the similar responses elicited by peptides YIGSR and YCGSR [117] and of the role of the serine residue is even
less important [118]. In biomaterials applications, attaching YIGSR onto polymers supported the adhesion and spreading of ECs including HUVECs [80, 94, 119-122]. Despite the well-documented impact of RGDS, IKVAV, YIGSR, and REDV on the attachment and growth of endothelial cells, they have not previously been explored in combination, primarily because doing so would be prohibitively complex within previous biomaterials systems. The materials described in this thesis allow, for the first time, such a combinatorial investigation in synthetic systems.

Beyond non-native designed peptides such as RAD16 and Q11, native amyloid fibrils have also received attention as engineered materials for applications in synthetic scaffolds and other biomedical applications [26, 123-125]. Some of these are capable of presenting biofunctional amino acid sequences analogously to the engineered peptides described above. In a recent example, amyloid-forming peptides corresponding to residues 105-115 of the protein transthyretin were functionalized with an RGD sequence, and the ligand-bearing peptides formed fibrils exhibiting enhanced fibroblast adhesion [87]. The fibril morphology was completely preserved, indicating that the addition of the ligand did not significantly alter the assembly of the transthyretin peptide. In light of these studies and those discussed above, it is interesting that so many completely unrelated β-sheet fibrillizing peptides, native or de novo, form similar
Figure 2.3 Negatively stained TEM images of β-rich fibrils from different peptides and peptide derivatives. (a) fibrils formed from the peptide Q11; (b) fibrils formed from the peptide RGDS-Q11; (c) fibrils formed from the thiol-presenting peptide Cys-SGSG-Q11; (d) fibrils formed by mixture of two aromatic short peptides, Fmoc-FF and Fmoc-RGD (reproduced from Zhou et al. Biomaterials 30, 2523-2530 (2009) [126], with permission. Box represents magnified area in original publication).
fibrils that have the capacity to display bioavailable ligands on their surfaces (Figure 2.2 and 3). This suggests that there is considerable flexibility in designing fibrillar hydrogel materials for synthetic scaffolds as well as other bionanotechnological applications. For example, the fibril-forming sequences of these peptides may be selected or engineered to address specific biological requirements (e.g. non-toxicity, non-immunogenicity, specific rate of degradation) and mechanical requirements (e.g. stiffness and compliance) that may vary from application to application.

The primary focus of the sections above was on what self-assembly is, how the self-assembling peptide Q11 was designed, why β-sheet fibrillar materials are useful for synthetic scaffolds, and how cell-matrix binding can be achieved by attaching ligand peptides to β-sheet fibrillizing peptides. In subsequent subsections, matrix mechanics and release of soluble factors will be discussed (2.5.2 and 2.5.3, respectively).

2.6.2 Modulating matrix mechanics via chemoselective ligation

Beyond the presence of ligands for cell attachment, another consideration in biomaterials design is viscoelasticity. Recently, multiple pieces of evidence have shown that mechanotransduction is an important signaling pathway for controlling cell behaviors. This section will discuss how important the
mechanical properties of microenvironments are and the approaches investigated in synthetic scaffolds. This overview of matrix mechanics is followed by a section describing the chemoselective ligation chemistry to modulate stiffness of Q11-based hydrogels.

For the proper functionality of cells in synthetic scaffolds, the viscoelasticity of extracellular environments should be precisely controlled. The stiffness of living tissues varies many orders of magnitude from several hundred Pa (e.g. brain) to the range of MPa (e.g. tendon and cartilage) [127]. Cells under different physiological functions modify their microenvironments throughout embryogenesis and development, and the remodeling of cellular and extracellular environments is a continuous process that maintains tissue homeostasis. Recent studies on the relationship between ECM stiffness and cell differentiation showed that stem cell fate can be controlled by substrate stiffness. Mesenchymal stem cells (MSCs) grown on soft substrates that mimic the stiffness of brain tissues were found to be neurogenic, whereas substrates of intermediate stiffness were myogenic, and stiffer substrates mimicking the stiffness of bone were found to be osteogenic [128]. In other studies, the spreading and proliferation of embryonic stem cells (ESCs) improved with increasing substrate stiffness, while the attachment of cells were not affected by the substrate, illustrating that mechanical properties can play an important role in both early and terminal differentiation of
stem cells [129]. In pathological conditions, the stiffness of ECMs can be altered, and metabolic processes can become disregulated. For example, breast tumorigenesis is accompanied by collagen crosslinking which can stiffen ECMs up to 15-fold, resulting in breast malignancy [130]. In both cases, the mechanical properties are critical for directing cell behaviors appropriately when synthetic scaffolds or biomaterials are used for regenerative medicine applications.

Beyond needing to specify matrix stiffness to elicit specific biological responses, other more practical considerations also necessitate the adjustment of matrix stiffness. For example, the handling of self-assembled synthetic scaffolds is inherently limited by the fact that these hydrogels have a predominantly non-covalent nature of molecular entanglements and a proclivity to fail cohesively. This lack of strength and stiffness makes it difficult to consistently manipulate the materials \textit{in vitro}, and it makes it difficult to produce uniform and robust coatings from the materials in any ultimate \textit{in vivo} application.

In order to overcome the generally poor mechanical properties of peptide hydrogels, several strategies have been preliminarily investigated previously. First, the simplest way to tune the viscoelasticity of hydrogels has been to change the total peptide concentration. For example, the elastic moduli of RAD16 peptide hydrogels were increased by increasing peptide concentration [131]. This response was hypothesized to originate from an increase in physical crosslinking
points among the fibrils. However, changing peptide concentration could affect local fibrillization, network structures, or other biophysical properties, making total peptide adjustment somewhat imperfect. In other approaches, the viscoelasticity of hydrogels has been tuned by attempting to specify the lateral aggregation of peptide fibrils. Lateral aggregation can be modulated by controlling the rate of folding and self-assembly, where faster assembly rates have led to increases in elastic modulus. For example, fast-gelling β-hairpin peptidomimetics produced about 12-fold stiffer gels than slow-gelling β-hairpin molecules [132, 133]. Lateral aggregation of fibrils can be also triggered by enzymatic dephosphorylation [134]. Upon addition of alkaline phosphatase, Fmoc-tyrosine (phosphate) peptides were assembled to form self-supporting hydrogels [134]. The elastic modulus of hydrogels was increased approximately 3.8-fold upon an increase in the enzyme concentration. Additionally, the viscoelasticity of hydrogels can also be modulated by ionic complexation or electrostatics. The viscoelasticity of the aforementioned β-hairpin peptide has been modulated by the physical cross-link points between the lysine residue and a boric acid/borate anion [135]. In related molecules, peptide amphiphiles (PAs), which possess a β-sheet folding domain as well as a lipid tail, mechanical properties have been tuned using different charge screening agents. The elastic modulus of hydrogels formed from PAs in the presence of divalent ions was
approximately 1.5-fold higher than those formed in the presence of monovalent ions [136]. Lastly, the viscoelasticity of hydrogels has been modulated by covalent crosslinking.

Of the methods available for modulating mechanical properties of synthetic scaffolds, NCL chemistry was selected for the work described in this thesis. NCL was originally developed for chemically synthesizing large polypeptides containing more than 300 residues from two or more unprotected synthetic peptide segments [137], which overcomes the typical ~50 residue size limit of conventional peptide synthesis [138]. The chemistry proceeds via the reaction of a C-terminal thioester and an N-terminal amine to form a native peptide bond. It is the formation of this native bond and the fact that the reaction occurs in aqueous solution, without solvents, that makes NCL highly attractive for cross-linking peptide hydrogels intended as scaffolds for biological applications. The production of the native bond reduces any immune response that could occur against a non-native peptide structure, and the aqueous chemistry allows the cross-linking to occur directly in the tissue culture dish. Further, the reaction is exceptionally chemoselective, and side reactions between amino acids other than the terminal reactive groups do not occur. This makes the chemistry compatible with any potential amino acid sequence that may be chosen to be incorporated within the scaffolds. For these reasons, NCL was chosen to modulate the
mechanics of Q11-based systems. Prior to the work described in this thesis, NCL had never been used to stiffen biomaterials scaffolds, peptide-based or otherwise, so the work reported in Chapter 3 also served to validate this chemistry for this purpose.

2.6.3 Release of a soluble factor, nitric oxide

In the two previous sections, strategies focusing on the modulation of cell-matrix binding and matrix mechanics were discussed. The mechanism of action of each of these strategies centers on interactions made directly between cells and the scaffolds. These strategies, however, may be limited in their effectiveness because they reduce these interactions to a small area. In addition, they could be prone to interference or fouling of the physically bound signals by processes such as protein adsorption. In order to overcome these limitations of directly immobilized signals, soluble factors were also incorporated into hydrogels using the modular Q11-based system.

Nitric oxide (NO) is synthesized and released locally and transiently [139], and it is maintained at low concentrations by ECs (the arterial or venous concentration of NO is 3-1000 nM [140], and that of nitrite (NO$_2^-$) is 100-500 nM [141]). NO regulates the balance between vessel constriction and dilation (vascular tone) [142], stimulates EC growth, and inhibits leukocyte and platelet
adhesions [143, 144] in an anti-inflammatory process. Proinflammatory stimuli such as tumor necrosis factor-α (TNF-α) downregulate endothelial NO synthase (eNOS), leading to decreased NO production [145, 146] and the recruitment of blood-borne leukocytes to inflamed tissues [147, 148]. Thus, in a given microenvironment, NO requires a tight control of its release. By virtue of the chemical specificity of the Q11-based self-assembling system, I hypothesized that the incorporation and release of NO molecules could be tightly regulated by accurately dosing in NO-releasing Q11 derivatives. The goal was to produce a Q11-derivative that could co-assemble with the other peptides developed (e.g. ligand-bearing and ligatable peptides), and that would predictably release NO. Such an approach would enable the addition of NO-releasing components into the scaffolds in a modular way. Recent research has demonstrated that NO-donor compounds such as \(N\)-diazeniumdiolates (NANOate) or \(S\)-nitrosothiols (RSNO) integrated in synthetic polymers spontaneously release NO at physiological conditions [149, 150] and can be used for localized delivery of NO by choosing different functional groups [151, 152] (Figure 2.4). However, it has been shown that some \(N\)-diazeniumdiolates polymers leach out of the polymer matrices and form potentially toxic nitrosoamine [153]. RSNO is known to be detected \textit{in vivo} as \(S\)-nitrosoalbumin (AlbSNO) [154] and is involved in the synthesis, transport, and bioactivity of endogenous NO [155-157]. Structures of low molecular weight
Figure 2.4. Diazeniumdiolate structures and possible release mechanisms.
RSNOs such as S-nitrosoglutathione (GSNO) and S-nitrosocysteine (CySNO) found in blood are shown in Figure 2.5. Although the release of NO from RSNO has more potential medical use owing to being involved in endogenous NO synthesis, the main limitation of both approaches is the finite reservoir of NO that exists within polymer matrices [158]. In order to overcome the limited source of NO, the immobilization of L-cysteine in polymeric surfaces enables utilization of endogenous NO in the form of RSNO and/or nitrates (NO$_3^-$) from circulating blood [159, 160]. In the experimental work detailed in Chapter 6, a NO-release peptide in the form of CysNO is integrated into co-assembled hydrogels. Practically, the preparation of RSNO with a given peptide or polymer matrix is simpler than that of NANOate in that mixing of cysteine containing matrices with sodium nitrite (NaNO$_2$) in low pH forms NO-conjugated matrices, as shown in Figure 6.1. In this way, Q11 derivatives capable of both releasing an initial pool of NO and concentrating endogenous pools of NO can be envisioned.

2.7 Design of Experiment (DOE)

Once a family of co-assembling, modular peptides has been synthesized, the challenge becomes how to efficiently explore the nearly limitless combinations of them that most effectively produce the biological response of interest, in this case the rapid formation of an endothelium.
Figure 2.5. Molecular structures of low molecular weigh RSNO.
To accomplish this, Design of Experiment (DOE) approaches were utilized. Conceptually, DOE is based on a “black box” model, as shown in Figure 2.3 [161]. In this model, several “factors”, or independent variables are under direct control, and any number of uncontrolled co-factors may also be present. These factors then take part in a process that ultimately generates the “output” or “response” to be measured, in this case the quantification of the growth of endothelial cells. In the work described in this thesis, the “factors” are each co-assembling peptide, particularly the ones that bear ligands. The uncontrolled factors are always present, but conditions can be maintained consistently to minimize any influence from them.

Once factors (inputs) and responses (outputs) are chosen according to the variables of interest and the biological processes that are desired, the next steps involve designing a set of experiments that can efficiently explore the influence of the factors on the output. There are many different experimental designs possible, but I have largely employed two of them: 1) screening and 2) response surface modeling [161]. The primary purpose of a screening experiment is to identify the factors that have the strongest influence on the response. In this way, important factors can be focused upon, and factors that have small or negligible effects can be eliminated to focus on more promising parameters.
Figure 2.6 Black box model.
From the three factors discussed in Chapter 2, matrix-mechanics, cell-matrix binding, and the release of nitric oxide (NO), the important main effects should be screened out to reach the objective (the rapid growth of endothelial cells). The response surface methods are designed to allow the estimation of interactions and the local shape of the response surface in order to find improved or optimal combinations of factors. In the DOE approaches, Factorial Experiments (FEs) and Response Surface Methodology (RSM) are utilized for screening and response surface objectives, respectively. In the following section, those two DOE approaches will be discussed.

To begin with, a few of DOE terminologies will be defined:

*Factors* are process inputs that an operator manipulates to cause a change in the output. As shown in Figure 2.6 some factors cannot be controlled by the operator but may affect the responses (co-factors).

*Factors* may have associated discrete values called *levels* of variation. Each process in Figure 2.6, has a definite combinations of factor levels.

*Effects* are defined as a change in the response produced by a change in a factor. The effect of a single factor is called a *main effect*. *Interactions* occur
when the effect of one factor on a response depends on the level of another factor(s).

A simplified way of mathematically expressing the relationship between the response and the factors is:

\[ y = \varphi(X_1, X_2, \ldots, X_k) \]  

(2.1)

where \( y \) is the response, \( X_1, X_2, \ldots, X_k \) are controllable factors, and \( \varphi \) is the process.

Analytically, this function can be expanded into a polynomial model,

\[ y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j + \sum \beta_{ii} X_i^2 \ldots \]  

(2.2)

where the \( \beta \)'s are regression or polynomial coefficients of each factor \( (X_1, X_2, \ldots, X_k) \).

From regression coefficient values, the factor effects can be estimated. Eq. 2.2 can be represented by a response surface in \( k \)-dimensional space. In search for an
optimum point, the response surface can be cut two-dimensionally to obtain a contour surface.

The most widely used factorial experimental type is called a two-level design. In this design, each factor is assigned a high and a low level. Even with multiple factors, the factor space can be covered efficiently by selecting only two levels. In Figure 2.7, each apex represents the level selected, covering the entire space. This is how FEs efficiently explore factor spaces without bias. In k-dimensionality, the number of two-level \((m=2)\) experiments becomes \(2^k\). Additional levels and factors can be added in subsequent experiments to obtain more local information in the factor spaces, but in this case the number of experimental runs increases exponentially. Thus, the most important initial considerations at the outset of a FE are the careful selection of factors, the selection of appropriate levels for each factor, and the selection of the most appropriate experimental design that can accomplish the research objective. As shown in Figure 2.5, the number of factor levels is related to the functional form of the responses. With two levels (high and low), only linear terms are left, yielding

\[
y = \beta_0 + \Sigma \beta_i X_i + \Sigma \beta_{ij} X_i X_j \tag{2.3}
\]
Figure 2.7 Factor space in two-level (high and low) Factorial Experiments.
where $y$ is the response and $\beta_0$, the intercept of the function (this is the response when main effects are 0). As discussed later, the results of FEs are estimated by the coefficients $\beta_i$ or $\beta_{ij}$ in Chapter 5.

Response surfaces, in contrast to FEs, have a curvature in their surface, meaning that any quadratic, cubic or quartic terms are present in the polynomial mode (eq. 2.2). The advantage of introducing higher-order terms is that the response function (eq. 2.1) will have a curvature, which allows us to estimate interactions and even curvature effects. RSM therefore gives us an idea of the local shape of the response surface of interest [161]. In order to generate higher order terms, a curve with more than two points is required, as depicted in Figure 2.8 [161].

In order for utilizing Response Surface Methodology (RSM), multiple factor levels are needed. A design method called central composite design (CCD) contains an embedded factorial design with center points that is augmented with a group of star points that allow estimation of curvature as shown in Figure 2.9. Generation of a CCD forms five factor levels $A, +, 0, -$, and $a$ throughout the factor spaces, enabling the addition of higher order terms in the polynomial model (eq. 2.2). The star points, which allow estimation of curvature, are the distance ($\alpha$) from the center of the design space to a factorial point. As depicted in Figure 2.9, the CCD is rotatable around its center point, implying that the variance of the
Figure 2.8 Possible behaviors of responses as functions of factor levels.
Figure 2.9 Generation of a Central Composite Design for two factors. A: highest axial value. 0: center point. a: lowest axial value. High or low levels from factorial design can be denoted as + or −, respectively.
predicted response at any point depends only on the distance of the point from the design center point. Rotatability is a desirable property for response surface designs [161]. The distance $\alpha$ is

$$\alpha = \left[ \text{number of factorial runs} \right]^{0.25} \text{ with } |\alpha| > 1$$

For a two-level and two-factor factorial ($2^2$), $\alpha = 2^{0.5} = 1.414$.

Since the factors (peptides) that will be dealt in this thesis have physical limitations, the physical limit of factor space should be recognized. One limit is 0 mM concentration. Such a limit poses a small problem, when moving from FE to RSM, as usually in this case the factor levels should be expanded. However, if the low levels are already 0, they cannot be made any lower. In this case, a modified version of CCD can be utilized. In Figure 2.10, a typical “circumscribed” CCD is shown, indicating the star points that fall outside the original levels. In contrast, an “inscribed” CCD design confines the factor space within the FE factor space. In this way, predetermined FE levels are kept for a subsequent RSM. This design circumvents the issue posed when one of the levels cannot be adjusted further, for example the 0 mM case described above.

It is theoretically possible to analyze up to the cubic term, but in general the higher order terms beyond quadratic ones are often omitted for computational
Figure 2.10 Comparison of Central Composite Design (CCD). CCD circumscribed vs. CCD inscribed. A: highest axial value. 0: center point. a: lowest axial value. High or low levels from factorial design can be denoted as + or –, respectively.
purposes and to minimize the complexity of analysis [162]. Therefore, the addition of quadratic terms to the linear model used in FEs (eq. 2.3) yields

\[ y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j + \sum \beta_{ii} X_{ii}^2 \]  

(2.4)

where \( y \) is a response, \( \beta_0 \) is the intercept of the function (this is the response when main effects are 0), \( \beta_i \) is the coefficient of each main factor (\( X_i \)), \( \beta_{ij} \) is the coefficient of two-way interactions (\( X_{ij} \)), and \( \beta_{ii} \) is the coefficient of quadratic terms (\( X_{ii}^2 \)) for suspected curvature.

### 2.8 Summary for Chapter 2

In this chapter, the physicochemical nature of self-assembly and self-assembling Q11 was discussed. Also, a description of \( \beta \)-sheet fibrillization as a potential utility for developing biomaterials with multifunctionality was described. With the demand for a precise control and systematic optimization of embedded functionality for endothelialization, three functionalities, cell-matrix binding, matrix mechanics, and release of nitric oxide were described, and previous studies for utilizing each functionality was outlined. The next four chapters detail the experiments for utilizing the modular self-assembling peptide Q11 with different
functionalities and their optimization for improving the growth of endothelial cell growth.
Chapter 3

Modulating the mechanical properties of

self-assembled peptide hydrogels

3.1 Introduction

Stiffening hydrogels. Self-assembling hydrogels are constructed from peptide fibrils, which are primarily composed of non-covalent bonds. The non-covalent nature of self-assembly is useful for modular construction of materials, but the unfortunate consequence is that the relatively poor mechanical properties of self-assembling hydrogels limit their application within tissue engineering or cell culture. In this chapter, a novel strategy is described for increasing the stiffness of β-sheet fibrillar hydrogels in a manner that is chemoselective, that maintains fibril morphology, and that produces native peptide bonds between self-assembled peptides. This work was published in Jung et al., Biomaterials 29, 2143-2151 (2008) [85].

For a mammalian cell, the most common attachment site is another cell or the ECM, and these materials have elastic moduli spanning several orders of
magnitude, from around 10-100 Pa for the softest tissues such as brain, up to the MPa range for stiff tissues such as cartilage [163]. Forces generated by cytoskeletal motors and applied through membrane attachment sites can stiffen or deform materials further. The morphology of cells and the signaling related to cell morphology can strongly depend on the stiffness of cell scaffolds. The stiffness of any synthetic or natural scaffolds profoundly alter cell behaviors both in vivo and in vitro (for reviews, see [127, 164]). As discussed in Chapter 2.6.2, matrix stiffness significantly affects differentiation, spreading, proliferation, and metabolism [127-129, 166], so controlling matrix stiffness is critically important for directing cell behaviors appropriately when applying these materials as 3-D cell culture matrices or as biomaterials for tissue engineering. For biomedical applications, synthetic scaffolds must withstand laboratory handling in cell cultures or forces generated by placement and normal functioning in tissue sites.

Considering both the biological importance of matrix stiffness and the practical issues for implantation of self-assembling materials, non-covalent interactions and covalent crosslinking methods were discussed as strategies to improve the mechanical properties of these materials in Chapter 2.6.2. They include increasing the concentration of peptides, tuning the lateral aggregation of peptide fibrils, modifying the ionic strength, and covalent crosslinking. In Chapter 3, a novel method for rapidly increasing the stiffness of self-assembled β-
sheet fibrillar peptide hydrogels using native chemical ligation (NCL) is investigated. NCL, as discussed in Chapter 2.6.2, is a chemoselective approach that has been widely utilized for joining unprotected peptides, one with an N-terminal cysteine residue and with a C-terminal thioester [137]. For several chemical and biological reasons, it was hypothesized that this reaction would be advantageous for stabilizing peptide hydrogels for use as 3-D culture matrices or as scaffolds for regenerative medicine. First, because NCL is carried out in aqueous conditions at neutral pH with unprotected peptides, crosslinking would be able to take place directly in water-swollen gels. Second, crosslinking would be achieved through the formation of native peptide bonds between the sulfhydryl (SH) functional group of cysteine residue and thioester, which would not be expected to significantly disrupt the self-assembled fibrils and would avoid non-native structures that could potentially increase the materials’ immunogenicity. Third, the reaction is chemically specific, allowing for the incorporation of additional amino acid sequences within the matrix (e.g. cell-binding sequences, substrates for proteolysis, growth factor binding sequences, etc.).

In this work, I designed and investigated a hydrogel-forming system of peptide α-thioesters with N-terminal cysteine residues based on an amino acid sequence that forms β-sheet fibrillar networks, Q11 (Ac-QQKFQFQFEQQ-Am) [44, 52]. The objective of the experiments was to improve the stiffness of non-
covalently assembled Q11 hydrogels and to test the effect of increased stiffness on EC growth. Furthermore, I non-covalently incorporated a cell-attachment ligand, RGDS (in the form of RGDS-Q11) to investigate any potential additive or synergistic effects from both functionalities (matrix mechanics and cell-matrix binding) on EC growth. The ligation chemistry, Q11-based self-assembly and combining matrix mechanics with cell-attachment ligands are described in Figure 3.1.

3.2 Materials and methods

**Synthesis of peptides and peptide thioesters.** In order to produce peptides capable of first self-assembly, then undergoing NCL, I needed to design and synthesize a peptide thioester. Peptide synthesis reagents were purchased from NovaBiochem. Peptide Q11 (Ac-QQKFQFQFQQ-Am, \(m/z\) calc’d: 1527.7; found: 1527.7) was synthesized on a 0.25 mmol scale using a CS Bio 136 automated peptide synthesizer on Rink amide AM resin using standard Fmoc protocols and activation with 1-hydroxybenzotriazole (HOBt) and 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU). All peptides were double-coupled and cleaved (deprotected) with 95:2.5:2.5 TFA:triisopropylsilane (TIS):H\(_2\)O. Peptides were precipitated and washed several times with cold diethyl ether, dissolved in water, lyophilized on a Labconco.
Figure 3.1 Schematic of co-assembling peptides for controlling matrix mechanics (CQ11G-thioester) and enhancing cell adhesion (RGDS-Q11) (top, reproduced from Ref. [14], with permission).
times with cold diethyl ether, dissolved in water, lyophilized on a Labconco freeze drying system, and stored as lyophilized powders below -20ºC. CQ11G-thioester (Cys-(Q11)-Gly-COSR, R=CH₂CH₂CO₂C₂H₅) was synthesized on a 0.1 mmol scale with standard Fmoc protocols using H-Gly-Sulfamylbutyryl NovaSyn® TG resin (NovaBiochem Cat# 04-12-3714) and Boc-Cys(Trt)-OH. Following the synthesis, the peptidyl resin was activated with 1 M trimethylsilyldiazomethane in 1:1 THF:hexane for 2 h, washed with THF, and cleaved using ethyl-3-mercaptopropionate (50 eq.) and sodium thiophenolate (0.5 eq) in DMF. The resin was removed by filtration and washed three times with DMF, and the DMF was removed by rotary evaporation. The fully protected peptide ([M + K]⁺ calc’d: 3753.5; found 3756.5) was precipitated in cold diethyl ether and stored as a dry powder below -20ºC until deprotection and experimentation. RGDS ligand-bearing peptide, RGDS-Q11 (Ac-GGRGDSGGG-(Q11)-Am, m/z calc’d: 2227.3; found 2227.6), and its scrambled sequence, RDGS-Q11 (Ac-GGRDGSGGG-(Q11)-Am, m/z calc’d: 2227.3; found: 2227.0), were synthesized and cleaved with the same protocol of Q11.

**Hydrogel formation, ligation reactions, and disulfide formation.** For measurement of viscoelasticity and cell cultures, hydrogels were formed from peptide solutions. Q11 hydrogels were formed by dissolving 30-35 mM peptide in water and layering Dulbecco’s phosphate buffered saline (PBS, 0.2 g/L KCl,
0.2 g/L KH$_2$PO$_4$, 8 g/L NaCl, 1.15g/L Na$_2$HPO$_4$) over the peptide solution. With careful pipetting, mixing of the layers could be avoided, allowing the peptide layer to form a hydrogel under the PBS layer by diffusion of buffer constituents into the peptide layer. For CQ11G-thioester, ligation polymerization was prevented until experimentation by storing it as a fully protected peptide. Immediately prior to experimentation, CQ11G-thioester was deprotected for 1 h with 93.5:2.5:2:2 TFA:H$_2$O:TIS:EDT (1,2-ethanedithiol) precipitated in cold diethyl ether, and washed five to six times with ether. The fully deprotected peptide thioester ($m/z$ calc’d: 1763.0; found: 1762.2) was soluble in water up to concentrations of at least 35 mM. In aqueous solutions of the peptide thioester, ligation was significantly inhibited by low pH (pH 2-3), which most likely arose from residual TFA. To accelerate rapid ligation, ligation buffer (20 mM tris (2-carboxyethyl) phosphine (TCEP), 200 mM mercaptophenylacetic acid (MPAA) in PBS, pH 7.4) was layered on top of the peptide solution. To induce disulfide bond formation after self-assembly and ligation in CQ11G-thioester gels, the gels were incubated under 100 mM iodine in PBS, pH 7.4. Penetration of the colored iodine solution into the gel was assessed visually and took between 40 min and 1 h, depending on geometry. The gels were then washed in PBS until the iodine diffused from the gels by visual inspection, about 1 h, and complete oxidation was verified using Ellman’s reagent.
**Monitoring the course of ligation polymerization.** In order to verify the status of oligo- or poly-merization from NCL, the products from various time points were analyzed with SDS-PAGE and mass spectrometry (MS). SDS-PAGE identified approximate molecular weights on the order of 1-10 kDa and MS gave exact molecular weights of species. After ligation of CQ11G-thioester gels for time periods between 5 s and 2 h, the ligation buffer was removed and reactions were terminated by adding 1 M iodoacetamide for quenching NCL (by covalently binding to SH) and vortexing vigorously to fragment the gel and allow rapid iodoacetamide penetration. Samples were diluted to 0.43 mM in SDS-PAGE sample buffer (2% SDS, 40% glycerol, 0.25 mg/mL bromophenol blue, 8 M urea in 200 mM Tris buffer, pH 6.8), in which the presence of 8 M urea prevents non-covalent peptide oligomerization [169]. Samples were analyzed on 10-20% Tris-tricine gels (Biorad), which were then fixed with 40% methanol and stained with Coomassie G250. Polymerization products were also analyzed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry on a Bruker Biflex III instrument using α-cyano-4-hydroxycinnamic acid (α-CHCA) as the matrix. A pipette tip was used to remove small pieces of gel, which were mixed vigorously with matrix and spotted onto the MALDI plate. Dehydration of the spot on the plate effectively stopped ligation reactions, so iodoacetamide was not used in MALDI analyses.
**Measuring viscoelasticity of hydrogels.** Bohlin Gemini rheometer (Malvern Instruments, Worcestershire, UK) was utilized to measure gel viscoelasticity. As depicted in Figure 3.2, the top plate of the rheometer rotates and measures viscoelasticity simultaneously, while the bottom plate is fixed and maintains sample temperature. Oscillating rheometry measures elastic and viscous properties of materials, which are quantified as storage ($G'$) and loss ($G''$) moduli, respectively. To produce gels of consistent geometry, a glass fiber filter paper template with an 8 mm hole was centered on the bottom rheometer plate. This template was pre-saturated with different buffers, and 80 µL of peptide solution was pipetted into the template’s central hole. To Q11 gels, PBS or PBS supplemented with 500 mM NaCl were added. It was hypothesized that additional salt would increase the strength of self-assembly. To CQ11G-thioester gels, ligation buffer was added. Additional buffer was then gently layered over the peptide solution in excess, and gelation was allowed to progress for 40 min. After gelation, the filter paper template was removed, leaving a cylindrical gel on the lower plate. For oxidized gels, an additional step of incubation under a layer of 100 mM iodine in PBS for 45 min was added. Complete oxidation of gels under similar conditions was verified with Ellman’s reagent. To prevent evaporation, these steps were performed in a humidified chamber. After gelation and removal of the filter paper template, the upper plate (parallel plate, 8 mm
Figure 3.2 Schematic of oscillating rheometry; measuring viscoelasticity.
diameter) was lowered until it was in conformal contact with the top surface of
the hydrogel. This corresponded to gap distances of 1,010-1,500 µm with
standard deviations of each group of gels being less than 100 µm. Storage moduli
and loss moduli were measured at 0.1% strain, the temperature was maintained at
25°C, and at least three independent gels were measured for each formulation.

**Assessment of fibril morphology.** The linear morphology and thickness
of Q11 and CQ11G-thioester fibrils were assessed and compared by utilizing
transmission electron microscopy (TEM), which can visualize from nano-
to micron-scale objectives. Stock solutions of 0.5 M peptide in water were prepared
and mixed 1:2 with PBS (Q11) or ligation buffer (CQ11G-thioester), vortexed,
sonicated, and incubated overnight at room temperature. Oxidation of peptide
fibrils was achieved by adding 100 mM iodine in PBS, pH 7.4, for 1 h. Samples
were then applied to 200 mesh lacey carbon grids (Electron Microscopy Sciences),
stained with 1% uranyl acetate for 2 min, and analyzed immediately using a JEOL
1230 TEM (JEOL Ltd., Tokyo, Japan).

**Assessment of secondary structures of peptides.** Along with fibril
morphology, the secondary structures of Q11 and CQ11G-thioester peptides were
assessed by circular dichroism (CD) spectroscopy. The secondary structure is
critical to proceed self-assembly and subsequent fibrillization of peptides. An
AVIV 215 circular dichroism spectropolarimeter (Aviv Biomedical, Lakewood,
NJ) was used with 0.1 cm path length quartz cells. To eliminate any aggregates formed during storage, Q11 was solubilized and disaggregated in a small amount of TFA for 15 min immediately before CD experiments. CQ11G-thioester was deprotected immediately prior to experimentation. Peptides were then precipitated with cold diethyl ether, collected via centrifugation, washed an additional eight times with ether, and dried under a stream of nitrogen. Stock solutions of 1 mM peptide at pH 7 were prepared in degassed water, and peptide concentrations were determined by phenylalanine absorbance at 257 nm. Owing to the strong UV absorbance of MPAA, it could not be used in buffers for CD analysis, and chloride-containing buffers were avoided to minimize CD signal diminishment. Instead, samples were diluted to a working concentration of 0.3 mM and 0.5 mM in 4.3 mM KH$_2$PO$_4$, 1.4 mM Na$_2$HPO$_4$·7H$_2$O, 140 mM KF, pH 7, in which ligated dimers and small amounts of trimers were verified by MALDI-TOF-MS after overnight incubation. Triplicate scans were averaged at 25°C. In these conditions, I observed adequate signal strength and PMT dynode voltage values less than 500 V at wavelengths greater than 185 nm.

**Endothelial cell culture.** For potential applications of hydrogels as synthetic scaffolds, endothelial cells (ECs) were cultured on the ligated and self-assembled hydrogels. With the motivation of achieving rapid and functional endothelialization on synthetic surfaces, I started using primary human
endothelial cells over other cell types. HUVECs are highly characterized and have served as an excellent model for endothelium research [170]. Like most other cell types, however, they can lose their EC characteristics in prolonged culture [171], accordingly I limited the HUVEC subcultures to 15 population doublings. HUVECs, EGM-2 medium, and subculture reagents were purchased from Lonza. Prior to seeding onto peptide gels, HUVECs were maintained in EGM-2 medium at 37°C/95% relative humidity/5% CO₂ in T-75 flasks coated with 5% gelatin (Fisher Cat# G7-500) and subcultured according to the supplier’s protocols. Gels were produced by pipetting 60 µL of 30 mM peptide in water into 24-well culture inserts (0.4 µm pore size, 1.13 cm² area, Fisher Cat# PICM-01250). The plates were sealed and these solutions were maintained at 4°C overnight. This incubation step increased the viscosity of the peptide solution such that subsequent buffer addition did not disrupt the gel’s surface. I gently added PBS into the outer and inner chambers, inducing further assembly and producing a uniform, translucent gel about 500 µm thick. For CQ11G-thioester gels, I added ligation buffer instead of PBS, aspirated it after 40 min, and washed the ligated gel with sterile PBS at least 15 times over a 24-hour period to completely remove TCEP and MPAA. These gels were stable at 4°C for at least three days. To seed cells, the PBS was replaced with EGM-2 medium, and HUVECs were seeded on top of the gels at a density of 8,850 cells·cm⁻². After 64
h of incubation, MTS assay (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, Promega Cat# G3582) was performed according to the manufacturer’s protocol and fit to a standard curve constructed with known quantities of HUVECs. This time point was selected to reduce the number of medium change down to once over 3 days and to culture HUVECs up to the point of which the number of HUVECs was not overly confluent, especially on ligated gels. Metabolically active cells can reduce MTS to water soluble formazan salts, which change the color of cell culture media. The absorbance was read on a Molecular Devices SpectraMax M2 microplate reader at 490 nm after 4 h of development. For immunofluorescence staining, cultures were fixed with 2% paraformaldehyde, permeabilized with 0.1% Triton X-100, and blocked with 10% goat serum for one hour. The primary antibody was anti-human PECAM mouse IgG1 (R&D Cat# BBA7, 1:100 dilution), and the secondary antibody was AlexaFluor 488 goat anti-mouse IgG1(γ1) (Invitrogen Cat# A21121, 1:200 dilution). Cultures were counterstained with 1 μg/mL DAPI.

**Statistical analysis.** Proliferation data were analyzed by one-way ANOVA with Tukey’s HSD post-hoc comparisons. Results are reported as mean ± standard deviations. p-values <0.01 were considered significant.
3.3. Results and discussion

Ligation leads to gel stiffening approximately five-fold. Both Q11 and CQ11G-thioester were synthesized in good yield and purity. The peptide Q11 formed gels as previously described when dissolved in water and incubated under a layer of PBS [44, 52]. The storage modulus (G') of 30 mM (4.6% w/v) Q11 gels prepared in this manner was 10.5 kPa at 1 Hz (Figure 3.3a), and the storage modulus of 5 mM (0.76% w/v) Q11 gels was 1.2 kPa (Figure 3.3b). For clarity, all moduli are reported in this section at 1 Hz unless otherwise specified. The G' values observed for Q11 were consistent across replicate samples and were comparable to gels formed by other self-assembling peptides [36, 174, 175] and peptide amphiphiles [176] at similar concentrations and in similar conditions. In contrast, 30 mM CQ11G-thioester gels ligated for 40 min under ligation buffer had a storage modulus of 48.5 kPa, a nearly five-fold increase over 30 mM Q11 (Figure 3.3a), and ligated gels of 5 mM CQ11G-thioester had a storage modulus of 9.1 kPa (Figure 3.3b), a sixfold increase over 5 mM Q11. All peptides displayed rheological properties consistent with hydrogels, including frequency insensitivity, loss tangent values (\(\tan \delta = G''/G'\)) near 0.1, and no crossing of G' and G'' at any measured frequency. Loss tangent values were also relatively independent from concentration or ligation (\(\tan \delta \) was 0.135 for 30 mM Q11 gels, 0.105 for ligated 30 mM gels, 0.125 for 5 mM Q11 gels, and 0.113 for ligated
Figure 3.3  Viscoelasticity behavior of ligated CQ11G-thioester (G’ ●; G”○) and Q11 (G’ ■; G”□). Peptide concentration in the gels was 30 mM (a) or 5 mM (b); n=3, mean±SD).
5 mM gels). These similar tan δ values indicated that network connectivity did not likely change significantly upon ligation. It is possible that the slight decrease in tan δ for the ligated samples arose from a slightly higher cross-link density [177], however, changes in other aspects such as fibril morphology, fibril stiffness, or lateral aggregation could also have produced this effect. In prior work, adjustment of peptide concentration has been a significant means for modulating the stiffness of self-assembled peptide matrices [131]. By employing NCL, G′ could be adjusted independently of peptide concentration, a property that is advantageous for studying how matrix stiffness affects cell behavior without confounding stiffness with other experimental variables. Most importantly, however, the use of NCL to stiffen peptide matrices enables the production of gels with G′ in the range of 10-50 kPa. This stiffness is reflective of highly collagenous soft tissues but has not previously been accessible in fibril-forming peptide matrices, so the present strategy broadens the utility of self-assembled peptide biomaterials towards the engineering of such tissues.

**Oligomerized species formed upon NCL.** SDS-PAGE and mass spectrometry were utilized to investigate the molecular species responsible for gel stiffening. To prevent premature ligation, CQ11G-thioester was stored in its fully protected state and deprotected immediately before experimentation (see 3.2 Hydrogel formation). Both Q11 and CQ11G-thioester were soluble in water up to
at least 50 mM, and the initial pH of these solutions ranged from pH 2 to pH 4, presumably a result of residual TFA. Owing to the pH-dependence of NCL [137, 178, 179], ligation was almost completely inhibited in initial solutions of CQ11G-thioester. When CQ11G-thioester peptide solutions were pipetted into flip-top tubes and overlaid with PBS containing 200 mM MPAA and 20 mM TCEP, peptide trimers formed within seconds and polymerization proceeded to at least hexamers (78 amino acids) by 48 min, as observed with SDS-PAGE (Figure 3.4a). Identically treated Q11 control samples (left) did not demonstrate these higher molecular weight bands, indicating that the polymerization bands observed for CQ11G-thioester likely represented covalent polymers as opposed to non-covalent aggregates. Moreover, ligation reactions were performed in the presence of 20 mM TCEP, and polymerizations were terminated with iodoacetamide, both of which should eliminate disulfide bond formation during the reaction and analysis, respectively. For both Q11 and CQ11G-thioester, the smallest peptide in each lane ran with an apparent molecular weight of about 3.5 kDa, which is larger than the true molecular weight of the monomeric peptides (1.5 kDa for Q11 and 1.7 kDa for CQ11G-thioester). Such deviations in electrophoretic mobility are common for short peptides [165]. A number of reports have indicated that 8 M urea prevents multimerization of similar fibrillizing peptides such as Aβ fragments [169, 180, 181], but these peptides migrate anomalously owing to
Figure 3.4  SDS-PAGE (a) and MALDI-TOF mass spectrometry (b) indicated that trimers formed by 5 s, with species up to hexamers produced by 36 min. SDS-PAGE showed the persistence of dimer, and MALDI indicated that this dimer band was a mixture of 26-amino acid linear thioesters and 26-amino acid cyclic peptides.
variations in SDS binding [169] or peptide conformation [180]. Given the similar conditions used here and the absence of a multimeric ladder for Q11, it is likely that the slightly slower migration of these peptides was likewise caused by variable SDS binding or by conformational effects. During the first few minutes of the reaction, the monomer predominated, but by 12 min the monomer band began to diminish and the dimer band became the predominant species. This dimer continued to predominate through 48 min as trimers through hexamers were also formed. It was hypothesized that the persistent dimer was attributable to the formation of a 26-amino acid cyclic peptide composed of two CQ11G peptides, which would be favored by the anti-parallel arrangement of Q11 strands suggested by FTIR data in previous studies [44] and would terminate polymerization. To investigate this aspect, I utilized MALDI-TOF mass spectrometry (Figure 3.4b), which at 2 h of reaction time showed the presence of monomer ([M + H]$^+$ calc’d: 1763.0; found: 1764.4), dimer ([M + H]$^+$ calc’d: 3390.8; found, 3394.7), cyclic dimer ([M + H]$^+$ calc’d: 3256.6; found 3254.4), trimer ([M + H]$^+$ calc’d: 5018.6; found: 5022.5), and cyclic trimer ([M + H]$^+$ calc’d: 4884.4; found: 4881.0). Polymerization products and cyclic peptides were distinguishable from non-covalent aggregates because peptides polymerized via NCL would possess only one thioester of ethyl-3-mercaptopropionate (134.2 Da), whereas non-covalent aggregates or disulfides of unpolymerized peptides would
be expected to possess more than one of these groups. Similarly, the \( m/z \) ratios of peaks attributed to cyclic peptides did not reflect the presence of any thioesters. However, peaks identified as cyclic species could possibly represent disulfides, for example symmetric disulfides of cyclic monomers ([M + H]\(^+\) calc’d: 3256.6) or mixed disulfides between a cyclic monomer and a cyclic dimer ([M + H]\(^+\) calc’d: 4882.2). The presence of these species is possible but not likely given the absence of any cyclic monomer peak ([M + H]\(^+\) calc’d: 1627.8) and the presence of 20 mM TCEP in the reaction buffer. Smaller amounts of tetramer, pentamer, and hexamer were also observed when detection of lower molecular weight species was suppressed. A detailed measurement of molecular weight distribution (e.g. by GPC-MALS) is challenging for self-assembling peptides owing to their propensity to aggregate in standard mobile phases, but by using SDS-PAGE and MALDI-TOF mass spectrometry, it can be concluded that polymerization proceeds to at least hexamers (78 amino acids) and that the extent of polymerization is modulated by the formation of cyclic species. In this way, polymerization proceeds to an extent that increases the stiffness of the gels, but not to such a degree that the peptides precipitate.

**No significant contribution to viscoelasticity of hydrogels from ionic strength and disulfide bonds.** Experiments aimed at studying the effect of ionic strength and disulfide bond formation were performed to determine the extent to
which these aspects contribute to matrix stiffening in Q11-based gels. To assess the impact of ionic strength, I compared the viscoelastic properties of Q11 gels incubated under the same buffer as used for ligation (PBS containing 200 mM MPAA and 20 mm TCEP) and under PBS supplemented with NaCl (500 mM total). These buffers stiffened the matrix to 23 kPa and 25 kPa, respectively (Figure 3.5a), indicating that increased ionic strength can account for as much as 35% of the total increase in $G'$ observed for NCL-stiffened gels. Similar degrees of stiffening by increased ionic strength have been reported for other β-sheet forming peptides as well [41, 174]. However, a significantly smaller degree of stiffening is achievable with buffers of high ionic strength alone compared to NCL, and high ionic strength cannot be maintained in cell culture or for in vivo applications. In addition to investigating ionic strength, I investigated whether oxidation of the N-terminal Cys residues on CQ11G-thioester impacted the gel's mechanical properties. This aspect is important given that some degree of disulfide bond formation is expected in cell culture conditions. Cysteine oxidation had no effect on the viscoelasticity of ligated gels (Figure 3.5b), even with complete oxidation of all cysteine residues to disulfides as verified with Ellman's reagent. Collectively, these experiments indicated that the oligomerized peptide species produced via NCL contributed to most of the observed increase in $G'$ and that the accumulation of disulfide bonds over time in culture is not likely
Figure 3.5 Contribution of ionic strength (a) and Cys oxidation (b) to matrix viscoelasticity. Ligated CQ11G-thioester gels (G′ ⋄; G″ ○), Q11 gels in ligation buffer (G′ ▽; G″ ▼) and Q11 gels in PBS with 500 mM NaCl (G′ □; G″ △) (a). Ligated CQ11G-thioester (G′ ⋄; G″ ○) and ligated/oxidized CQ11G-thioester (G′ ▽; G″ ▼) (b). The concentration of all gels was 30 mM; n=3, mean ± SD.
to contribute to changes in the viscoelastic properties of the matrix.

**Ligation does not significantly disrupt fibrillization or secondary structures.** TEM with negative staining indicated that ligated CQ11G-thioester peptides formed fibrils (Figure 3.6). CQ11G-thioester peptides that had been assembled, ligated, and oxidized also formed fibrils, and both oxidized and unoxidized ligated fibrils shared a number of morphological features with Q11 fibrils. Fibrils of all groups were between 11 nm and 13 nm wide, and individual fibrils several hundred nanometers long were observed for each group. In addition, uranyl acetate preferentially stained the fibril periphery in all groups, producing dark edges and leaving less electron-dense fibril cores. The most significant difference between the morphologies of unligated and ligated fibrils was that Q11 fibrils appeared somewhat more laterally aggregated than ligated fibrils (Figure 3.6a); however, it is difficult to assess network connectivity or lateral aggregation in gels from negative-stained TEM samples because they have been adsorbed and dried onto the TEM grid. A more detailed measurement of the spatial arrangement of fibrils in the hydrogels could be provided with additional techniques such as cryo-TEM, but it is nevertheless clear from negative-stained TEM samples that the peptides retained the ability to fibrillize after ligation and oxidation, and the major morphological features of the fibrils remained similar.

Circular dichroism (CD) spectroscopy was utilized to evaluate secondary
Figure 3.6 Transmission electronic microscopy of Q11 (a), ligated CQ11G-thioester (b), and ligated/oxidized CQ11G-thioester (c).
structural changes induced by ligation. Q11 displayed a minimum at 224 nm and a maximum around 205 nm, attributable to β-turn structure (Figure 3.7) [183, 184] and consistent with the behavior of Q11 reported previously [44]. CD spectra of Q11 were independent of concentration in the range tested (0.3-0.5 mM). Upon ligation, CD spectra displayed small differences compared to Q11. The long-wavelength minimum shifted slightly to 222 nm and became more pronounced, particularly at 0.5 mM peptide concentration. The maximum at 205 nm remained, but at lower intensity. The most significant change induced by ligation was the appearance of a new maximum at 190 nm. This band may be attributed to β-sheet structure and is similar to the dichroic maxima exhibited by the “all-β” proteins superoxide dismutase and prealbumin [183]. This assignment is not unambiguous in the absence of additional analyses such as FTIR, but the bands at 205 nm and 222 nm and the fibrillization behavior observed by TEM make it likely that this peak arises from β-sheet structure. This additional β-sheet content by CD may be contributed by the cyclic peptide observed in SDS-PAGE and mass spectrometric analyses, or it may arise from longer β-sheet segments between turns that would be produced by the ligation of adjacent β-sheet strands. Collectively, TEM and CD indicated that fibril morphology is subtly but not significantly altered by ligation polymerization, and the structure remains predominantly β-sheet.
Figure 3.7 Circular dichroism of ligated CQ11G-thioester (0.3 mM ■; 0.5 mM □) and Q11 (0.3 mM ▼; 0.5 mM ▽).
Gel stiffening significantly enhances HUVEC growth. Growth of HUVECs was slow on Q11 gels, and less than half of the originally seeded cells remained attached to the gels by the third day in culture (Figure 3.9). This poor ability of Q11 to support HUVECs on its gels' surfaces was possibly a result of cohesive failure in the gel leading to cell detachment, or it may have arisen from a lack of engagement of integrins or other cell attachment proteins. Despite Q11's otherwise useful property of forming chemically defined self-assembled gels in culture medium, this aspect has previously hindered its use as a defined nanostructured coating for supporting cells. In contrast, ligated CQ11G-thioester gels supported significantly higher rates of HUVEC growth and proliferation. By 72 h, HUVECs on ligated gels were nearly confluent and expressed the cell–cell adhesion protein PECAM (platelet endothelial cell adhesion molecule) (Figure 3.8a and b). Cells on ligated gels adopted a cobblestone morphology by phase microscopy (Figure 3.8c) and expressed PECAM at cell-cell contacts (Figure 3.8a). PECAM (CD31) is an important mediator of wound healing, angiogenesis, and inflammatory processes, and its expression at cell-cell contacts is expected in normally functional endothelial cells [185]. Cells on unligated Q11 gels were in contrast more spindle-shaped, significantly less confluent, and did not consistently express PECAM at cell-cell contacts (Figure 3.8b and d). Quantification of cell growth by MTS assay indicated that HUVECs on ligated
Figure 3.8 PECAM expression was greater at 72 h in culture for HUVECs on ligated CQ11G-thioester (a) than on Q11 (b); green, PECAM; blue, DAPI. HUVECs were nearly confluent on CQ11G-thioester (c), but spindle-shaped and sparse on Q11 (d).
Figure 3.9 HUVEC proliferation at 64 h. *p < 0.01, ANOVA with Tukey's HSD post-hoc test compared to Q11; **p < 0.01, ANOVA with Tukey's HSD post-hoc test compared to all others; n=4, mean ± SD.
gels are more than 150% of their originally seeded number of cells by 64 h, whereas fewer than 50% of the originally seeded cells remained on unligated Q11 gels (Figure 3.9).

**RGDS and ligation have additive effects on HUVEC growth.** In addition to this enhancement in growth over time produced from ligation polymerization, the chemoselectivity of this approach was demonstrated by incorporating an RGDS-functionalized Q11 (RGDS-Q11) peptide into the ligated gels. Enhancement of HUVEC growth significantly above the level induced by ligation alone and abrogation of this response with scrambled RDGS-functionalized Q11 (RDGS-Q11) indicated that the RGDS sequence was functionally presented by the ligated gels and was not compromised by the ligation chemistry (Figure 3.9). This result is important for the development of Q11-based gels with additional functionality. Since it is not certain whether this phenomenon arises from specific engagement of cell-matrix mechanotransduction [127, 164, 186] or whether this is simply attributable to a reduction in cohesive failure of the material that would otherwise lead to cell detachment, additional work is required to determine the increase of HUVEC growth from ligated gels.

3.4 Conclusions
This work provides a means for stiffening self-assembled peptide hydrogel culture substrates in a way that maintains the advantageous chemical definition and modularity inherent in self-assembling systems. Reactions were aqueous and chemically specific, and polymerization was self-limited by the formation of cyclic oligomers, so the peptides did not grow to such molecular weights as to become insoluble. In addition, stiffening by NCL could be achieved at multiple peptide concentrations, effectively decoupling matrix elasticity from peptide concentration. Ligation of the gels significantly improved HUVEC growth over time, and the chemoselectivity of NCL was capitalized upon to produce gels with added biofunctionality, as illustrated through the incorporation of RGDS-Q11 and its additive enhancement of HUVEC growth.

Matrix mechanics is a non-specific stimulus [187, 188], but the impact on cell behaviors was significant enough to drive faster growth of HUVECs in the self-assembling system described here. For specific interactions between cells and synthetic scaffolds (matrices), variants of self-assembling Q11 peptides with appended ECM-derived ligands (RGDS, IKVAV, YIGSR, and REDV, see Section 2.6.1) were required. In Chapter 4, the functional presentation of such bioactive ligands and the effect of ligand presentation on cell behaviors were tested.
Chapter 4

Co-assembling ligand-bearing peptides as defined matrices for endothelial cells

4.1 Introduction

Cell-matrix binding via ECM-derived ligands. The experiments in Chapter 3 demonstrated that native chemical ligation introduces covalent crosslinks into self-assembled peptide hydrogels. Ligation increased the stiffness of self-assembled peptide hydrogels and enhanced HUVEC proliferation over time. Interestingly, the mechanism governing enhanced HUVEC growth was non-specific, as the increased stiffness of self-assembling peptide hydrogels crosslinked via native chemical ligation did not modulate specific intracellular signaling pathways [189]. For this reason, I hypothesized that self-assembled hydrogels presenting ECM-derived peptides would mediate cell adhesion via integrin receptors [190], and provide a specific mechanism to modulate HUVEC behavior.
Cell attachment to the ECM is mediated by specific interactions between cell surface receptors and biomolecules present throughout the matrix. For example, integrin receptors bind to ligands present within ECM proteins, such as fibronectin and laminins. In turn, integrin-ligand binding initiates intracellular signaling cascades that are involved in directing cell growth, survival, differentiation, and apoptosis [190-192]. A key challenge in engineering biomaterials as synthetic scaffolds is establishing spatial and temporal control over cell attachment and signaling cascades to enhance cell growth in vitro or aid natural healing processes in vivo. To date, numerous ECM-derived ligands have been incorporated into biomaterials to modulate cell behavior, and these examples have been discussed in Section 2.6.1. Of interest for this work are the peptides RGDS and REDV derived from fibronectin, and IKVAV and YIGSR derived from laminin, as they represent the minimal amino acid sequence required to modulate cell behavior analogous to the ECM protein from which they are derived. The advantage of using a peptide, rather than the ECM protein itself, is related to maintaining the biofunctionality of the ligand upon incorporation into a biomaterial. ECM proteins can be anchored or physically adsorbed to synthetic scaffolds or substrates. However, these processes often influence protein folding, which can alter ligand orientation and decrease ligand functionality. The functionality of short peptides, on the other hand, is typically not dependent on
folding and, as such, peptides are less susceptible to loss of functionality when incorporated into a biomaterial. Additionally, synthetic peptides can readily be produced in large quantities using established synthetic methods [193].

The key advantage of the self-assembling peptide Q11 is that the synthesis can be modified to incorporate a short, biofunctional peptide onto the N-terminus, without adversely affecting self-assembly via the Q11 domain. For example, results presented in Chapter 3 demonstrated that RGDS-Q11 self-assembles via non-covalent interactions between Q11 domains to form scaffolds. Additionally, CQ11G-thioester and RGDS-Q11 peptides were co-assembled by simple mixing of the two peptides to enhance hydrogel stiffness and introduce the integrin receptor-ligand, RGDS. From a recent report, in contrast, incorporating RGDS, IKVAV, and YIGSR to crosslinked poly(ethylene glycol) (PEG) hydrogels affected stiffness, swelling ratio, mesh size, and diffusion of bovine serum albumin (BSA)[194]. In this chapter, this approach has been extended to incorporate other cell adhesion peptides, into self-assembling Q11 peptide (represented by X-Q11, X=RGDS, IKVAV, YIGSR, or REDV). Importantly, varying the amount of X-Q11 peptides present in solution prior to hydrogel assembly provides control over the concentration of each cell adhesion domain present in the hydrogel, similar to the examples previously provided in Chapter 3. Additionally, due to the modular nature of Q11 system, any of the additional
types of functionality described in Chapter 2 can be added directly to the self-assembled Q11 peptides.

Here, in Chapter 4, I investigated hydrogels co-assembled from Q11 with peptides having Q11 at the C-terminus and RGDS, IKVAV, YIGSR, or REDV at the N-terminus. The objective of the experiments was to test the biofunctionality of such ligands conjugated Q11 peptides. First, the gelation and incorporation of the ECM-derived functional peptides into backgrounds of the basic self-assembling peptide was quantified. Then, the extent to which the ligands are presented on the surface of the self-assembled fibrils was determined. Also, the effect of ligand-bearing peptide incorporation on hydrogel stiffness was assessed. Lastly, endothelial cell attachment, spreading, and growth on hydrogels containing ECM-derived sequence-bearing peptides were evaluated.

Co-assembly of Q11 with RGDS-Q11, IKVAV-Q11, or YIGSR-Q11 was quantified by high-performance liquid chromatography (HPLC). Quantitative co-assembly of peptides is described by the complete incorporation of peptide present in solution into the self-assembled hydrogel, and is a key measure of quality control of modular self-assembling biomaterials. The fibrillization and secondary structures of Q11 co-assembled with RGDS-Q11 or IKVAV-Q11 were verified with TEM and CD, respectively, using the methods described in Chapter 3. The stiffness co-assembled hydrogels was investigated with oscillating
rheometry. I hypothesized that the amino acid sequence of the biofunctional peptide will not influence the stiffness of a self-assembled hydrogel. Streptavidin-gold labeling was used to assess the physical presentation of RGDS or IKVAV. HUVECs cultured on the hydrogel were used to test the biofunctionality of RGDS and IKVAV. This work was published in Jung et al., *Biomaterials* **30**, 2400-2410 (2009) [86].

### 4.2 Materials and methods

**Design of ligand-bearing peptides.** All Q11 peptides functionalized with ECM-derived ligands had the same basic construction: two N-terminal Gly residues, the ligand sequence, a triglycine spacer, and a C-terminal Q11 sequence (Table 4.1). For streptavidin-gold labeling, biotinylated versions of these peptides included N-terminal biotinylation with biotin-ONp (NovaBiochem Cat# 01-63-0116) and a Ser-Gly-Ser-Gly spacer. All other methods of peptide synthesis are the same as described in Section 3.2.

**Hydrogel Formation.** Hydrogels were formed in culture inserts for HUVEC culture or on the bottom plate of a rheometer for viscoelasticity measurements, as described in Section 3.2.

**Quantitative incorporation of peptides.** Quantitative assessment of the peptide concentration in self-assembled Q11 hydrogels provides an evaluation of
Table 4.1 Peptides synthesized.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>$m/z$ (calc'd)</th>
<th>$m/z$ (found)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q11</td>
<td>Ac-QQKFOFQFEQQ-CONH$_2$</td>
<td>1526.7</td>
<td>1527.7</td>
</tr>
<tr>
<td>RGDS-Q11</td>
<td>Ac-GGRGDSGGG-(Q11)-CONH$_2$</td>
<td>2227.3</td>
<td>2227.6</td>
</tr>
<tr>
<td>RDGS-Q11 (scrambled)</td>
<td>Ac-GGRDGSGGG-(Q11)-CONH$_2$</td>
<td>2227.3</td>
<td>2227.0</td>
</tr>
<tr>
<td>IKVAV-Q11</td>
<td>Ac-GGIKVAVGGG-(Q11)-CONH$_2$</td>
<td>2322.6</td>
<td>2322.4</td>
</tr>
<tr>
<td>VAKVL-Q11 (scrambled)</td>
<td>Ac-GGVAKVIGGG-(Q11)-CONH$_2$</td>
<td>2322.6</td>
<td>2321.9</td>
</tr>
<tr>
<td>Biotin-RGDS-Q11</td>
<td>Biotin-SGSGRGDSGGG-(Q11)-CONH$_2$</td>
<td>2585.8</td>
<td>2586.2</td>
</tr>
<tr>
<td>Biotin-IKVAV-Q11</td>
<td>Biotin-SGSGIKVAVGGG-(Q11)-CONH$_2$</td>
<td>2681.0</td>
<td>2681.3</td>
</tr>
<tr>
<td>YIGSR-Q11</td>
<td>Ac-GGYIGSRGGG-(Q11)-CONH$_2$</td>
<td>2388.6</td>
<td>2389.1</td>
</tr>
<tr>
<td>REDV-Q11</td>
<td>Ac-GGREDVGGG-(Q11)-CONH$_2$</td>
<td>2311.5</td>
<td>2311.8</td>
</tr>
</tbody>
</table>

Ac: acetylated
the efficiency of self-assembly. As shown in Figure 4.1, if peptides are completely self-assembled into fibrils there will be no free peptide present in buffer after washing the hydrogel. For hydrogel self-assembly experiments, peptides were dissolved in water at specified concentrations and sonicated with a probe-type ultrasonicator to ensure complete dissolution. Peptide solutions were overlayed with PBS for 40 min without mixing to induce complete gelation of the peptides. Gels were cast in microcentrifuge tubes for bulk analysis of peptide entrapment. To measure the entrapment of ligand-bearing peptides, solutions were prepared that contained 20 mM total peptide, with 5-50% of the total peptide being RGDS-Q11, IKVAV-Q11, or YIGSR-Q11. After hydrogel formation, the gels were washed three times with PBS for 30 min each, dissolved by adding TFA at a ratio of 1:2.3, and analyzed on a C4 HPLC column. Relative peptide concentrations were determined by measuring peak areas at 215 nm, and gelled samples were compared with ungelled solutions to determine if any peptide leaching or unpredictable incorporation occurred. Gels were formed and measured in triplicate.

Assessment of fibril morphology and physical ligand presentation. The introduction of a biofunctional domain, such as RGDS or IKVAV, could disrupt fibrillization of the self-assembling peptide Q11. Using the same TEM negative staining protocol described in Section 3.2, but with a different electron
Figure 4.1 Schematic of HPLC experiments for quantitative assessment of mixed peptides.
microscope (FEI Tecnai F30), fibril morphology of a binary mixture of 10% RGDS-Q11 or IKVAV-Q11 in the presence of 90% Q11 was prepared for TEM. To visualize the presentation of ligands using colloidal gold staining, grids with adsorbed fibrils from biotinylated peptides were floated on droplets of 1% bovine serum albumin (BSA) to block non-specific binding, washed with distilled water, floated on droplets of 1 µg/mL streptavidin-colloidal gold conjugate (Invitrogen Cat# A-32360), washed again, and stained with 1% uranyl acetate.

**Assessment of secondary structures of peptides.** Circular dichroism (CD) was used to evaluate the secondary structures of peptide mixtures consisting of 10% RGDS-Q11 or IKVAV-Q11 with 90% Q11. The secondary structure of Q11-based functionalized peptides is β-sheet and I hypothesized that RGDS-Q11 and IKVAV-Q11 will not disrupt the secondary structure of Q11 peptide fibrils. The experiments were performed with the same methods in Section 3.2, but with a different spectrometer (An AVIV 202 CD spectrometer, Aviv Biomedical, Lakewood, NJ, USA). Solutions of Q11 and mixtures containing 10% RGDS-Q11 or IKVAV-Q11 and 90% Q11 were diluted to a working concentration of 300 µM in CD-compatible buffer (4.3 mM KH₂PO₄, 1.4 mM Na₂HPO₄, 140 mM KF, pH 7.4) and analyzed immediately.

**Measuring hydrogel viscoelasticity.** I used oscillating rheometry to test the viscoelasticity change resulting from incorporating RGDS-Q11 or IKVAV-
Q11 into self-assembled Q11 hydrogels. The protocol for viscoelasticity measurements was the same as described in Section 3.2.

**Cell attachment, spreading, and growth.** HUVEC culture protocols were the same as described in Section 3.2. Here, the HUVECs were cultured on hydrogels containing 10% RGDS-Q11 or IKVAV-Q11 to test the biofunctionality of the ligand-bearing peptides. For attachment and spreading assays, HUVECs were fed with serum-free medium (EBM-2, Lonza Cat# cc-3156) for 24 h to synchronize cell cycles and plated on hydrogels. In all other experiments, HUVECs were fed with serum and growth factor-supplemented medium (EGM-2). HUVEC attachment and spreading was assessed at 1 h, and HUVEC growth was quantified at 64 h. HUVECs were seeded at a density of 8,850 cells/cm² on top of Q11 gels or on uncoated inserts. As a positive control, Q11 hydrogels lacking RGDS-Q11 or IKVAV-Q11 were incubated under a solution of human plasma FN (Sigma cat# F2006, 100 µg/mL in EBM-2) overnight. After one hour, the medium was removed, and unattached or loosely adherent cells were removed with serial PBS washes. For immunofluorescence staining, cells were fixed with 3.7% paraformaldehyde and permeabilized with 0.1% Triton X-100 for 30 min. Nuclei were stained with 10 ng/mL 4',6-diamidino-2-phenylindole (DAPI), and f-actin was stained with phalloidin. Using epifluorescence microscopy, six non-overlapping images per gel were collected, and nuclei per gel were counted.
Average projected areas were calculated by dividing the total phalloidin-stained area by the number of nuclei using ImageJ software. The measurement of HUVEC growth was analyzed with an MTS-based proliferation assay as the same methods in Section 3.2

**Assessment of peptide cytotoxicity.** Q11 and the functionalized peptides are significantly shorter than neurotoxic amyloid beta (Aβ) or cytotoxic polyglutamine peptides. Although the predicted cytotoxicity from their structures is low, this basic characterization is necessary to rule out potential complications resulting from synthetic Q11 scaffolds. Peptide cytotoxicity was measured using the same MTS assay after incubating confluent monolayers of HUVECs on 96-well TCPS for 24 h in EGM-2 containing 0.01-1.0 mg/mL peptide. Higher peptide concentrations than these were not possible because gels were formed in the culture medium and disrupted the subsequent MTS assay. Controls included cultures without peptide, cultures that had been fixed with absolute ethanol, and wells without cells.

**Statistical analysis.** Attachment, spreading, proliferation, cytotoxicity, and rheometry experiments were analyzed by one-way ANOVA with post-hoc comparisons. Experiments having residuals with equal variances (attachment, spreading, proliferation, and rheometry) were analyzed using Tukey’s HSD post-hoc comparisons, and experiments with normal but unequal variances
(cytotoxicity) were analyzed using Tamhane’s T2 post-hoc comparisons [195, 196].

4.3 Results and discussion

**Formation of hydrogels from peptide solutions.** All Q11-based peptides were soluble in water at concentrations up to 40 mM. Self-supporting, translucent gels were formed when aqueous solutions of 10-40 mM Q11 were overlaid with PBS. With careful pipetting, the underlying peptide layer and overlying PBS layer did not mix, and the peptide layer gelled within 1 h as a result of buffer constituent diffusion from the PBS into the peptide layer. After gelation, the PBS layer could be removed and replaced with additional buffer or media. Importantly, incubating Q11 solutions overnight at 4°C prior to the PBS overlay increased the viscosity of the Q11 solution slightly, resulting in an extremely smooth gel surface. Gels with a smooth surface allowed for subsequent cell attachment, spreading, and proliferation experiments to be conducted without variability in gel topography.

**Quantitative incorporation of peptides.** To investigate the co-assembly of multiple Q11-based peptides into hydrogels on a bulk scale, the composition of peptide mixtures with HPLC before and after gelation was measured (Figure 4.2). To determine if any of the co-assembled peptides leached out of the gels during
PBS washes, I compared the peptide ratio of a pre-gelled solution to the ratio in solutions collected from hydrogels that had been washed with PBS and disassociated with TFA. My results demonstrated that RGDS-Q11 was quantitatively incorporated into Q11 matrices at every concentration tested, up to 44% RGDS-Q11/56% Q11. For IKVAV-Q11, no deviation was observed up to 21% IKVAV-Q11/79% Q11. At 38% IKVAV-Q11/62% Q11, the concentration of IKVAV-Q11 was 3.2% higher in gels than in pre-gelled solutions, suggesting that at higher concentrations IKVAV-Q11 may disrupt the assembly of Q11. For YIGSR-Q11, no deviation was observed as high as 24% YIGSR-Q11/76% Q11, but at 40% YIGSR-Q11/60% Q11, the concentration of YIGSR-Q11 was 2% higher in gels than in peptide solutions. The observation that ligand-bearing Q11 peptides are quantitatively incorporated into self-assembled hydrogels indicates that mixing peptides in solution at a desired ratio and subsequently inducing gelation provides gels with well-defined peptide composition. Of note, another ligand-bearing peptide, REDV-Q11, was tested using an identical method. However, the absorbance peak of REDV-Q11 overlaps with that of Q11, which prevented quantitative assessment of REDV-Q11 incorporation into the gel. To work within the range of quantitative peptide incorporation, the following experiments used 10% RGDS-Q11 or IKVAV-Q11.

**RGDS or IKVAV incorporation does not affect secondary structures**
Figure 4.2 Quantitative bulk co-assembly of Q11 with RGDS-Q11 (a) IKVAV-Q11 (b), YIGSR-Q11 (c) as determined by comparing peptide ratios in gels with those of their corresponding pre-gelled peptide solutions using HPLC. The dotted line represents perfect matching between gels and solutions. Mean ± SD with 9 gels represented per panel; 3 independent gels for each formulation.
Cellular responses to ligand-presenting materials can be strongly affected by material stiffness [164, 166], so independent control of ligand incorporation and viscoelasticity is important to systematically optimize materials for driving a desired cell phenotype [85]. I employed TEM, circular dichroism, and oscillating rheometry to characterize the influence of ligand-bearing Q11 peptides on fibril morphology and stiffness of Q11-based gels. I chose a 1:10 ratio of ligand-bearing peptides to Q11 based on the results in Chapter 3 demonstrating that this level of RGDS incorporation significantly increased HUVEC proliferation in gels stiffened by native chemical ligation [85]. In addition, HPLC analyses (Figure 4.2) demonstrated that this peptide ratio was quantitatively incorporated, with no leaching or disruption of self-assembly. Scrambled versions of the ligand-bearing peptides (RDGS-Q11 and VAKVI-Q11) were also evaluated, as they will later be employed as negative controls in HUVEC culture experiments.

Using TEM with negative staining, I observed that none of the four peptides significantly altered the fibrillization of Q11 when combined in a 1:10 ratio with Q11 (Figure 4.3). Each peptide mixture containing 90% Q11 and 10% RGDS-Q11, IKVAV-Q11, RDGS-Q11 or VAKVI-Q11 formed fibrils having widths of approximately 10-20 nm and morphologies, similar to 100% Q11 fibrils. There was no evidence of morphologically distinct subpopulations of fibrils that
Figure 4.3 TEM of Q11-based peptide mixtures. Q11 (a), 10% RGDS-Q11/90% Q11 (b), 10% IKVAV-Q11/90% Q11 (c), 10% RDGS-Q11/90% Q11 (d), and 10% VAKVI-Q11/90% Q11 (e). Circular dichroism (f) of 100% Q11 (○, blue), 10% RGDS-Q11/90% Q11 (▲, green), and 10% IKVAV-Q11/90% Q11 (■, red). 300 µM peptide in 140 mM KF, 4.3 mM KH₂PO₄, 1.4 mM Na₂HPO₄, pH 7.4; 0.1 cm path length.
might indicate segregation of the peptides into mutually exclusive fibrils, so it was assumed that the mixed peptides formed co-assembled fibrils. The observed fibril morphologies were also consistent with other previously reported β-sheet fibrillar peptides [183, 184]. Interestingly, significant lateral aggregation was observed for all samples, although it is difficult to conclusively relate the observed interfibrillar interactions to those that may occur in the gels given that the samples were adsorbed and dried onto TEM grids.

Using circular dichroism (CD) spectroscopy, I investigated whether the incorporation of 10% RGDS-Q11 or IKVAV-Q11 significantly changed the secondary structure of Q11 fibrils. Q11 displayed a positive band at 206 nm and a negative band at 224 nm (Figure 4.3f), indicating a predominantly β-turn structure, which is consistent with previous reports for Q11 [44, 52] and the related peptide Ac-QQRFWQFEQQ-Am (DN1 [45, 46], discussed in Section 2.3), but is distinct from other self-assembling β-sheet peptides such as RADA16-I, which exhibits a more classical β-sheet structure [36]. The secondary structure of Q11 was maintained for mixtures of 10% RGDS-Q11 or IKVAV-Q11 with 90% Q11. As a further confirmation of the TEM results, these CD data indicated that the presence of the ligand-bearing peptides did not significantly alter the morphology of Q11 fibrils, as an altered β-sheet folding of Q11 mediated by ligand-bearing Q11 would be noted by significant spectral changes. Additionally, if the ligand-
bearing peptides assembled into distinct fibrils, one would expect to see additional spectral features added to the baseline of Q11’s spectrum. Note that the CD spectra have been plotted in terms of ellipticity (mdeg) rather than mean residue ellipticity owing to the fact that samples were mixtures of different peptides.

**Ligand-bearing peptide incorporation does not alter the stiffness of hydrogels.** Having established that ligand incorporation did not alter fibril morphology, the viscoelasticity of Q11-based gels was subsequently investigated. In Chapter 3, the stiffness of Q11 gels demonstrated a dependence on peptide concentration, with storage moduli ranging from 1-10 kPa for gels having peptide concentrations between 5-30 mM, respectively [85]. Such concentration-dependent stiffness has also been observed for other self-assembling peptide gels [131]. In this experiment, gels containing 30 mM total peptide and 10% RGDS-Q11, 10% IKVAV-Q11, or 5% RGDS-Q11/5% IKVAV-Q11 were compared with unmodified Q11 gels. All four formulations demonstrated strain-insensitive behavior at oscillation frequencies between 0.01 Hz and 10 Hz, loss moduli ($G''$) that were about one order of magnitude smaller than storage moduli ($G'$), and no crossing of $G'$ and $G''$ at any frequency (Figure 4.4a). That is, each formulation exhibited the rheological characteristics of a cross-linked gel. Average storage moduli varied less than 2.5 kPa between groups, and evaluation of multiple gels did not reveal statistically significant differences between Q11 and ligand-bearing
Figure 4.4 Oscillating rheometry. Frequency sweep (a) and statistical comparison of multiple gels at 1 Hz (b). G’ (filled symbols) and G” (open symbols) are shown for Q11 (●, ○, blue, labeled “Q” in (b)), 10% RGDS-Q11/90% Q11 (▼, ▽, green, “R”), 10% IKVAV-Q11/90% Q11 (■, □, red, “K”), and 5% RGDS-Q11/5% IKVAV-Q11/90% Q11 (◆, ◆, gray, “R/K”). All gels had 30 mM total peptide. *p < 0.05 by ANOVA with Tukey’s HSD post-hoc test. Mean±SD; n=3 independent gels per group.
gels (Figure 4.4b). It is possible that statistical significance may be revealed with a larger sample size, but even if this were to be the case, the differences between groups are smaller than those that have previously been found to affect cell behavior [128]. As shown in Figure 4.4b, the only statistically significant difference observed was for the loss modulus of the 10% RGDS-Q11/90% Q11 gels, which was slightly higher compared with the other gel formulations. However, small variances in $G''$ have not been previously associated with differential cell behavior. The limited impact of incorporated ligand on the gel viscoelasticity allows for the simultaneous and independent adjustment of ligand presentation and gel mechanics (as shown previously in Chapter 3). Importantly, the ability to independently adjust these aspects enables systematic adjustment of gel properties to achieve the desired biological responses. Moreover, RGDS and IKVAV are significantly different in terms of their chemical properties, RGDS being neutrally charged and hydrophilic, and IKVAV being positively charged and comparatively hydrophobic, suggesting that it would be possible to co-assemble a wide range of other peptide sequences within Q11 gels.

**RGDS and IKVAV ligands are presented on fibril surfaces.** Ligands displayed on peptide fibrils were visualized using N-terminally biotinylated peptides, avidin-conjugated colloidal gold, and TEM. Fibrils of biotin-RGDS-Q11, biotin-IKVAV-Q11, and mixtures of the ligand-bearing peptides with Q11
Figure 4.5 Ligands were displayed on the surface of Q11 fibrils, as evidenced by the labeling of biotinylated ligand-bearing peptides with streptavidin-colloidal gold. Q11 fibrils showed minimal background gold staining (a), whereas 10% biotin-RGDS-Q11/90% Q11 (b), 100% biotin-RGDS-Q11 (c), 10% biotin-IKVAV-Q11/90% Q11 (d) and 100% biotin-IKVAV-Q11 (e) bound significant levels of avidin-gold.
all stained specifically and strongly with avidin-gold compared to Q11 (Figure 4.5). This indicated that the N-termini of these peptides, and presumably their N-terminal ligands, were located on the surface of the fibrils. In the TEM images, some amount of resolution was sacrificed during blocking and gold labeling (see methods), but the fibrils were clearly visible. The gold labeling was distributed along the surface of the fibrils for each of the biotinylated groups, and for mixtures of peptides there was not significant evidence of segregation into distinct fibrils. This indicated that the peptides co-assembled into fibrils containing both peptides. Although it was clear that the RGDS and IKVAV ligands were present on the fibril surface, it was not clear whether all of them were present or whether there existed some degree of heterogeneity in fibril structure. However, the morphologic similarities between the ligand-bearing co-assemblies and pure Q11 (Figure 4.5), as well as the gold labeling, suggested that most of the ligand sequences were not buried within the peptide fibrils. Such burial might be expected to induce greater morphological changes than those observed. Subsequent cell culture experiments, described below, further supported that these ligands were available on the fibril surface.

**RGDS and IKVAV are functionally presented by Q11 fibrils.** The behavior of HUVECs on Q11 gels was modulated by the inclusion of RGDS-Q11 and IKVAV-Q11 in the matrix. One hour after seeding, the attachment of
HUVECs to 30 mM peptide gels containing 10% RGDS-Q11 was five-fold greater than on Q11 gels. In addition, cell attachment was as extensive on RGDS-bearing gels as on fibronectin-adsorbed Q11 gels, a highly adhesive surface (Figure 4.6a). HUVECs did not attach, spread, or proliferate to any degree on unmodified culture inserts. The response to RGDS-Q11 was specific, as the scrambled RDGS-Q11 peptide abolished this effect. Collectively, these cell attachment data in conjunction with the TEM and CD data indicated that the RGDS ligand was presented by peptide fibrils and available for specific cell binding, even in serum-containing medium. Including of 10% IKVAV-Q11 also increased HUVEC attachment, but to a lesser degree than RGDS-Q11. This increased attachment was statistically significant by t-test compared with Q11 and the scrambled VAKVI-Q11 (p=0.002 and 0.01, respectively), but was no longer statistically significant when compared within the experiment as a whole by ANOVA (p=0.06 with Tukey’s HSD post-hoc test). These results are interpreted to mean that IKVAV-Q11 had a detectable but small effect on HUVEC attachment. This small effect of IKVAV on HUVEC adhesion was not unexpected, as it has previously been demonstrated that surface-bound IKVAV only slightly increased endothelial cell attachment, while also inducing a more spindle-shaped, migratory morphology [108, 109, 112]. The similar response induced by IKVAV-Q11 on HUVECs, along with the sequence-specificity
Figure 4.6 HUVEC behavior on Q11 gels. Attachment (a) and spreading (b) at 1 h (n=4 independent gels; mean±SD). Cell numbers (c) at 64 h (n=5, mean±SD). *p < 0.05 by ANOVA with Tukey’s HSD post-hoc test.
indicated by the scrambled peptide control, suggested that the IKVAV sequence was also displayed by the Q11 fibrils. To observe a more dramatic effect of this peptide on different cell types, it may be interesting in future work to investigate Q11-based matrices in cultures of primary neurons or neuronal cell lines, as IKVAV has been shown to modulate neuronal cell behavior in a variety of different biomaterials contexts [111, 202-204].

Cell spreading at short time points was less affected than overall attachment by the inclusion of ligands (Figure 4.6b). At one hour, only 10% RGDS-Q11 gels showed a significant increase in spreading over unmodified Q11, and this difference was relatively small. At later time points, however, larger differences in cell spreading and morphology were observed by phase contrast microscopy. After three days, FN-coated and 10% RGDS-Q11 gels showed significantly more cell coverage in comparison to unmodified Q11, the scrambled RDGS-Q11 control surface, or IKVAV-containing gels (Figure 4.7). On FN-coated and RGDS-Q11-containing matrices, HUVECs exhibited morphologies approaching a normal cobblestone appearance, whereas the IKVAV-Q11-containing surfaces promoted a more spindle-shaped, elongated morphology with far fewer cells. An MTS-based assay of cell number after 3 days of culture (3 days, Figure 4.6c) demonstrated that 10% RGDS-Q11 gels supported cell growth at a rate between that of cells cultured on Q11 or fibronectin-coated Q11. Similar
Figure 4.7 Phase contrast images of HUVEC cultures at 64 h on Q11 (a), fibronectin-adsorbed Q11 (b), 10% RGDS-Q11/90% Q11 (c), 10% RDGS-Q11/90% Q11 (d), 10% IKVAV-Q11/90% Q11 (e), and 10% VAKVI-Q11/90% Q11 (f).
to cell attachment, the scrambled RDGS-Q11 did not produce this effect. Since MTS signals are dependent on metabolic activity of cells, it was not explicitly determined whether the increased cell numbers were a result of improved early adhesion or an increase in proliferation over time, however, both mechanisms likely contributed. Looking more closely at the RGDS-containing gels, additional cultures were followed for up to seven days, after which both RGDS-containing and fibronectin-adsorbed gels exhibited confluent monolayers approaching a normal cobblestone morphology (Figure 4.8). This was in contrast to unfunctionalized Q11 gels, which showed only sparse clusters containing a few cells each. Taken together, the cell attachment, spreading, and growth data indicated that RGDS-Q11 assembled in such a way that the RGDS ligand was available on the surface of the fibrils and could interact with cell surface receptors to modulate cell behavior. The effect of IKVAV was more subtle, as proliferation was not increased, but similar to previous observations [108]. HUVECs on IKVAV-containing gels exhibited a more spindle-shaped morphology, contrasting with the cobblestone appearance on FN or RGDS-containing gels.

**RGDS-Q11 and IKVAV-Q11 are not cytotoxic to HUVECs.** Glutamine-rich β-sheet-forming peptides that are significantly longer than Q11 can be cytotoxic, especially when the polyglutamine stretches are longer than 37 residues [205, 206]. Understanding the cytotoxicity of Q11 and its derivatives is
Figure 4.8 Phase contrast images of HUVEC cultures on Q11, 10% RGDS-Q11/90% Q11, and fibronectin-adsorbed Q11 (FN) at days 1, 2, 4, and 7.
necessary for guiding the future development of Q11-based peptides as biomaterials, and also for understanding the influence of cytotoxicity on the variable cell attachment, spreading, and growth of HUVECs observed in the present study. In confluent cultures of HUVECs, none of the Q11-based peptides were found to be cytotoxic at any concentrations tested (Figure 4.9). This observation eliminates cytotoxicity as a possible mechanism for the improved attachment, spreading and cell growth observed on ligand-bearing Q11 gels and suggests that hydrogels formed from these peptides may be suitable for *in vitro* culture applications.

### 4.4 Conclusions

In Chapter 4, I prepared a set of peptides based on the self-assembling sequence Q11 functionalized with ECM-derived cell adhesion ligands, RGDS, REDV, IKVAV, and YIGSR. My results demonstrated that varying the ratio of peptides in the pre-gelled solution provides control over the ratio of peptides co-assembled into the gel. RGDS-Q11, IKVAV-Q11, and YIGSR-Q11 were quantitatively incorporated into Q11 hydrogels in a wide range of peptide ratios. Ligands incorporated at 10 mol% had no significant impact on fibril morphology or secondary structure. The viscoelasticity of hydrogels containing ligands was minimally changed, allowing ligand incorporation to be adjusted independently of
**Figure 4.9** Cytotoxicity of Q11-based peptides. In (a), controls included no peptide (“n”), dead cells fixed with ethanol (“d”), and medium only (no cells, “m”). *p<0.05 compared to no peptide control by ANOVA with Tamhane’s T2 post-hoc test (n=6, mean±SD).
gel mechanics. Both RGDS-Q11 and IKVAV-Q11 were physically presented on the surface of fibrils, and these ligands modulated HUVEC behavior \textit{in vitro}. RGDS-Q11 significantly affected HUVEC attachment, spreading, and growth, while IKVAV-Q11 had a small effect on cell attachment and a subtle influence on cell morphology. Scrambled peptides RDGS and VAKVI (for RGDS and IKVAV, respectively) abolished these effects, indicating that these responses were specific, even in serum-containing medium. Finally, none of the peptides investigated were cytotoxic.

This biophysical and biofunctional characterization demonstrates that the Q11-based system allows for modular construction of self-assembled peptide hydrogels presenting multiple different functionalities. As discussed in Chapter 2, multifunctionality will be increasingly important for designing biomaterials with complex, yet tunable, bioactivity. Consequently, the next step is to incorporate all of the ligand-bearing peptides characterized in Chapter 4, as well as other functionalities, such as variable matrix mechanics or soluble factor release. In Chapter 5, I will describe the influence of self-assembled peptide hydrogels co-assembled from systematically varied ratios of multiple ligand-bearing peptides on HUVEC growth.
5.1 Introduction

**Modular construction of biomaterials.** In Chapter 4, the main focus was on how Q11 peptides can be functionalized with ECM-derived ligand-bearing sequences capable of modulating the behaviors of HUVECs, while maintaining the stiffness of the hydrogels. As mentioned earlier in Section 2.6, naturally existing scaffolds for cells are much more complex in their architecture and functions, bearing many different ligands whose relative concentrations are highly specified. Since such extremely complex and multifactorial ECMs are not synthetically accessible, a means for systematically optimizing the available factors at hand is necessary. Currently, one of the major limitations of synthetic scaffolds for Regenerative Medicine applications is that materials designed for such scaffolds tend to be extremely reductionist, bearing only one [86, 87], or at most, two [80, 82] biological functionalities. One of the reasons for this
reductionism is that previous synthetic scaffolds have been predominantly constructed from covalent polymer networks, and within such materials it is difficult to incorporate multiple functional moieties such as peptide ligands in a systematic, controllable, and predictable manner. For example, in polymerization reactions involving multiple co-monomers, simple mixing of the monomers often leads to a polymerized product whose final composition has drifted significantly from the initial ratio of the reactants [208, 209]. It is often possible to measure such compositional drifts, but this must be done for each new ratio of monomers to ascertain the final composition of each new material. This aspect greatly hinders the investigation of many different formulations, especially in biological experiments, which themselves are time consuming to begin with. This is the main rationale for why we need modular construction in biomaterials: to enable the systematic and precise compositional refinement of several cell attachment factors so as to be able to experimentally maximize a desired biological response.

The work described in this chapter capitalizes upon the inherent modulatity of the non-covalent self-assembly approach developed in this thesis, focusing on the systematic adjustment of multiple ligand-bearing peptides to maximize the growth of HUVECs.

**Systematic adjustment of materials’ properties.** Creating modular materials is a key step towards systematic adjustment of multiple scaffold
properties. In Chapters 3 and 4, Q11 peptides were functionalized with a viscoelasticity-modulating component and ligand-bearing components, respectively. In those two cases, the ability of the Q11 domain to self-assemble into fibrillar structures was not compromised, enabling predictable co-assembly of the functionalized molecules. Rather, the mechanical properties (Chapter 3) and the presentation of cell binding ligands (Chapter 4) were modulated by co-assembling functional Q11 variants into the matrix. This modular approach is utilized here, but with more factors, and with many more compositions.

Then, the question becomes how can systematic adjustment of ligand-bearing peptides be achieved? The experimental design method called Design of Experiment (DOE) approaches can be utilized to achieve systematic adjustment for many factors (see Section 2.7). This methodology was proposed by Ronald A. Fisher in 1935 [210]. The main goal is to efficiently design experiments to understand the interactions among different input factors in order to identify optimal combinations. This approach has not been extensively investigated in biomaterials research but there are a few recent examples in neural tissue engineering [211], biomechanics [212], and microarray platforms [213]. Still, it is not an unusual practice in many industries and scientific fields, including chemical engineering and pharmaceutical development [214]. Using DOE approaches, the systematic adjustment of modular Q11-based functionalized
peptides is feasible for maximizing desired properties of gels or for achieving a specific target property. In addition, the complex interactions of multiple factors can be predicted by this method in polynomial models in DOE (eq. 2.2) that is not achievable through conventional analysis of experiments such as multiple comparisons.

\[ y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j + \sum \beta_{ii} X_i^2 \cdots \]  

(2.2)

\( y \), response, where \( \beta \)'s are theoretical regression or polynomial coefficients of the factors (X’s)

The working hypotheses for this chapter are that 1) DOE approaches will identify optimum combinations of peptides that differ from those that would be selected using more conventional approaches. Such conventional approaches are represented by selecting individual single-factor optima for each peptide, and combining them without further multifactorial optimization. 2) Materials designed through DOE approaches will elicit more rapid HUVEC growth (the targeted response) than could be achieved without systematic optimization through DOE.
Systematic adjustment of multiple ligand-bearing peptides to maximize the growth of HUVECs. For clarity, the DOE-related terms that were generally defined in Chapter 2 will be more specifically defined here, in the context of the experiments actually performed:

Factors are each peptide, RGDS-Q11, IKVAV-Q11, YIGSR-Q11, REDV-Q11, and Q11.

Levels are the concentration of each factor (peptide). For example, low and high levels of RGDS-Q11 were initially set at 0 and 6 mM, respectively. For different DOE approaches, the concentrations of each peptide were varied.

The main Response was cell growth as measured by MTS assay (absorbance at 490 nm). After each factor was combined in a hydrogel formulation, HUVECs were cultured for 64 h on those gels. Then, an MTS assay was conducted to obtain absorbance values. Through all DOE approaches described here, the response was this MTS reading.

Effects are the changed responses from changing the levels of a factor. The effect of a single factor is called a main effect.

Interactions occur when the effect of one factor depends on the level of another factor(s). For example, it was found that the response from RGDS-Q11 was dependent on the levels of YIGSR-Q11, meaning they have a certain type of
interaction. Using these definitions, the flow of the work performed is more clearly understood, as shown in Figure 5.1.

The first experiments were single-factor dose-dependent experiments of each factor (ligand-bearing peptide). The factors were RGDS-Q11, IKVAV-Q11, YIGSR-Q11, and REDV-Q11. The purpose of this single-factor dose-dependent experiment was two-fold: 1) to verify the bioactivity of each peptide, and 2) to initially select the high and low levels for the first multifactorial experiments. A series of binary mixtures of each ligand-bearing peptide and Q11 were prepared, effectively dosing each ligand-bearing peptide at various levels into a background of Q11. From these experiments, the levels and factors for the subsequent DOE were identified (Figure 5.1). Since one purpose of DOE approaches is to run a minimal number of experiments (cost-effective) and to maximize information (time-saving), discrete levels should be identified to explore all possible and continuous ranges (see Section 2.7 and Figure 2.7). The most popular FE designs are two-level (high and low levels) designs (Section 5.3.2 in Ref. [161]). These design is simple, economical (k; factors, m; levels, then FE needs m^k experimental runs), and gives most of the information required to then advance to a multilevel response surface methodology (RSM). As discussed in Section 2.7, the search for optimal conditions needs multilevel experiments. RSM is designed to find improved or optimal process settings by allowing us to estimate interaction
Figure 5.1 Flow of the experiments using DOE approaches.
and quadratic effects, and therefore give us an idea of the (local) shape of the responses (Section 5.3.3 in Ref. [161]). As graphically shown in Figure 2.9, a central composite design (CCD) method was used to conduct multilevel experiments. The important main factors were the same, but their levels were varied to search the improved responses using CCD. Three CCDs were ultimately performed to arrive at an optimized formulation. After this formulation was reached, I also verified the validity of the RSMs by directly comparing the optimized formulation with several control formulations representing non-optimized materials. These final experiments also served to test the hypothesis that DOE approaches can be used to engineer materials that promote EC growth better than those not optimized using DOE.

5.2 Materials and methods

Ligand-bearing peptides. Instead of adding all three different functionalities (matrix mechanics, cell-matrix binding, and release of soluble factors), Chapter 5 focused on four ligand-bearing peptides (factors). The only difference among them is the ligand and their co-assemblies are more straightforward than viscoelasticity modulating element or NO-release peptides. Those four ligand-bearing peptides were introduced in Chapter 4.2 and their sequences and mass spectrometry results are listed in Table 5.1.
<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>m/z (calc’d)</th>
<th>m/z (found)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q11</td>
<td>Ac-QQKQFQFQFEQQ-CONH₂</td>
<td>1526.7</td>
<td>1527.7</td>
</tr>
<tr>
<td>RGDS-Q11</td>
<td>Ac-GGRGDSGGG-(Q11)-CONH₂</td>
<td>2227.3</td>
<td>2227.6</td>
</tr>
<tr>
<td>RDGS-Q11 (scrambled)</td>
<td>Ac-GGRDGSGGG-(Q11)-CONH₂</td>
<td>2227.3</td>
<td>2227.0</td>
</tr>
<tr>
<td>IKVAV-Q11</td>
<td>Ac-GGikitAVGGGG-(Q11)-CONH₂</td>
<td>2322.6</td>
<td>2322.4</td>
</tr>
<tr>
<td>VAKVI-Q11 (scrambled)</td>
<td>Ac-GGVAKVI GGGG-(Q11)-CONH₂</td>
<td>2322.6</td>
<td>2321.9</td>
</tr>
<tr>
<td>YIGSR-Q11</td>
<td>Ac-GGYIGSRGGG-(Q11)-CONH₂</td>
<td>2388.6</td>
<td>2389.1</td>
</tr>
<tr>
<td>RYGSI-Q11 (scrambled)</td>
<td>Ac-GGRYGSGGG-(Q11)-CONH₂</td>
<td>2388.6</td>
<td>2387.4</td>
</tr>
<tr>
<td>IGSE-Q11 (YIGSR control)</td>
<td>Ac-GGIGSEGGG-(Q11)-CONH₂</td>
<td>2198.3</td>
<td>2199.1</td>
</tr>
<tr>
<td>REDV-Q11</td>
<td>Ac-GGREDVGGG-(Q11)-CONH₂</td>
<td>2311.5</td>
<td>2311.8</td>
</tr>
<tr>
<td>EVDR-Q11 (scrambled)</td>
<td>Ac-GGEVDRGGG-(Q11)-CONH₂</td>
<td>2311.5</td>
<td>2311.2</td>
</tr>
</tbody>
</table>
Hydrogels with multiple different ligand-bearing peptides. Single factor dose-dependent experiments were employed to select the high and low factors for factorial experiments. Of the four ligand-bearing peptides, only 3 mM of RGDS-Q11 or 3 mM of IKVAV-Q11 in the context of 27 mM of Q11 had previously been tested. The total peptide concentration was fixed at 30 mM for consistency, as in Chapters 3 and 4, and the concentration of each ligand-bearing peptide was varied from 0 to 30 mM. That is, the concentrations of X-Q11 (X=RGDS, REDV, IKVAV, and YIGSR) were 0.0, 0.3, 1.5, 3.0, 6.0, 15, 30 mM, and Q11 concentrations were 30, 29.7, 28.5, 27.0, 24.0, 15, 0 mM, respectively. As a control to confirm the specificity of the responses from the ECM-derived ligands, analogous scrambled sequence-bearing peptides (RDGS-Q11, EVDR-Q11, VAKVI-Q11, and RYGSI-Q11) were synthesized and evaluated for comparison. All peptide sequences are included in Table 5.1.

From the single-factor dose-dependent experiments, high and low levels (concentrations) were identified for the next step, Factorial Experiments (FEs). FEs identify main effects for the all tested factors (peptides), and they also provide information for additionally screening factors. The difference between single factor experiments and FEs is that the single-factor experiments only identify whether there are any discrete levels (concentrations) that affect the responses with only one factor (peptide). In the FEs performed here,
unfunctionalized Q11 again served a background for ligand incorporation, with ligand-bearing peptides being dosed into the matrix at various high and low concentrations.

The levels and factors for RSM were set by a Central Composite Design (CCD). As depicted in Figure 2.9, the levels for CCD are augmented on top of the FEs. For the simplest FEs, two levels, high and low, are required. In CCD, additional levels are extrapolated from the levels of FEs so as to create multiple levels. The rationale for creating factors with multiple levels is described in Section 2.7 and Figure 2.8. For each experiment, tables containing levels and factors are provided for clarity. In these tables, one experimental run (a row in the table) means the formulation of one hydrogel.

**Measuring viscoelasticity of hydrogels.** In order to rule out any effect from mechanical properties of hydrogels on the response (MTS reading), the viscoelasticity of the hydrogels was measured utilizing a Bohlin Gemini rheometer (Malvern Instruments Ltd., Malvern, UK) with an 8 mm parallel plate configuration. Instead of testing every single factor formulation, only two levels, 1.5 and 6.0 mM, were tested. In Chapter 4, incorporating 3 mM (referred as 10 mol% in Chapter 4) ligand-bearing peptide did not alter the stiffness of hydrogels. At 6 mM, 20 mol% equivalent, of each ligand-bearing peptide can be added to 100 mol% (30 mM) of a total of five peptide concentration. For this reason, 6 mM
of each ligand-bearing peptide with 24 mM of Q11 hydrogels were prepared for oscillating rheometry. For lower levels, 1.5 mM concentration was chosen since incorporating 0.3 mM is unlikely affect the stiffness of hydrogels based on the fact that 3 mM incorporation did not change the stiffness of 30 mM hydrogels.

Hydrogels were incubated for a longer period of time to match the time of incubating gels for cell culture experiments. The time for incubating hydrogels in PBS for cell cultures is around 3 days from casting in inserts. In the methods utilized for the initial rheometry experiments described in Chapter 4, hydrogels were prepared in situ on the lower plate of the rheometer and only incubated for 40 min. This significant time difference could result in changes in stiffness. Gels were produced on a glass slide using a filter paper template with an 8 mm diameter hole. The template was pre-saturated with PBS, and peptide solutions were pipetted into it. The peptide solution was incubated in a sealed Petri dish overnight. Then, the peptide gels were gently overlaid with PBS and enclosed in the dishes to minimize drying for two days. To conduct rheometry, the PBS and template were removed, leaving a circular gel of 8 mm diameter on a slide glass. The top plate was lowered onto the gel until the top plate was completely and uniformly contacted, and frequency sweep measurements were performed from 0.01-10 Hz at 0.1% strain. Three independent gels were tested at each formulation.
Endothelial cell cultures and measurement of HUVEC growth and attachment. The method for HUVEC culture, subculture, attachment and growth is the same as described previously in Section 4.2. After the formulations that significantly enhanced HUVEC growth at 64 h were determined, two control peptides for YIGSR-Q11, RYGSI-Q11, and IGSE-Q11 (Table 5.1), were used to further clarify the role of YIGSR-Q11. The measurement of HUVEC growth was repeated at 1, 24, 48 and 64 h in order to assess if there is any difference in the rate of HUVEC growth over the time period.

Immunofluorescence staining of endothelial cell-specific marker (PECAM). Although the measured responses were the growth of HUVECs over 64 h period, primary cells are prone to changes to culture conditions. For this reason, EC-specific markers needed to be identified. This method is described in Chapter 3.2 for immunofluorescence staining of HUVECs on hydrogels, but with different time points and minor changes to reduce the background staining of Q11-based gels. Cultures were fixed with 2% paraformaldehyde for 1 h and permeabilized with 0.1% Triton X-100 for 5 min. In order to prevent excessive adsorption of primary and secondary antibodies, gels were blocked with 10% goat serum for 2 days at 4°C. The primary antibody was mouse IgG1 anti-human PECAM (R&D Cat# BBA7) diluted 1:100 in the blocking buffer, and the secondary antibody was AlexaFluor 488 goat anti-mouse IgG1(γ1), diluted 1:200
in the blocking buffer. Nuclei were stained with 100 ng/mL of 4',6-diamidino-2-phenylindole (DAPI).

**Statistical Analysis of the Experiments.** Single-factor dose-dependent experiments and the validation experiments of HUVEC attachment and growth were analyzed by one-way ANOVA using Tukey’s HSD post-hoc comparisons. After the factors (peptides) and the levels (concentrations) were identified from the single factor dose-dependent experiments to conduct FEs, a corresponding set of formulations were generated by JMP software (SAS Inc.). The principles to generate formulations were described in Section 2.7 and Figure 2.7. The results were reported as parameter estimates (the coefficient of the fit to equation 2.3, standard error, t-ratio (parameter estimate divided by its standard error), and probability tested against a null hypothesis (parameters=0). For simplicity, all the interactions were tested only between two factors (the 2\textsuperscript{nd} order interactions or two-way interactions). The result of the FEs was summarized as Summary of Fit and ANOVA. After FEs identified main factors, RSM was utilized and the levels were generated from CCD (see Figure 2.9). The results of RSM were reported as parameter estimates, standard error, t-ratio, and probability tested against a null hypothesis, as in the FEs. Contour plots were generated using JMP software to visualize the interactions between two factors (peptides) in RSM results. All samples were prepared in triplicate for each formulation.
5.3 Results and discussion

RGDS-Q11, IKVAV-Q11, and YIGSR-Q11 were main factors for FE.

Towards the goal of identifying a combination of multiple peptides that can significantly enhance HUVEC growth beyond what can be achieved with only one or two peptides, four ECM-derived peptides were functionalized at the N-terminus of Q11 peptide. Although there are a few number of reports that utilized multiple ligands presented on a scaffold for cell culture [94, 215-217], none of these previous combinations of ligands were systematically adjusted to derive a maximal effect towards a specific biological function.

Four ligand-bearing peptides (RGDS-Q11, REDV-Q11, IKVAV-Q11, and YIGSR-Q11) plus Q11 were selected to enhance the growth of HUVECs. The portion of ligand-bearing peptide was varied from 0 to 30 mM with the fixed total concentration of peptide at 30 mM, in order to identify which factors (peptides) are important and how much of the important factors are required to modulate the response (MTS reading, HUVEC growth at 64 h). This step was not yet technically a DOE approach, but it was required to utilize DOE subsequently. It was found that 6 mM RGDS-Q11, 1.5 to 6 mM IKVAV-Q11, and 6 mM of YIGSR-Q11 significantly enhanced the growth of HUVECs for 64 h over Q11 and their scrambled control peptides (Figure 5.2). Out of the four peptides tested, RGDS-Q11 enhanced the growth of HUVECs more significantly than IKVAV-
Figure 5.2 Dose-dependent responses of each ligand-bearing peptide in the context of Q11. The measured responses were HUVEC growth for 64 h with MTS-based proliferation assay determined by absorbance of the media at 490 nm. The total concentration of gels was 30 mM. ■ represents biologically active sequences, □ corresponding scrambled sequences. ** p<0.01 and * p<0.05 compared to Q11 by ANOVA with Tukey’s HSD post-hoc test, n=3, mean±SD.
Q11 or YIGSR-Q11, and over a wide range of concentrations from 1.5 to 30 mM. This is not unexpected, as the RGD sequence has been widely utilized for receptor-ligand mediated cell adhesion due to its strong and well documented biological effects [91, 94-96]. IKVAV-Q11 in a range of concentrations improved the growth of HUVECs over Q11 and over its corresponding scrambled controls. Interestingly, 30 mM of IKVAV-Q11 significantly diminished the response (MTS reading), below that for Q11 or for 30 mM VAKVI-Q11. However, the mechanism for this response is unclear at this time. YIGSR-Q11 did not show any significant increase of the response up to 3 mM YIGSR/ 27 mM Q11, but at 6 mM YIGSR-Q11, the response was significantly increased over Q11 and its negative control. Interestingly, the fibronectin-derived REDV-functionalized peptide, REDV-Q11, did not enhance HUVEC growth at any concentration, despite its having been shown to be able to promote EC growth in previous studies [102, 103]. None of the Q11 derivatives functionalized with scrambled sequences (RDGS, VAKVI, RYGS, or EVDR) enhanced the growth of HUVECs, indicating the specificity of the ligand-bearing peptides.

From these single factor dose-dependent experiments, three peptides, RGDS-Q11, IKVAV-Q11, and YIGSR-Q11 were selected as the factors for subsequent FE experiments. As discussed in Section 5.1, discrete levels should be selected to efficiently cover a reasonable range of interest. For maximal
coverage with two given points, a low and a high point should be selected, as shown in Figure 2.7. The low levels for the subsequent FEs were 0 mM for each of the three ligand-bearing peptides, and the high levels were 6 mM RGDS-Q11, 1.5 mM IKVAV-Q11, and 6 mM YIGSR-Q11. The rationale for choosing 1.5 mM IKVAV-Q11 was that the three IKVAV-Q11 concentrations, 1.5, 3.0, and 6.0 mM, did not show any significant difference between them, the gradient of the HUVEC growth was higher at 1.5 mM than any other two concentrations, and it conserved expensive peptides.

Storage modulus (G’) of the hydrogels was not changed by including 1.5 mM and 6.0 mM factors. In order to rule out the effect of stiffness in gels incorporating different numbers of ligands or different types of ligands, oscillating rheometry was utilized to measure the viscoelasticity of gels. Previously in Chapter 4, 3 mM of ligand-bearing peptides in the context of 27 mM of Q11 did not alter the stiffness of gels, as determined with a frequency sweep test at 0.1% fixed strain [86]. Since the high levels of each ligand-bearing peptide were selected either at 1.5 or 6 mM, I decided to test both concentrations.
Figure 5.3 Incorporation of RGDS, IKVAV, or YIGSR did not significantly affect gel rheology in the concentration regimes explored in this chapter. The total concentration of gels was 30 mM. ● or ▼; closed symbol, storage modulus ($G'$), ○ or ▽; open symbol, loss modulus ($G''$). ** $p<0.01$ and * $p<0.05$, compared to both loss moduli. Mean±SD; $n=3$ independent gels per group.
to determine if there were any significant changes in stiffness. As shown in Figure 5.3, the incorporation of RGDS-Q11, IKVAV-Q11, or YIGSR-Q11 at either 1.5 mM or 6 mM did not change the storage modules (G') of the gels. However, the loss modulus of IKVAV-Q11 and YIGSR-Q11 varied with different amounts of incorporated ligand-bearing peptides. Currently, the understanding of how loss modulus affects cell behavior is extremely limited, but to my knowledge there is no report indicating that small changes in loss modulus can lead to variations in HUVEC cell growth or the growth in any other cell type [218]. With the previously published studies and the results shown in Figure 5.3, the data strongly suggested that gel stiffness is not significantly altered for ligand-bearing peptide incorporation up to at least 6 mM.

**RGDS-Q11, IKVAV-Q11, and YIGSR-Q11 were main effects to the response, not Q11.** From the single factor dose-dependent experiments, three ligand-bearing peptides (factors) were identified, and the low and high levels were selected. Additionally, Q11 was selected as a factor since the concentration change of a gel is one and simplest ways to modulate cell growth [131]. Although Q11 is not functionalized with any ECM-derived ligand, it was hypothesized that a 10-fold change in concentration would modulate HUVEC growth in the context of other ligand-bearing peptides. The experimental runs are summarized in Table 5.2. The groups were randomized to remove any possible bias during
Table 5.2 Factors and levels were used for the FEs.

<table>
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<tr>
<th>Factors (mM)</th>
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<th>Low (−)</th>
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<tr>
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<tr>
<td>YIGSR</td>
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<td>0</td>
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<tr>
<td>Q11</td>
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</table>
One extra center point (3, 0.75, 3, 11) (mM) = (RGDS, IKVAV, YIGSR, Q11) was added for statistical analysis purpose since the predicted response between the low (−) and high (+) levels of each factor is not linear for all of the factors as evidenced by the single factor dose-dependent experiments (Figure 5.2). In other words, the responses at 3, 0.75, and 3 mM (center points) of RGDS-Q11, IKVAV-Q11, and YIGSR-Q11, respectively, are not exactly half of the responses at 6, 1.5, and 6 mM (high levels) of RGDS-Q11, IKVAV-Q11, and YIGSR-Q11. This non-linearity is more dominant for YIGSR-Q11, having as it does a significant increase at 6 mM concentration (level).

FEs showed that each factor, RGDS-Q11, IKVAV-Q11, and YIGSR-Q11, showed significant and positive effects to HUVEC growth, but Q11 did not (Figure 5.4). RGDS-Q11 showed the most positive effect, followed by YIGSR-Q11 and IKVAV-Q11, as quantified by estimates, indicating that those ligand-bearing peptides are candidates for gels with multiple ligand-bearing peptides. Q11 did not show any significant and positive effect to the response, though the concentration was varied 10-fold in this FE. For this reason, Q11 was not selected as a factor in the following optimization procedures in a series of response surface methodology (RSM). However, 2 mM of Q11 was added as background to make sure hydrogels formed under any combination (formulation) of ligand-bearing peptides. Interaction effects were also identified by the t-ratio
<table>
<thead>
<tr>
<th>Run</th>
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**Table 5.3** Experimental runs for Factorial Experiments. RGDS; RGDS-Q11, IKVAV; IKVAV-Q11, and YIGSR; YIGSR-Q11. The unit of each factor is mM. In pattern, + and − means high and low levels, respectively. 0 indicates a center point.
Figure 5.4 Results of Factorial Experiments. The plot of the t-ratios identifies significant main and interaction effects up to the 2nd degree. Blue lines indicate the level ±1.96 which corresponds to significant probability values 0.05.

Estimates are the coefficients of the linear form of the polynomial model (eq. 2.2) found by regression.

t-Ratio is a statistic that tests whether the true parameter is zero. t-Ratio and the bar graphs together shows the effect of the factors.

Prob > |t| is the probability of getting an even greater t-statistics (absolute value), given the hypothesis that the parameter is zero.
plot in Figure 5.4. In eq. 2.2, they are $X_iX_j$ (total order of the term is second for both $X_iX_j$), where they are RGDS*YIGSR, RGDS*Q11, IKVAV*YIGSR, RGDS*IKVAV, IKVAV*Q11, and YIGSR*Q11 in Figure 5.4. Surprisingly, all the two-way or second-order interaction effects turned out to be negative or antagonistic. Only RGDS*YIGSR, RGDS*Q11, and IKVAV*YIGSR were significant. The interpretation of the two-way interactions is easier with contour plots (Figure 5.5). The interactions of RGDS-Q11 with others were positive with respect to the increase of RGDS-Q11 concentration (Figure 5.5a, c, and d). The interactions of IKVAV-Q11 with YIGSR-Q11 or Q11 showed the most positive responses in their middle concentrations (Figure 5.5b and e). This trend was observed for the interaction of YIGSR-Q11 with Q11 (Figure 5.5f). The detailed statistical analysis was summarized in Table 5.4. The $R^2$ of the fit by linear regression was 0.791591. The ANOVA results showed that the fit was significant against the null hypothesis that all coefficients are zero. From FEs, the three positive main effects, RGDS-Q11, IKVAV-Q11, and YIGSR-Q11, were identified.

**Improving the response from RGDS-Q11, IKVAV-Q11, and YIGSR-Q11 factors.** Now, with these three factors (peptides) and new levels (concentrations), this three-peptide formulation can improve the growth of HUVECs. As discussed in Sections 2.7 and 5.1, Response Surface Methodology
Figure 5.5 Contour plots showing the interactions between two peptides (factors) from the Factorial Experiments. Scales bar: values of the responses, absorbance at 490 nm. Higher values indicate higher responses.
Table 5.4  Summary of the statistical analysis of FEs.

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<td></td>
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<td>prob &gt; F</td>
<td>&lt;0.0001*</td>
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Summary of Fit: summaries of the response for the multiple regression model
Observation: the number of observations used in the fit

Source: the sources of variation
DF: degree of freedom
SS: sum of squares for each source of variation
MS: mean of squares, SS divided by its associated DF

Model DF: the number of parameters used to fit the model
Error DF: difference between the C.Total DF and the Model DF
C. Total DF: one less than the number of observations

F-ratio: model MS divided by error MS, testing the hypothesis that all parameters are zero

prob > F: the probability of obtaining a greater F-value by chance alone if the specified model fits no better than the overall response mean.
(RSM) allows us to find improved or optimal process setting when the number of factors are 2-4 (Section 5.3.3 in Ref. [161]). To begin with, the same levels were selected with the three main effects, RGDS-Q11, IKVAV-Q11, and YIGSR-Q11 (Table 5.2). In this design, the low levels were 0 mM, yielding negative concentrations by augmenting star points (Figure 2.9). This setting can be changed by scaling down with the value $\alpha = 2^{0.25k}$ ($k$, the number of factors), see Section 2.7. From the three factors ($k=3$), $\alpha = 1.682$. The scaled down CCD is called CCD inscribed (CCDI) as depicted in Figure 2.10. In Table 5.5, the CCDI formulations were laid out. Interestingly, this new design generated two additional levels (concentrations), A and a. These are augmented “star points” in Figure 2.9, resulting in 5 levels in each factor, A, +, 0, −, and a. In Figure 5.6, RGDS-Q11 had the most positive and significant effect to the response (MTS reading). YIGSR-Q11 showed the most negative contribution to the response, meaning that the levels tested in the CCD Inscribed design, YIGSR-Q11 antagonized the growth of HUVECs. The interactions of IKVAV-Q11 with YIGSR-Q11 (IKVAV*YIGSR) also had negative effects on the response. Between 0 and 1.5 mM, IKVAV-Q11 alone did not change the response significantly in Figure 5.6 and 7a and b. Based on the results from the first RSM, it cannot be concluded whether the maximum of the response exists in the levels tested. The response from RGDS-Q11 increases regardless of the other two
Table 5.5 Experimental runs for CCD Inscribed design (the same levels from FEs). RGDS; RGDS-Q11, IKVAV; IKVAV-Q11, and YIGSR; YIGSR-Q11. The unit of each factor is mM. In pattern, + and − means high and low levels, respectively. 0 indicates a center point. A and a means the highest and lowed axial levels, respectively (See Figure 2.9).

<table>
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<td>1.2</td>
<td>0.3</td>
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</table>
Figure 5.6 Results of RSM with CCD Inscribed design (the same levels from FEs). The plot of t-ratio identifies significant main and interaction effects up to the 2nd degree. Blue lines indicate the level ±1.96 which corresponds to significant probability values 0.05.

Estimates are the coefficients of the linear form of the polynomial model (eq.2.2) found by regression.

t-Ratio is a statistic that tests whether the true parameter is zero. t-Ratio and the bar graphs together show the effect of the factors.

Prob > |t| is the probability of getting an even greater t-statistics (absolute value), given the hypothesis that the parameter is zero.
Figure 5.7 Contour plots showing the interactions between two peptides (factors) from the 1st Response Surface Methodology with CCD Inscribed design (the same levels from FEs). Scales bar: values of the responses, absorbance at 490 nm. Higher values indicate higher responses.
factors, IKVAV-Q11 and YIGSR-Q11, as shown in Figure 5.7a and c. In addition, those second order terms (RGDS*RGDS or IKVAV*IKVAV or YIGSR*YIGSR in Figure 5.6) were not significantly different from zero, meaning that the overall response surface did not show significant curvature. This result led to the investigation of two subsequent RSMs; 1) expanding the levels of each factor up to their limit and 2) recentering the levels around the high levels identified from single dose-dependent experiments, i.e., 6 mM of RGDS-Q11, 1.5 mM of IKVAV-Q11, and 6 mM of YIGSR-Q11. The first conditions zoom out the levels to see all possible improvements on the responses; in contrast, the second conditions zoom in to find more precise local levels (concentrations) for best HUVEC growth.

**Exploring wider and focused levels of RGDS-Q11, IKVAV-Q11, and YIGSR-Q11, separately.** Upon expanding the levels of each factor, RGDS-Q11 and IKVAV-Q11 showed positive and significant effects to the response (Figure 5.8). In the levels tested for YIGSR-Q11, it did not show any significant effects the response except the interaction with RGDS-Q11. The interaction of YIGSR-Q11 with RGDS-Q11 (RGDS*YIGSR in Figure 5.8) was barely significant and limited in the concentration range, 0 to 6 mM. In Figure 5.9a and c, the response was dominated by RGDS-Q11, however, the difference was that IKVAV-Q11 showed a synergistic effect with increasing the concentrations (levels) of RGDS-
Table 5.6 Experimental runs for CCD with expanded levels. RGDS; RGDS-Q11, IKVAV; IKVAV-Q11, and YIGSR; YIGSR-Q11. The unit of each factor is mM. In pattern, + and − means high and low levels, respectively. 0 indicates a center point. A and a means the highest and lowed axial levels, respectively (See Figure 2.9).
Figure 5.8 Results of the RSM with expanded levels. The plot of t-ratio identifies significant main and interaction effects up to the 2nd degree. Blue lines indicate the level ±1.96 which corresponds to significant probability values 0.05.

*Estimates* are the coefficients of the linear form of the polynomial model (eq. 2.2) found by regression.

*t-Ratio* is a statistic that tests whether the true parameter is zero. t-Ratio and the bar graphs together show the effect of the factors.

*Prob > |t|* is the probability of getting an even greater t-statistics (absolute value), given the hypothesis that the parameter is zero.
Figure 5.9 Contour plots showing the interactions between two peptides (factors) from the 2\textsuperscript{nd} Response Surface Methodology with expanded levels. Scales bar: values of the responses, absorbance at 490 nm. Higher values indicate higher responses.
Q11 while YIGSR-Q11 did not. The response became antagonistic below 3 mM of YIGSR-Q11 and above around 8 mM of RGDS-Q11. In Figure 5.8, the estimate of RGDS*RGDS was significant and negative, meaning that the coefficient of RGDS*RGDS term in equation 2.3 was negative. This indicated that the response surface had a maximum between the levels 0 and 15 mM of RGDS-Q11. Collectively, at least one local maximum of RGDS-Q11 existed between 0 to 15 mM, and IKVAV-Q11 should be synergistic for the response in the levels between 0 to 6 mM. The effect of YIGSR-Q11 was limited and its levels needed to be changed in the subsequent RSM.

By recentering the levels around (6 mM, 4 mM, 6mM) = (RGDS-Q11, IKVAV-Q11, YIGSR-Q11), the analysis of the response was more focused than the RSM with expanded levels. In Figure 5.10, YIGSR-Q11 showed significant and negative effects to the response, as did the interaction between YIGSR-Q11 and RGDS-Q11 (RGDS*YIGSR). In Figure 5.11b and c, a decrease of the response is accompanied with an increase of YIGSR-Q11 levels in the context of both RGDS-Q11 and IKVAV-Q11. RGDS-Q11 (RGDS*RGDS in Figure 5.10) showed positive effects and indicated the existence of a maximum exists between levels 3 and 9 mM. IKVAV-Q11 showed negative effects and the negative estimate of the second order IKVAV-Q11 term (IKVAV*IKVAV in Figure 5.10) indicated that the maximum of IKVAV-Q11 existed between 2 and 9 mM.
### Table 5.7 Experimental runs for CCD with recentered levels. RGDS; RGDS-Q11, IKVAV; IKVAV-Q11, and YIGSR; YIGSR-Q11. The unit of each factor is mM. In pattern, + and − means high and low levels, respectively. 0 indicates a center point. A and a means the highest and lowed axial levels, respectively (See Figure 2.9).

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<td>000</td>
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</table>
Figure 5.10 Results of RSM with recentered levels. The plot of t-ratio identifies significant main and interaction effects up to the 2nd degree. Blue lines indicate the level ±1.96 which corresponds to significant probability values 0.05.

Estimates are the coefficients of the linear form of the polynomial model (eq. 2.2) found by regression.

$t$-Ratio is a statistic that tests whether the true parameter is zero. $t$-Ratio and the bar graphs together show the effect of the factors.

$\text{Prob} > |t|$ is the probability of getting an even greater $t$-statistics (absolute value), given the hypothesis that the parameter is zero.
Figure 5.11 Contour plots showing the interactions between two peptides (factors) from the 2nd Response Surface Methodology with recentered levels. Scales bar: values of the responses, absorbance at 490 nm. Higher values indicate higher responses.
Combining the results from the two RSMs with expanded and recentered levels, one outstanding result was that YIGSR-Q11 showed a significant antagonistic effect for the growth of HUVECs (response). This result was confirmed by the first RSM (Figure 5.6, CCDI) and the RSM with recentered levels (Figure 5.10). For this reason, YIGSR-Q11 was removed from the pool of factors. This is somewhat unexpected because this peptide has been used as a cell-adhesive ligand for endothelial cells in previous studies [80, 121, 122, 219, 220]. None of the literature reported antagonistic effects of the YIGSR peptide in the context of multiple cell-interactive ligands presentation to date, illustrating that the DOE method utilized here was capable of uncovering synergies and antagonisms between peptides that have heretofore been unknown. The levels of RGDS-Q11 and IKVAV-Q11 were determined by the highest responses in Figures 5.9a and 11a, suggesting that RGDS-Q11 from 8 to 11 mM and IKVAV-Q11 from 2 to 6 mM represented the region of interest. The levels for all the RSM experiments are summarized in Figure 5.12, including the last RSM without the factor YIGSR-Q11.

**Determination of final formulation.** In the preceding RSM, RGDS-Q11 and IKVAV-Q11 showed strong synergistic and positive effects on the response, as evidenced by the t-ratio (Figure 5.13) and the increase of the high-end response values from 0.35 to 0.42. From the contour plot (Figure 5.14), the highest
Figure 5.12 Factor levels tested for the Factorial Experiments and the series of Response Surface Methodology experiments.
Table 5.8 Experimental runs for CCD with two factors. RGDS; RGDS-Q11 and IKVAV; IKVAV-Q11. The unit of each factor is mM. In pattern, + and − means high and low levels, respectively. 0 indicates a center point. A and a means the highest and lowest axial levels, respectively (See Figure 2.9).

<table>
<thead>
<tr>
<th>Run</th>
<th>Pattern</th>
<th>RGDS</th>
<th>IKVAV</th>
</tr>
</thead>
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<td>1</td>
<td>++</td>
<td>13.0</td>
<td>8.0</td>
</tr>
<tr>
<td>2</td>
<td>0a</td>
<td>10.5</td>
<td>2.0</td>
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<td>5</td>
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<td>5.5</td>
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<tr>
<td>6</td>
<td>−+</td>
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</tr>
<tr>
<td>9</td>
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<td>13.0</td>
<td>3.0</td>
</tr>
<tr>
<td>10</td>
<td>−−</td>
<td>8.0</td>
<td>3.0</td>
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</tbody>
</table>
Figure 5.13 Results of RSM with two factors. The plot of t-ratio identifies significant main and interaction effects up to the 2nd degree. Blue lines indicate the level \( \pm 1.96 \) which corresponds to significant probability values 0.05.

\[
\begin{array}{|c|c|c|c|c|}
\hline
\text{Term} & \text{Estimate} & \text{Std Error} & \text{t Ratio} & \text{Prob>|t|} \\
\hline
\text{RGDS(8,13)} & -0.034246 & 0.008023 & -4.27 & 0.0003^* \\
\text{RGDS*IKVAV} & 0.0353 & 0.011346 & 3.11 & 0.0048^* \\
\text{IKVAV(3,8)} & 0.0047975 & 0.008023 & 0.60 & 0.5555 \\
\text{IKVAV*IKVAV} & -0.003452 & 0.010614 & -0.33 & 0.7478 \\
\text{RGDS*RGDS} & 0.0025396 & 0.010614 & 0.24 & 0.8129 \\
\hline
\end{array}
\]

*Estimates* are the coefficients of the linear form of the polynomial model (eq. 2.2) found by regression.

*t-Ratio* is a statistic that tests whether the true parameter is zero. t-Ratio and the bar graphs together shows the effect of the factors.

*Prob > |t|* is the probability of getting an even greater \( t \)-statistics (absolute value), given the hypothesis that the parameter is zero.
Figure 5.14 A contour plot showing the interactions between two peptides (factors) from the last Response Surface Methodology with two factors. Scales bar: values of the responses, absorbance at 490 nm. Higher values indicate higher responses.
responses were shown at the lower levels of RGDS-Q11 and IKVAV-Q11 factors, 8 mM RGDS-Q11 and 3 mM of IKVAV-Q11, respectively. This was indicated in t-ratio plots (Figure 5.13) in that RGDS-Q11 effects decreased from the levels 8 to 13 mM. In contrast, IKVAV-Q11 did not show the similar effect to RGDS-Q11, indicating the significant portion of the response was affected by RGDS-Q11 rather than IKVAV-Q11. Instead of adjusting the levels of the two factors, RGDS-Q11 and IKVAV-Q11, further to improve the response by an unknown magnitude, I hypothesized that the formulation identified out of these RSM experiments would significantly enhance the response (HUVEC growth) over the conventional formulation, as mentioned in Section 5.1, i.e., 6 mM of RGDS-Q11, 1.5 mM of IKVAV-Q11, and 6 mM of YIGSR-Q11.

Multiple comparisons showed the results of RSM improved the response. The determined levels (concentration) from the DOE approaches were 8 mM RGDS-Q11, 3 mM IKVAV-Q11, and 2 mM Q11. The response predicted from that specific formulation was solely determined by DOE approaches. The last step was a validation process to confirm whether that formulation indeed improved the response. The formulations tested during the validation process are summarized in Table 5.9. In Figure 5.15a, the identified formulation (Opt) showed a significantly higher response, compared to all other groups except the positive control fibronectin-adsorbed Q11 (FN) and Opt/IGSE gels. This is a
Table 5.9 A summary of gel formulations prepared for validation processes (MTS, attachment, and immunofluorescence staining experiments)

<table>
<thead>
<tr>
<th>Name</th>
<th>RGDS-Q11 (mM)</th>
<th>IKVAV-Q11 (mM)</th>
<th>YIGSR-Q11 (mM)</th>
<th>Q11 (mM)</th>
</tr>
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<tbody>
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<td>6</td>
<td>6</td>
<td>6</td>
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<tr>
<td>Q11</td>
<td>6</td>
<td>1.5</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Opt</td>
<td>8</td>
<td>3</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Pre-opt</td>
<td>6</td>
<td>1.5</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>RGDS</td>
<td>8</td>
<td>6</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Opt/YIG</td>
<td>8</td>
<td>3</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Opt/RYG</td>
<td>8</td>
<td>6 (RYGS)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Opt/IGSE</td>
<td>8</td>
<td>6 (IGS)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Human plasma fibronectin adsorbed on Q11 gels.

<sup>b</sup> Instead of YIGSR-Q11, RYGSI-Q11 was added.

<sup>c</sup> Instead of YIGSR-Q11, IGSE-Q11 was added.
product of the DOE and the results suggested that the response (the growth of HUVECs for 64 h) is comparable to the positive control. During the DOE approaches, the cell-adhesive ligand factor, YIGSR-Q11 was removed owing to its antagonistic effects on HUVEC growth. It was not clear how the cell adhesive ligand was antagonistic for HUVEC growth. The detailed mechanisms need to be investigated to understand whether that result is due to biochemical cues from the simultaneous presentation of multiple different, cell-interactive ligands, RGDS and/or IKVAV. Another possible explanation is physical interference from the YIGSR sequence. It is known that the bulky tyrosine and arginine residues are mainly responsible for binding of cells, and that they should be separated by an intervening glycine residue [116]. In the scrambled sequence peptide RYGSI, those two bulky tyrosine and arginine residues are still present and that could be why the addition of RYGSI-Q11 did not recover the response (the HUVEC growth at 64 h) (Figure 5.15a). With a new control sequence for YIGSR peptide, IGSE [115], the inclusion of 6 mM of IGSE-Q11 in 8 mM RGDS-Q11/ 3 mM IKVAV-Q11 did recover the HUVEC growth as shown in Figure 5.15a. To further confirm the MTS assay results whether the difference was due to initial attachment or not, the same attachment assay (method described in Section 4.2) was performed. In Figure 5.15b, all other groups showed similar degrees of attachment within 1 h except Q11, which bears no ligands. The extent of HUVEC
Figure 5.15 Experimental validation of identified gel formulations in cultures of HUVECs. An MTS-based growth measurement at 64 h (a), attachment assay after 1 h of seeding (b), and time-course MTS-based growth measurements (c). ** p<0.01 and * p<0.05 compared to “Opt” formulation by ANOVA with Tukey’s post-doc test (mean±SD); n=5 (a), n=4 (b), and n=3 (c).
attachment onto RGDS only gels was less than any of the multiple ligands or FN
gels. This could be due to the display of only one type of ligand. Others except
Opt/IGSE have comparable degree of attachment to Opt gels. IGSE is a random
sequence and does not support attachment of cells, which could explain the lower
degree of HUVEC attachment on Opt/IGSE gels. However, Opt/IGSE gels did
support or at least did not diminish the growth of HUVECs over 64 h, requiring
further investigation of possible signaling pathways initiated by ECM cues. In
monitoring the growth of HUVECs over time using the MTS assay, it was found
that at short time point (1 h), RGDS only gels actually supported significantly
improved cell numbers, but this advantage was diminished over time, resulting in
significantly inferior growth to the optimized gels beyond 24 h (Figure 5.15c, blue
line). The YIGSR-containing gels also showed significantly less degree of
growth from 48 to 64 h. (Figure 5.15c, purple line). It is not clear why the early
time point MTS measurements and attachment assays do not agree with each
other for the RGDS only and YIGSR-containing gels, but it could have arisen
from the different methods the washing steps between the two assays.

This approach could be utilized further in order to adjust multiple
responses such as differentiation or controlled homeostasis of endothelial cells
monolayers. From a materials perspective, this DOE approach is only possible
with a modular construction of materials, which enables a systematic and
independent adjustment of multiple factors. Otherwise, the adjustment of targeted properties could lead compositional drift or significant deviation and inconsistency from predicted responses.

**HUVECs expressing PECAM on the identified gel formulation.** The identified formulation maximally increased the growth of HUVECs for a given time frame; however, an assessment of endothelial cell function was still needed. I wanted to know if the HUVECs on the identified formulation expressed endothelial cell-specific markers such as PECAM to a greater or lesser degree than on unoptimized gels. Immunofluorescence (IF) staining of PECAM was already performed in Section 3.2, and was simply adapted to address this question. Besides using the established 64 h time point, the HUVEC cultures were also extended up to 5 days (120 h). In Figure 5.16, HUVECs on fibronectin-adsorbed Q11 gels (FN) and the identified formulation (Opt) gels did not show any significant difference in PECAM expression or the number of cells presented. The level of PECAM expression was not quite pronounced yet since PECAM is expressed at cell peripheries upon cell-cell contact. The Preopt gels (6 mM RGDS-Q11/1.5 mM IKVAV-Q11/ 6 mM YIGSR-Q11) showed fewer cells and a lesser degree of PECAM expression compared to fibronectin-coated or optimized gels. At 120 h, HUVECs on Opt or FN gels stained with higher levels of PECAM and those on Preopt gels, which showed a lesser degree of PECAM expression.
Figure 5.16 Phase-contrast microscopy images of HUVEC cultures at 64 h (left), immunofluorescence staining of HUVEC cultures at 64 h (middle) and 120 h (right). Green; PECAM, blue; DAPI.
Although IF staining is semi-quantitative, it nevertheless can be concluded that the difference in growth between the optimized gel formulation and the pre-optimized gel formulation is significant at later time points.

5.4 Conclusions and further studies

Using a modular biomaterial construction and Design of Experiment (DOE) approaches, multiple ligand-bearing peptides were integrated into one biomaterial and systematically optimized. With the motivation of providing coating materials for prosthetics, the desired response was to improve the growth of endothelial cells. Four ECM-derived ligand-bearing peptides were designed, synthesized, and characterized to form individual components of the modular system. From single factor dose-dependent experiments, three of them, RGDS-Q11, IKVAV-Q11, and YIGSR-Q11, enhanced the response individually. In Factorial Experiments (FEs), all of them were identified as main factors that controlled HUVEC growth. Through a series of response surface methodology (RSM), YIGSR-Q11 was identified to be antagonistic with respect to the other two peptides, whereas RGDS-Q11 and IKVAV-Q11 synergistically improved the response. These findings of synergism and antagonism would be challenging to uncover in experimental approaches, as evidenced by the previous lack of knowledge of them in the literature. A validation process followed, involving
MTS assays, attachment assays, and immunofluorescence staining aimed at comparing unoptimized and optimized hydrogels. Through this validation process, it was confirmed that the DOE-identified formulation (8 mM RGDS-Q11/ 3 mM IKVAV-Q11/ 2 mM Q11) significantly improved the response over a formulation obtained without the DOE approaches (6 mM RGDS-Q11/ 1.5 mM IKVAV-Q11/ 6 mM YIGSR-Q11).

Interestingly, YIGSR-Q11 has been used as a cell-binding peptide for endothelial cells previously. However, in the context of other ligands such as RGDS and IKVAV, its presence was found to be antagonistic to HUVEC growth. This interesting finding would benefit from a subsequent mechanistic investigation to understand its basis. At present, there are many possible mechanisms, including steric hindrance, where the YIGSR ligand physically occludes the other ligands, or a specific ligand-binding event that results in intracellular signaling that is antagonistic to HUVEC growth. Furthermore, these methods could be used to optimize the materials towards many other responses. As it stands, DOE was only used to optimize the materials for maximal MTS signal at 64h. These methods could be easily extended to other time periods of growth or other biological outputs such as cell survival, expression of specific markers, or quality of the barrier formed by the endothelium. What the research presented in this chapter does indicate is that through DOE approaches and
modular biomaterials, each of these outputs is likely to yield new information regarding the interactions between the factors that drive the responses.
Chapter 6

Nitric oxide-releasing self-assembled peptide hydrogels

6.1 Introduction

Nitric oxide to modulate endothelial cell behaviors. This chapter discusses the synthesis and characterization of a Q11 variant that binds and releases nitric oxide (NO). A brief review of the biological and chemical nature of NO is provided, followed by a description of previous chemistries used to conjugate NO-donor compounds to polymeric or peptide-based materials for the controlled release of NO in therapeutic applications. Unlike other chapters, the work described here is to an extent preliminary, and it needs further resolution for publication. I believe, however, that this ground work and initial characterization of NO-releasing peptides is helpful for adding soluble factors as an additional functionality in synthetic scaffolds.

Nitric oxide is synthesized by nitric oxide synthase (NOS). NOS oxidizes the guanidine group of L-arginine, forming L-citrulline and nitrite (NO$_2^-$), in a process originally reported in 1987 by Hibbs et al. [221]. That same year, two other independent groups identified that the endothelium-derived relaxing factor (EDRF) which is produced and released by the endothelium is nitric oxide [222,
Since then, NO has been known to be a major player in a variety of physiological and pathophysiological pathways [224]. An important biological reaction of NO is S-nitrosylation, which converts a sulphydryl (SH) group to an S-nitrosothiol (RSNO), a reaction that is significant in the post-translational regulation of proteins [225]. In the cardiovascular system, NO is known to act through the stimulation of soluble guanylate cyclase, with the subsequent activation of cyclic guanosine monophosphate (GMP)-dependent signaling, resulting in relaxation of vascular smooth muscles (vasodilation) and increased blood flow [226, 227]. There are also cGMP-independent pathways including the post-translational modification of proteins at reactive cysteine residues to form RSNO [228-230]. An example of the role of S-nitrosylation on cell functions is the inhibition of EC apoptosis. A proapoptotic caspase (caspase-3) was shown to be S-nitrosated at Cys163 as well as other cysteine residues, which was functionally related to NO-mediated inhibition of the caspase signaling cascade [231, 232]. These data suggest the role of NO-mediated signaling regulates vasodilation and cell death via S-nitrosylation. From a pharmacological standpoint, exogenous supply of NO sources via NO-donor compounds or NO-releasing devices would compensate for not only NO shortage, but also local control of blood flow and cell growth/death.
**NO-releasing biomaterials.** A number of biomaterials intended for effective delivery of NO have been designed using two types of donor compounds, NONOates and RSNO (already discussed in Chapter 2.6.3) [233-235]. The carcinogenic byproduct nitrosamine from NONOates limits its potential application as NO-donor compound *in vivo*, but its versatile chemistry for modulating NO-flux and half-life keeps overcoming such limitations to date [236]. RSNO as a NO-donor compound has several advantages. The chemistry to conjugate RSNO to a peptide or polymer is simple compared to that of NONOates. Also in contrast to NONOates, RSNO is produced endogenously and can be converted to NO with catalysis. For this reason, RSNO has a greater potential for therapeutic use, and recently, RSNO has been used in small clinical trials [237]. In biomaterials research, the two donor compounds have both been incorporated into polymers or peptide-amphiphiles to achieve local delivery of NO *in vivo and in vitro*. Upon balloon inflation during angioplasty, the endothelium and underlying smooth muscle cells (SMCs) are often damaged. Such damage can result in occlusion of the blood vessels due to thrombosis or restenosis, which is in part caused by migration and proliferation of SMCs and their secretion of matrix proteins to form occlusive neointimal layers [238-240]. NO has been shown to reduce platelet adhesion and SMC proliferation, while stimulating EC proliferation [241-244]. Therefore, the local delivery of NO to the injured
endothelium may serve a useful tool to reduce thrombosis and restenosis. The conjugation and delivery of NO from polyethylene glycol (PEG) hydrogels has been tested previously for the efficacy of platelet adhesion and SMC proliferation [245]. This system was shown to inhibit neointima formation in a rat balloon-injury model [151]. Towards the same objectives, NONOate was incorporated into polyurethanes [246] or peptide-amphiphiles [247]. In a rat carotid artery injury model, NONOate inhibited neointimal hyperplasia [248]. To promote both the proliferation of ECs and the inhibition of SMCs, laminin-derived YIGSR sequence and NONOate donors were incorporated to polyurethanes [249] or peptide-amphiphile [80].

Given the usefulness of NO-releasing materials for modulating endothelial cell behavior, and given the relative lack of knowledge regarding how NO release may modulate cell responses to other biomaterials-mediated cues such as cell-binding ligands, I aimed to devise a Q11 derivative that could be easily incorporated within Q11 scaffolds and that would predictably release NO. The first step was the conjugation of NO from sodium nitrite to Cys-Q11 peptide as depicted in Figure 6.1. After this, the products of NO-conjugation were verified, and the release of NO from NO-CysQ11 hydrogels was measured by a colomic assay.
6.2 Materials and methods

**Synthesis of NO-donor peptide.** The NO-releasing peptide CysQ11 (Ac-Cys-SGSG-Q11-Am, *m/z* calc’d: 1918.1; found: 1919.3) and control peptide GlyQ11 (Ac-Gly-SGSG-Q11-Am, *m/z* calc’d: 1872.0; found: 1873.1) were synthesized. GlyQ11 had a similar structure to CysQ11 peptide except that it had a sulfhydryl (SH) group on the N-terminal cysteine residue. The absence of sulfhydryl group did not allow NO-conjugation. The protocol for Fmoc-based syntheses and cleavage were the same as described earlier in Section 3.2. The hydrogel formation on inserts as the same as described in Chapters 3-5. Water and buffers were degassed with a stream of nitrogen immediately before experimentation. For fixed concentration experiments, 2 mM of CysQ11 or GlyQ11 in the context of 28 mM Q11 (total 30 mM) were prepared. For dose-dependent experiments, 1.5, 3.0, and 6.0 mM of CysQ11 or GlyQ11 were prepared and the total concentration of each binary mixture was 30 mM. After one day of incubation at 4°C, pH 2 PBS was added to the hydrogels to prevent the formation of disulfides. The PBS added to inserts included 1 mM of tris(2-carboxyethyl)phosphine (TCEP) to further reduce disulfide bonds.

**Coupling NO to CysQ11 and measuring NO-release.** CysQ11 and GlyQ11 (negative control) were reacted with 40 mM sodium nitrite (NaNO₂) in 100 mM HCl for 1 h on a rocker table at room temperature. TEM was utilized to
Figure 6.1 Schematic of NO-conjugation and release from self-assembling peptide.
investigate whether fibrillization was altered before and after NO-conjugation. The conversion of sulfhydryl groups to RSNO was measured by using Ellman’s Reagent [250], which measures the amount of free cysteines. After washing the hydrogels 10 times over 1 h with PBS (pH 2), warm (37°C) HUVEC medium (EGM-2) was added to hydrogels. The plate containing hydrogels on inserts were wrapped with several layers of parafilm and placed in CO₂ incubator at 37°C for multiple NO-release measurements. For fixed concentration (2 mM CysQ11 or GlyQ11) experiments, the production of NO was measured at 0, 24, 48, 72, and 240 h. For dose-dependent experiments, the time points were 0, 0.4, 24, and 48 h. As discussed later in Section 6.3, most of the NO was released within 24 h. In the dose-dependent experiments, therefore, the time points were limited to within 48 h, and a shortest time point that was experimentally feasible from t₀ was added (0.4 h or 24 min) to detect the release NO early in the release process. The production of NO was assessed using the Griess assay (Promega, Cat# G2930), which detects nitrite (NO₂⁻) in the supernatant colorimetrically [251].

### 6.3 Results and discussion

**Reaction conditions for coupling NO to CysQ11.** The chemistry of NO-conjugation to a cysteine residue is based on the work reported by Masters et al. [245]. Unlike the method for conjugating NONOate to lysine containing peptides
this method is based on simple chemistry. The efficiency of the coupling reaction is strongly dependent on pH [252]. The results in Grossi et al. showed that a 1:1 ratio of SH (from cysteine) and NO$_2$ (from sodium nitrite, NaNO$_2$) reacted most efficiently below pH 3.5, and the rate of CysNO production was not changed after 1 h. In these previous experiments, the stoichiometry of CysQ11 and NaNO$_2$ was equimolar. This method has been used to conjugated NO (from NaNO$_2$) to cysteine containing proteins such as bovine serum albumin [253], tissue-type plasminogen activator [254], and cathepsin B, [255]. These methods previously resulted in yields ranging from 85-90% [256], and they were adapted for use with Q11-based hydrogels. Previously these reactions were conducted with the NaNO$_2$ and the protein to be modified dissolved in 500 mM HCl. For hydrogels, the HCl concentration was lowered to 100 mM so as not to disrupt the gels, and the cysteine to NaNO$_2$ ratio was increased an order of magnitude so as to overcome any physical barriers for conjugation.

**Identification of the reaction products (NO-CysQ11).** After selecting the reaction conditions, the chemistry was tested 1) in-tube and 2) on-gel. The in-tube method was easy to conduct since the reactant CysQ11 peptide and NaNO$_2$ were in solution. However, prevention of gelation was not possible even in the absence of salt (using phosphate-citrate buffers instead of PBS). Furthermore, this method required centrifugation and sonication of the samples during washing
steps and after coupling reactions. With this method, no conjugated NO was detected, so an on-gel method was utilized. Previously in Chapter 3, the chemoselective ligation was conducted by adding ligation buffer onto pre-formed CQ11G-thioester hydorgels. Using a similar method, NaNO₂ was added to CysQ11 (or negative control GlyQ11) hydorgels after the formation of hydorgels in inserts. TEM samples were taken from these gels and diluted in PBS (pH 2). The NO-conjugation did not disrupt fibrillization as evidenced by TEM in Figure 6.2. For definitive confirmation of NO conjugation to CysQ11, mass spectrometry was used. However, the significant ionic strength of the samples prevented reliable mass spectrometric analysis, and dialysis was unsuccessful at removing the contaminating salts and NaNO₂.

Since the direct identification of NO-CysQ11 formation was not possible, an indirect method was employed. The measurement of free cysteine or its functional group, SH, was conducted. A colorimetric method using Ellman’s reagent (5,5′-dithio-bis-(2-nitrobenzoic acid) or DTNB) was utilized. A solution of this compound produces a colorimetrically measurable yellow-colored product when it reacts with free SH groups [250]. The result of free cysteine measurement is shown in Figure 6.3a. After 1 h of conjugation of NO to 2 mM CysQ11 or GlyQ11, the amount of free cysteine from both peptides is around 0.25 mM, yielding approximately 88% conversion. GlyQ11 does not possess any SH
Figure 6.2 Transmission electron micrographs of CysQ11 and GlyQ11, showing fibrillization of each peptide before and after NO conjugation with 40 mM NaNO₂ in 100 mM HCl.
Figure 6.3 The measurement of free cysteine concentrations after NO-conjugation 40 mM NaNO$_2$ in 100 mM HCl for 1 h (a) and nitrite concentration at $t_0$ (after 10 washes with PBS (pH 2)) (b). Each peptide, 2 mM in the context of 28 mM Q11. mean±SD, n=3. $t$-test showed no difference.
groups on the residue, and thus it can conclude that the reaction reached near completion as evidenced by almost no available free cysteine from DTNB reaction. To further confirm nearly quantitative completion of the conjugation, initial NO release ($t_0$) was measured. The formation of nitric oxide can be measured by detecting either of two primary, stable, and nonvolatile breakdown products of NO, nitrite ($\text{NO}_2^-$) or nitrate ($\text{NO}_3^-$). Griess assay employs a colorimetric reaction, whereby a pink colored product is formed from a diazotization reaction when sulfanilic acid reacts with nitrites [257]. As discussed earlier in the introduction of the chapter, NO reacts with oxygen species and biological molecules to form a variety of end products, including nitrite ($\text{NO}_2^-$) and nitrate ($\text{NO}_3^-$). Griess reaction detects nitrite ($\text{NO}_2^-$) easily without expensive reagents.

For a more accurate measurement of all degradation products, bacterial nitrate reductase or metals such as cadmium [258, 259] can be additionally employed but were not used here. After extensive washing of CysQ11 and GlyQ11 hydrogels with PBS (pH 2), Griess assay was performed. This washing step was to remove any residual nitrite from NaNO₂. At $t_0$, no detectable amount of nitrite was released from both hydrogels as shown in Figure 6.3b. Collectively, although the conjugation product was not confirmed by a definitive method (mass spectrometry), indirect assays showed that the yield of conjugation was relatively
high (88%) and there was no detectable amount of NO from gels after washing of residual nitrite.

**Release of NO from NO-CysQ11 hydrogels.** After confirming NO-conjugation to CysQ11 hydrogels, a time-course measurement of NO-release was conducted. The first experiment was to measure the duration of NO-release. As shown in Figure 6.4a, the release was biphasic, releasing most of the NO within 24 h. The measured nitrite concentration from the control GlyQ11 hydrogels was significantly lower than CysQ11 hydrogels. The measured nitrite concentrations from both inside and outside of the inserts did not show any difference over the time points (data not shown). Due to its low molecular weight and fast diffusion through barriers (diffusion coefficient estimated 3,300 µm²/s [140]), the equilibrium was reached rapidly.

Instead of investigating the exact kinetics and mechanisms of NO-release from CysQ11 hydrogels, the release profile from CysQ11 hydrogels with different concentrations was sought. In the second experiment, the concentration of CysQ11 was varied from 1.5 to 6 mM at three discrete concentrations. Figure 6.4b shows a dose-dependent response with respect to the concentration of CysQ11 peptide in the hydrogels with balance Q11 peptide. In general, the release primarily occurred in less than 0.4 h, exhibiting an initial burst of NO-release regardless of the concentration of CysQ11. Taken together, the release of
Figure 6.4 Cumulative nitrite concentration from 2 mM CysQ11 or GlyQ11, measured from inside and outside of inserts (a) and multiple concentrations, 1.5, 3.0 and 6.0 mM (b). Total peptide concentration, 30 mM for both (a) and (b). Mean±SD, n=3.
NO from NO-CysQ11 peptide is controllable by varying the amount of CysQ11 peptide in a formulation of a hydrogel. However, the release is biphasic, with an initial burst occurring within an hour. The ideal profile of such release would be more sustained over a desired period of time or a rapid burst at a designated time for effective and timely delivery of NO.

6.4 Conclusions

In this discrete set of experiments, I developed a Q11 derivative that could bind and release NO. The follow-on experiments that would naturally proceed from this work include 1) investigating NO-CysQ11 as a factor in combination with the cell binding ligands, 2) determining whether the NO released by this peptide is biologically active, possibly by suspending NO-releasing Q11 gels over endothelial cells in transwell cultures and conducting growth, apoptosis, and proliferation assays, and 3) seeking strategies to extend the release of NO beyond the initial burst.
Chapter 7

Discussions, future directions, and recommendations

7.1 Introduction

In this section, I will highlight several outstanding issues, unresolved questions, and recommendations for future work. I have broken the topics down roughly by chapter.

7.2 Matrix mechanics (Chapter 3)

Potential disruption of fibrillization by free cysteines. Ellmans’s reagent analysis indicated that there are a number of free cysteines left in the CQ11G-thioester hydrogels after NCL. The disulfide bonds did not significantly contribute to the mechanical properties of CQ11G-thioester hydrogels. However, the influence of unreacted cysteines on gel mechanics should be considered in the presence of cysteine-reactive species, e.g. in vivo, which may disrupt fibrillization by anchoring high molecular weigh proteins onto the gel.

The stiffening mechanism of intra-fibril crosslinking. The exact mechanism mediating increased gel mechanics after NCL was not investigated in detail during this work. Monitoring the course of oligomerization and identifying
the oligomerized species indicated that linear or cyclic dimers were the predominant species. This result suggests that entanglement of linear and cyclic dimers inhibited the sliding of fibrils, resulting in stiffening of the hydrogel fibril networks. This stiffening mechanism can be further investigated.

The limit of linear elasticity of CQ11G-thioester and Q11 hydrogels. Common biopolymer networks such as fibrin clots or actin filaments stiffen as they are strained (strain-stiffening), but this phenomenon is not typically observed with synthetic polymers [260]. Further, coating materials are subject to shear and strain changes during service or placement. Although it would be challenging to completely mimic the mechanical properties of biopolymers from chemoselectively crosslinked hydrogels, it is beneficial to know the limits of the linear elastic behavior of these hydrogels.

7.3 Cell-matrix binding (Chapter 4)

Other potential functionalities to add. In Chapter 4 (and Chapter 5 for this aspect), only endothelial cell-binding sequences were considered, and the utility of Q11-based materials was only tested with this cell type. Other candidates that modulate other cell functions could be considered within the broad scope of tissue engineering applications. For example, most of the cited work in Chapter 3 and 4 described synthetic biomaterials that promote cell adhesion and
growth. A recent study, in contrast, used synthetic biomaterials to provide a cell death ligand to transformed (cancer) breast epithelial cells [261].

**Aortic endothelial cells or stem cells for broad understanding of cell-matrix interactions.** I have also cultured Madin-Darby Canine Kidney (MDCK) cells on ligand-bearing gels or ligated gels. Although no specific change in cell behavior was observed with either functionality, MDCK monolayers were maintained significantly better on Q11 hydrogels when compared to HUVECs. The human ovarian cancer cell line (SKOV-3) did not show any detectable growth difference between 20 and 40 mM Q11 gels. Based on these two cases, I cannot exclude the possibility that the results discussed in this dissertation are limited to HUVECs. To consider the application of Q11-based self-assembling materials in vascular tissue engineering, future projects should characterize aortic cell behavior during culture on Q11, rather than vein cells. In most vascular pathologies requiring surgical intervention, arteries must be repaired or replace, rather than veins, so any targeted model system should focus on arteries, along with their relevant hemodynamic and physiological conditions. Due to their developmental potential, stem cells could also be considered.

**Immunological responses to Q11-based materials.** The immunological response to Q11-based peptides was not addressed in Chapter 4, however, results from immunological experiments were published with the work discussed in
Chapter 4 [86]. Briefly, no immunogenicity was observed for Q11, RGDS-Q11, and 10% RGDS-Q11/ 90% Q11 even in the presence of an adjuvant. It is an encouraging result for potential \textit{in vivo} applications, and more mechanistic studies of immune responses to Q11 system are currently being pursued in our laboratory.

\textbf{7.4 Multiple different functionalities (Chapter 5)}

\textbf{Adding more than two functionalities.} The experiments described in Chapter 5 only incorporated two factors. Based on the results from Chapter 3, CQ11G-thioester can be incorporated without any further characterization. The improved HUVEC growth was from 8 mM RGDS-Q11 and 3 mM IKVAV-Q11 with 2 mM Q11 background, suggesting that the higher concentration of materials is not always favored in cell-material interactions, compared to ECs cultured on 30 mM hydrogels. Again, this formulation is only valid for the growth of HUVECs culture for 64 h. If one intended to target a different response, the statistical adjustment methods should be reapplied from the beginning.

\textbf{Mechanisms of multi-ligand signaling.} A significant finding from this project was the antagonistic effect of YIGSR-Q11 peptides. The scrambled sequence RYGSI-Q11 also showed similar antagonistic behavior in the context of 8 mM RGDS-Q11/ 3 mM IKVAV-Q11/ 2 mM Q11. Another YIGSR-control sequence, IGSE, was able to recover the response (Figure 5.15). The gap in
knowledge here is that intracellular signaling mechanisms are not known. A certain combination of ECM-derived ligands was able to promote HUVEC growth, but these results do not indicate whether a survival or proliferation signal was triggered by the combination of ligands. To understand this, I attempted Western Blotting of Akt and phospho-Akt proteins collected from HUVECs culture on Q11 hydrogels. The serine/threonine kinase Akt has a critical regulatory role in diverse cellular processes including survival, growth, proliferation, angiogenesis, metabolism, and migration [262]. In cells, the ratio of phospho-Akt to Akt is related to the status of proliferation or survival. Ideally, there would be a correlation between the level of phospho-Akt protein expression and the growth of HUVECs on hydrogels with different formulations. The problem, however, was that the total amount of cellular proteins was below the detectable ranges in Western Blotting. On average, I was only able to collect 3-4 µg of proteins per sample, while 10-20 µg of protein is usually required for SDS-PAGE. As an alternative, a sandwich ELISA kit targeting key signaling proteins in pathways controlling growth (Cell Signaling Technology, Cat# 7239) was utilized to detect Akt, phospho-Akt (Ser473 and Thr308), phospho-ERK 1/2 since a sandwich ELISA, in general, is significantly more sensitive than Western Blotting. However, the amount of proteins extracted from HUVECs cultured on hydrogels was not enough to observe any changes in phosphorylation of Akt and
ERK 1/2. Typically, 500 µL of cell lysis buffer is added to a culture in 10 cm dish (area=78.5 cm²) whose surface area is approximately 70 times larger culture inserts (area=1.13 cm²) used for casting hydrogels. This technical challenge can be resolved more easily by modifying the culture conditions such seeding density or gel casting methods rather than assay protocols or sample preparation steps. Additionally, to understand the contribution of each ligand on the increase of HUVEC growth, blocking of integrin receptors with antibodies during HUVEC cultures would identify the integrins involved in the signaling mechanism. However, growth and proliferation is a complex cascade of multiple pathways and crosstalk among many proteins, and involvement of specific integrins would only represent a part of the underlying mechanism.

7.5 Release of soluble factors (Chapter 6)

**Initial burst release of NO.** The initial burst release of NO was the major problem associated with NO-CysQ11 hydrogels. This observation challenges the application of this hydrogel in biomaterials intended for localized and controlled delivery of NO. Re-designing NO-donor compounds and CysQ11 would be challenging. With the Q11-based fibrillizing system, encapsulation of NO-CysQ11 hydrogels in order to add physical barriers may retard and maintain the release of NO. Also, the pH-sensitivity of NO-release may be useful to effect a
NO-release switch in response to changes in local pH. Additionally, as discussed in Section 6.4, the concentrated initial release of NO from NO-CysQ11 hydrogels could be purposely utilized for an NO depot.
Bibliography


31. Holmes, T.C., et al., *Extensive neurite outgrowth and active synapse formation on self-assembling peptide scaffolds*. Proceedings of the


50. Yang, W., et al., *Aggregated polyglutamine peptides delivered to nuclei are toxic to mammalian cells.* Human Molecular Genetics, 2002. 11:2905-2917.


256. Stamler, J.S., et al., *S-nitrosylation of proteins with nitric oxide: synthesis and characterization of biologically active compounds*. Proceedings of the


