INHERENTLY ANTIMICROBIAL HYDROGELS

ALTERING ACTIVITY VIA TRYPTOPHAN/ARGININE INTERACTIONS

by

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A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Honors Bachelor of Science in Biology with Distinction.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Hydrogels are heavily hydrated, viscoelastic, porous materials that show promise as artificial extracellular matrices for use in tissue regenerative therapies. Unfortunately, not only can injected hydrogels make a wound site into an ideal environment for cell proliferation, but for opportunistic bacteria as well. To combat the threat of infection, hydrogels are often modified to display antibacterial activity, usually by impregnating the gel with antibiotic agents or covalently attaching them to the gel surface. The development of hydrogels that are inherently antibacterial has been of great interest to the hydrogel research community. We have developed MAX1, a self-assembling, twenty amino acid peptide hydrogel whose surface exhibits inherent antibacterial activity against several gram-negative and gram-positive bacteria prevalent in hospital settings. Under physiological conditions, MAX1 folds into an amphiphilic β-hairpin and subsequently self-assembles into a highly-crosslinked hydrogel network composed of fibrils rich in β-sheet, but the process is too slow to homogenously encapsulate cells for delivery . Previous attempts to speed self-assembly by reducing the peptide's cationic charge led to a loss of antibacterial activity. This study aims to design, synthesize, and characterize a peptide hydrogel with favorable folding and self-assembly kinetics and potent antibacterial activity through incorporation of cation-π interactions, which are common in antibacterial peptides found in nature. A new peptide sequence (RWMAX1) was designed and synthesized, incorporating a cross-strand Tryptophan/Arginine pair into the MAX1 sequence. The folding and self-

assembly properties were assessed using circular dichroism and rheology and the antibacterial activity was investigated against representative gram-positive and gram-negative bacterial strains *Staphylococcus aureus* and *Escherichia coli*, respectively. RWMAX1 hydrogels were found to possess both rapid folding and self-assembly and potent antimicrobial activity against both bacterial strains, suggesting that Tryptophan/Arginine substitution may be a viable strategy for the development of injectable hydrogel therapies.

Chapter 1

INTRODUCTION

1.1 Peptide hydrogels

Peptide hydrogels represent a novel technology with enormous potential for implanted tissue regenerative therapies(1-7). These therapies aim to improve wound convalescence, especially in tissues – like avascular cartilage – that do not heal or heal poorly on their own. At present, tissue regenerative therapies depend upon one of two general strategies – implantation of either a foreign substance or alternately of cells isolated from other parts of the patient's body. In both strategies the scaffolding used to deliver the payload is of critical importance, and for many therapies appropriate scaffolding has not yet been developed.

Peptide hydrogels are attractive options from both a utility and design standpoint(11). A properly-designed self-assembling peptide hydrogel can act as an artificial extracellular matrix, giving implanted cells a scaffolding on which to adhere and conditions conducive to cell proliferation and wound healing. Such a peptide could be used to deliver cells along with growth factors, antibiotics, or other payloads in a minimally invasive manner, and subsequently keep them localized at the wound site.

From a design standpoint, peptide hydrogels offer a number of distinct advantages. First of all, peptide chemistry is relatively well-understood, with many thousands of naturally-occurring proteins from which to draw inspiration. The twenty natural amino acids offer a great deal of flexibility (as, again, the abundance and

diversity of natural proteins attests), including several well-studied secondary structures. More importantly, however, designing peptide hydrogels as a bottom-up system, where the complex self-assembly of the final material depends upon properties of a simple monomer, gives an enormous amount of control over the final material. As the bulk hydrogel's properties descend directly from the primary sequence of its constituent peptide, alterations to the sequence can allow the resultant hydrogel to be adjusted to adopt certain behaviors or characteristics.

1.1 MAX1 structure, self assembly, and physical properties

MAX1 (VKVKVKVKVPP^{D}TKVKVKVKV-NH_2), the Schneider lab's parent peptide, exhibits many properties that could be valuable in a peptide hydrogel (8). MAX1 is a 20-residue oligopeptide consisting of alternating valine and lysines flanking a type II' turn-forming tetrapeptide sequence. In solution at neutral pH, MAX1 is fully soluble and adopts a random-coil conformation due to repulsion between its protonated lysine residues (figure 1). With the addition of a folding trigger – which can include increasing pH to deprotonate lysines and reduce repulsion (9) or increasing local ionic concentrations to screen the repulsive charges(10), among others – the peptide folds into an amphipathic β-hairpin with a defined valine-rich hydrophobic side and lysine-rich hydrophilic side. In this conformation, the peptide rapidly self-assembles into a fibrillar hydrogel. Self-assembly is driven by intermolecular facial interactions between hairpins in the hydrophobic collapse of valine-rich regions and intermolecular lateral interactions in hydrogen bonds between the peptide backbones and Van Der Waals contacts between valines on adjacent hairpins(9). MAX1 β-hairpins assemble into bilayered fibrils, which themselves form the fibrillar hydrogel network. Entanglements between fibers and branch points due to packing errors lead

to non-covalent crosslinks, increasing the hydrogel's rigidity. Self-assembly can happen in minutes or take hours, depending on the peptide's concentration and environment(9).

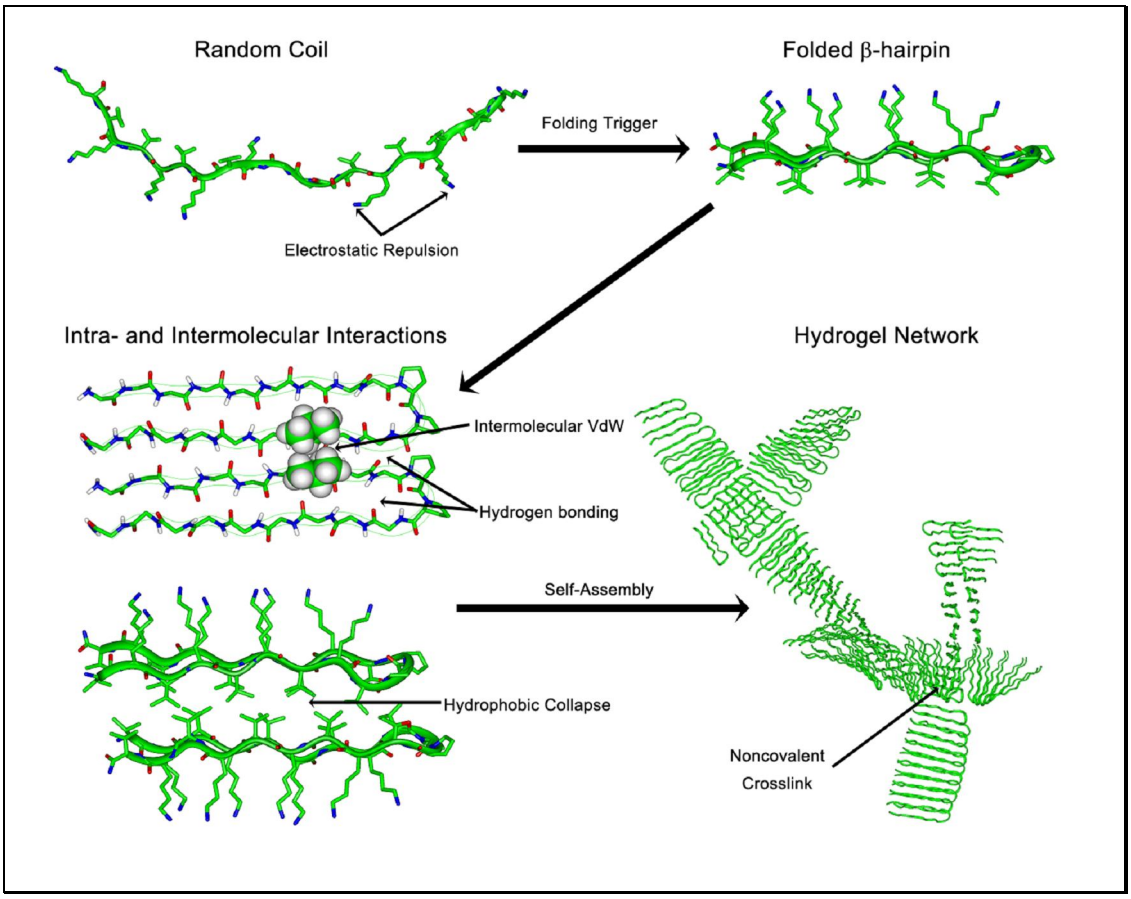

Figure 1 The self-assembly scheme and major forces driving assembly in MAX1.

MAX1's self-assembly scheme gives it valuable self-healing properties. Shear strain can disrupt a preformed hydrogel network, breaking fibrils and reducing the gel's rigidity enough that it can flow, but upon removal of the strain, MAX1 hydrogels will quickly recover to 80% or greater of their original rigidity(11). This has

important ramifications – MAX1 hydrogels can be premade in syringes and then injected into wound sites, where they will rapidly reform without permanent damage to the hydrogel network. This non-invasive delivery mechanism is a distinct advantage over many implant systems, which require surgical implantation that carries greater risk for infection and longer convalescence times.

MAX1 has several useful biofunctional properties. Most importantly, murine mesenchymal stem cells introduced onto MAX1 hydrogels adopt healthy morphologies and grow indefinitely(8). Further, cells encapsulated in this way are not damaged by the shear forces involved in syringe injection(11), opening up the possibility that MAX1 hydrogels could be used as stem cell delivery vehicles. Stem cells, growth factors, drugs, and other payloads could be mixed into a peptide solution inside of a syringe, the peptide given time to self-assemble into a hydrogel, and then ultimately injected into a wound site where the hydrogel would recover and keep the cells localized in a hydrated, biocompatible environment conducive to cell growth(16). MAX1 in a wound site would also be biodegradable and could steadily be replaced by nascent tissue.

1.3 MAX1's antibacterial activity

One potential complication for all implantation-based therapies is the threat of bacterial infection. Biomaterial-centered infections represent more than half of nosocomial infections(12), and are often very difficult to treat, not only due to interference from the body's foreign body response but also due to the formation of durable bacterial biofilms on implant surfaces(16). Implant-associated bacterial infections lead to chronic inflammation and often necessitate surgical removal of the implant(16). This issue remains relevant for hydrogel-based systems which, by virtue

of providing environments conducive to cell proliferation, provide environments conducive to bacterial proliferation as well(13). Some scaffolding systems attempt to resolve this problem by covalently attaching antibiotic species to an implant or encapsulating antibiotics within a hydrogel network(14,15). MAX1, however, is inherently antibacterial, displaying potent, broad-spectrum activity against both gram-negative and gram-positive bacteria(16). Obviously this is a very valuable property for implanted materials and could be invaluable in avoiding implant-associated infections and the development of quick-and-dirty therapies, especially for use in nonsterile conditions.

The mechanism of MAX1's antibacterial activity is not presently known. The molecule resembles many natural antibacterial peptides in its size, amphiphilicity, and cationic charge – most notably poly-lysine, known to have moderate antibacterial activity(17,18). MAX1's cationic charge may contribute towards its antibacterial activity by helping the peptides interact with and bind nonspecifically to negatively-charged bacterial surface components. Most antibacterial peptides are believed to kill bacteria by inserting into the membranes and forming pores, thereby lysing the target cell(19), and we believe MAX1 may operate much the same. Experiments measuring the leaking of intracellular (and pericellular, in the case of gram-negative bacteria) components suggest that bacterial membranes exposed to MAX1 are disrupted(16), though it remains unknown if this is the killing action or secondary to cell death. It is critical to note, however, that unlike most antibacterial peptides (including poly-lysine), MAX1's antibacterial activity appears to be dependent upon a surface interaction between the bacteria and an assembled hydrogel. The soluble peptide and semi-assembled soluble peptide aggregates display no antibacterial activity(16). Confocal

microscopy live-dead assays (figure 2) suggest that MAX1 hydrogel surfaces lose activity only when inoculated with so many bacteria that the cellular debris remaining after initial bacteria contact the surface becomes extensive enough to shield later cells. Perhaps the surface limitation of MAX1's activity reflects a need for many simultaneous peptide/membrane interactions.

A second commonly-cited mechanism for cationic antibacterial peptides involves the disruption of the cell walls of bacteria. Both gram-negative and gram-positive bacteria are surrounded in a thick layer of negatively-charged protein or polysaccharide (lipopolysaccharide and teichoic acid, respectively) stabilized by salt bridges with divalent cations(18). Some cationic antibacterial peptide families, such as lactoferricin derivatives, defensins, magainins, cecropins, melittins, and indolicidins, have been found to bind to LPS or teichoic acid and either displace or sequester the stabilizing cations (18,20-21). This may destabilize the cell wall as well as the membrane to which cell wall components are anchored. Other evidence exists that suggests that the cell wall plays a critical role in antibacterial peptide activity – antibacterial peptide-resistant *Salmonella typhimurium* strains were found to have mutated LPS with reduced negative charge, while mutants with higher negative charge were highly sensitive (19).

While membrane and/or cell wall disruption are the most commonly proposed mechanisms for antibacterial peptide activity, evidence for intracellular targets exists as well. In many cases, while membrane penetration is evident, it nonetheless lags significantly behind actual bacterial death (22-23), suggesting that membrane permeabilization merely allows the peptides access to an intracellular target.

Some classes of antibacterial peptide, like the defensins, have been shown to be largely inactive in metabolically-deactivated cells (19).

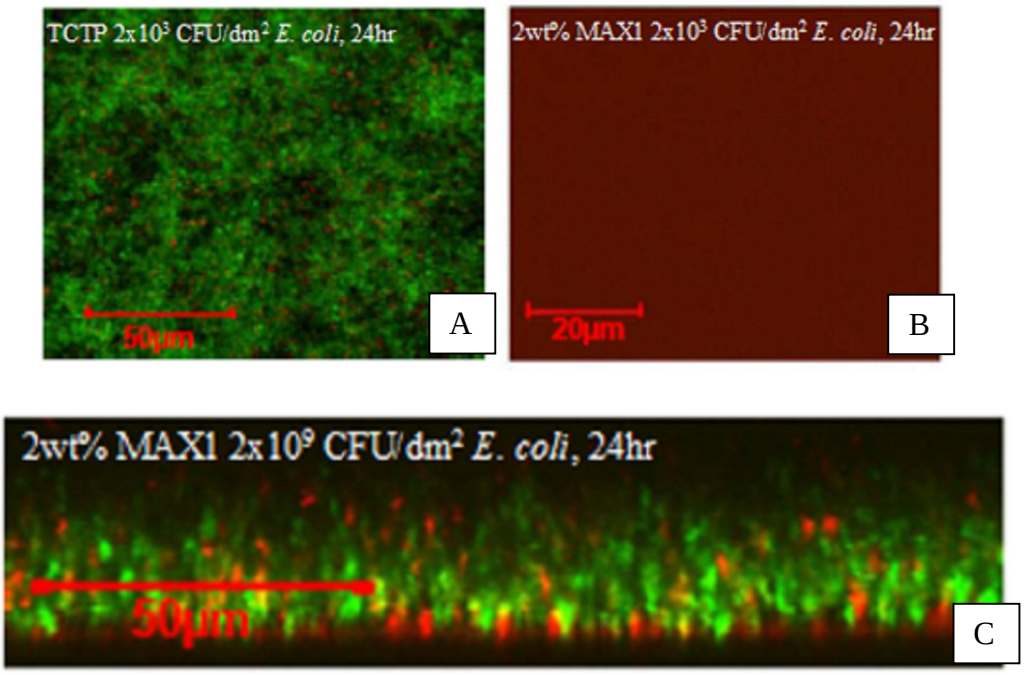

Figure 2 LSCM images of $2x10^3$ CFU's/dm^2 *E. coli* on TCTP (A) or 2wt% MAX1 hydrogel (B) surfaces. Live/dead assay where green cells (stained with Syto 9) are alive and red cells (propidium iodide) are dead cells with compromised membranes. (C) LSCM projection of $2x10^9$ CFU's/dm^2 *E. coli* overwhelming a 2wt% MAX1 hydrogel surface, viewed parallel to the gel. (16)

MAX1's antibacterial activity is selective – coculture experiments wherein murine and bacterial cells were simultaneously plated onto MAX1 hydrogels showed potent antibacterial activity without harming the eukaryotic cells(16). As with naturally occurring antibacterial peptides, this selective activity is believed to reflect differences

in the membrane composition of eukaryotic and prokaryotic cells(24-26) – the outer leaflets of eukaryotic cell membranes are generally made of neutrally-charged lipids like phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin, compared to the negatively-charged bacterial membrane lipids phosphatidylglycerol, cardiolipin, and phosphatidylserine, and thus the cationic peptides' initial interactions may be weaker, leading to reduced membrane-disrupting ability(16). It is worth noting, however, that bacterial and eukaryotic cells alike sport neutral and negatively-charged species on their surfaces, and so another mechanism may be critical for selectivity(23, 27-28). This possibility is further supported by the fact that some antibacterial peptides show greater action against neutral bacterial membranes than negatively-charged eukaryotic membranes (19).

Antibacterial peptides offer a distinct advantage over traditional antibiotics in being relatively difficult for bacteria to develop resistance against(26). Traditional antibiotics often depend upon specific interaction with receptors or the active sites of enzymes to disrupt cell wall synthesis, DNA replication, translation, or other critical processes(19). The specificity of these mechanisms often makes the evolution of antibacterial resistance trivial – mutation of a few residues on the target protein may disrupt antibiotic binding while retaining function for the bacterium. Antibacterial peptides may offer some reprieve from this behavior. Though it varies, many antibacterial peptides appear to have nonspecific mechanisms, acting upon broad and highly conserved cellular structures like the lipid bilayer(19). As these structures cannot simply be mutated, evolving resistance to these mechanisms is likely to be much more difficult and rarer, allowing these peptides to be used more widely without fear of them growing obsolete. As the evolution of antibacterial resistance is a serious medical

issue, especially with fewer and fewer antibiotics being discovered, this is a very valuable property. It is important to note, however, that while many antibacterial peptides do not appear to be highly sequence specific, many do(19). Resistance can be developed against antibacterial peptides, generally via development of enzymes to degrade them, so care would still need to be taken in how antibacterial hydrogels are employed(29).

1.4 Two design concerns

The criteria for a successful hydrogel scaffolding for therapeutic use are of course many, but self-assembly kinetics and antibacterial activity may be of especial significance. Future descendents of MAX1 must be built with these two properties in mind.

Adequately swift self-assembly kinetics are critical for proper hydrogel function. Hydrogels that cannot self-assemble (or recover after disruption) quickly enough run the risk of leaking out of a wound site into the surrounding tissues, unless a very well-defined cavity is made available(9). A critical worry as well comes in encapsulation of cells. Cells are encapsulated into MAX1 hydrogels by mixing them into a peptide solution and allowing the gel to set. If setting takes too long, the cells sediment out and a heterogeneous encapsulation results (figure 3). As cell behavior is strongly influenced by local density(30-31), this is to be avoided(11).

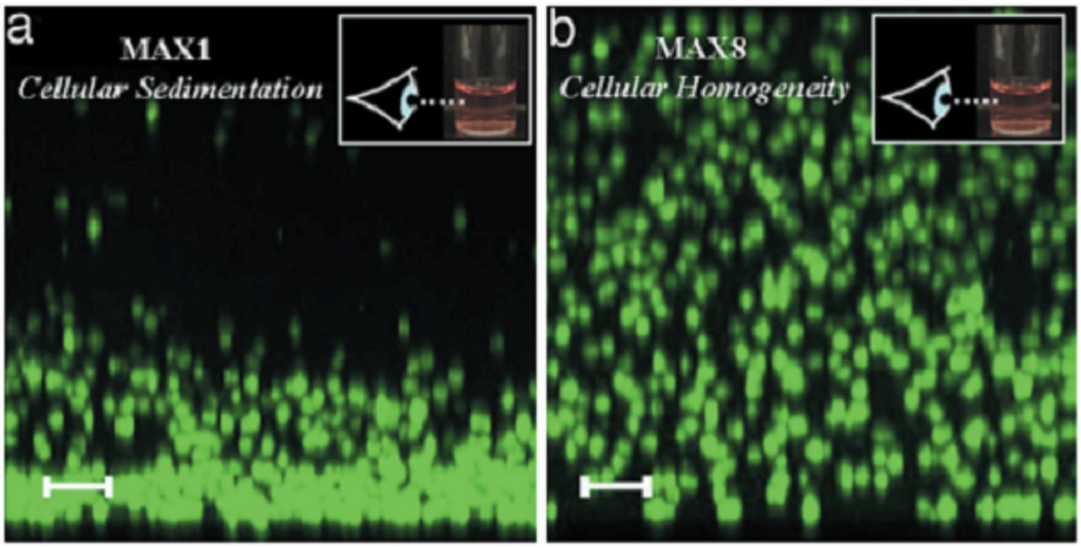

Figure 3 LSCM projection of cell encapsulation of C3H10t1/2 stem cells in .5wt% MAX1 (A) and MAX8 (B) hydrogels, showing heterogeneous and homogenous encapsulation, respectively. Scale bar represents 100μm (11).

In physiological conditions, MAX1 does not fold or self-assemble rapidly enough to homogenously encapsulate cells(11). Repulsion between protonated lysines at pH 7.4 is only barely overcome by charge screening by local ionic concentrations. In light of this shortcoming, the Schneider lab developed MAX8 ($VKVKVKVKVPP^{D}TKV\underline{\mathbf{E}}VKVKV\text{-}NH_2$), which replaces the lysine at MAX1's position 15 with a glutamic acid, reducing the peptide's charge from +9 to +7 (11). This change resulted in a marked increase in self-assembly speed, as the positive charge repulsion between lysines was much reduced. MAX8 hydrogels self-assemble much faster than MAX1 hydrogels, even at physiological conditions, and are considerably more rigid (figure 4), perhaps owing to more extensive self-assembly or new

intermolecular salt bridges between negatively charged glutamic acid residues and positively charged lysines(11).

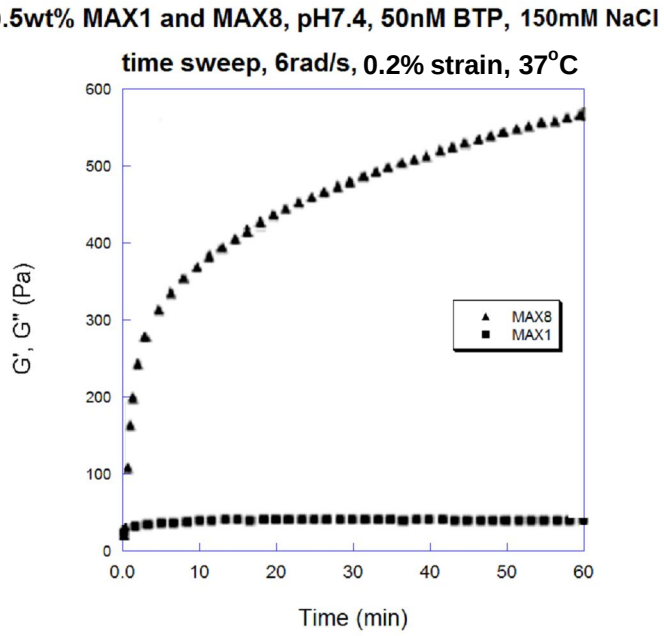

Figure 4 Oscillatory shear rheology of 0.5% MAX1 and MAX8 hydrogels, monitoring storage modulus (G') as a function of time. (11)

Unfortunately, while MAX8 could homogeneously encapsulate cells, the substitution came at the cost of its antibacterial activity (unpublished data). The reason for this loss of activity is not known, but may relate to the reduced cationic charge. Cationic charge is associated with many naturally occurring antibacterial peptides, where it may function to help attract the peptides to the negatively-charged bacterial surfaces(19).

MAX1 and MAX8 thus both possess attractive properties for a therapeutic peptide hydrogel. The tradeoffs between MAX1 and MAX8 may hinge primarily upon their cationic charge, and so a new strategy is necessary in order to design a peptide hydrogel that can incorporate the advantages of both parents - both the rapid folding and self-assembly kinetics of MAX8 and the potent antibacterial activity of MAX1.

1.5 RWMAX1

RWMAX1 (VKV**R**VKVKVPP^{D}TKVKVKV**W**V-NH_2) was designed in an attempt to balance both the physical and antibacterial properties of a hydrogel. An arginine and a tryptophan residue were incorporated on the solvent-exposed hydrophilic side on MAX1's residues 4 and 19, respectively, resulting in a peptide with a +8 charge, intermediate between MAX1 (+9) and MAX8 (+7). The arginine and tryptophan residues were placed on the peptide's terminii in the interest of producing a solvent-exposed cation-π interaction. Exact positioning was decided based upon computer modeling and partly inspired by the staggered RW-containing β-sheet designs developed by the Waters' lab(32).

It was hypothesized that RWMAX1's design would self-assemble faster than MAX1 and into a more rigid gel based upon its reduced cationic charge, which, as in MAX8, would reduce the repulsion between protonated lysines and stabilize the folded peptide at lower pH values. The presence of a cross-strand cation-π interaction was also expected to stabilize the folded hairpin – interactions of this type in proteins typically contribute a stabilization energy of 0-2.4kcal/mol (33), roughly equivalent to that of a hydrogen bond.

Due to the reduction in cationic charge, one might expect RWMAX1's antibacterial activity to be reduced. The inclusion of tryptophan and arginine – as opposed to reducing the peptide's charge with some other substitution – was intended to compensate for this expected loss. Tryptophan and arginine are very common residues in antibacterial peptides found in nature– one class in particular, the indolicidins, are particularly rich in tryptophan and arginine (and in fact have the highest tryptophan content of any known natural peptide) (34). Numerous experiments have shown that the introduction of tryptophan and arginine (and to a lesser extent tryptophan and other cationic residues capable of forming cation-π interactions with it like lysine) into a peptide can increase its antibacterial activity, even in peptides as short as five or six residues(20, 23, 35-39). Some of these experiments suggest that the tryptophan/arginine activity is highly specific (36), while others suggest the opposite (37).

The mechanism of tryptophan and arginine's effect on antibacterial activity is not known, but it is believed to relate to membrane permeabilization(21). Tryptophan has an interesting interaction with membranes – while a hydrophobic residue, it tends to burrow only partway into lipid bilayers, into the 'interfacial' region between their hydrophobic tails and hydrophilic heads(40) and, in fact, has been shown to burrow deeper into more negatively-charged bacterial membranes than neutral eukaryotic membranes, perhaps enhancing an antibacterial peptide's selectivity. In antibacterial peptides, tryptophan may thus act as an anchor, affixing peptides to bacterial surfaces. Further, however, tryptophan's large π electron systems can be used to 'shield' a cationic charge from the hydrophobic environment(21). Though all cationic residues appear to be capable of this interaction, the arginine-tryptophan interaction is

most stable, as arginine's delocalized cationic charge retains the ability to hydrogen bond with other residues while interacting with tryptophan. Exactly what the shielded arginine may do at the membrane's interfacial region is unknown, but it is presumed that it plays a role in destabilizing the membrane perhaps, as aforementioned, by displacing stabilizing cations or contributing to the formation of a pore. Some tryptophan-containing antibacterial peptides, at least, seem to depend upon interaction with the negatively-charged inner core of LPS in order to kill gram-negative bacteria, such that mutant bacteria lacking LPS are resistant (23). Tryptophan/arginine interactions have also been shown to stabilize antibacterial indolicidins at bacterial surfaces (41), and may also play key roles in selectivity, especially against the durable gram-negative bacteria *Pseudomonas aeruginosa* and most gram-positive bacteria (35).

Of further interest, however, are penetratins, a class of peptides capable of freely crossing cell membranes and, in some cases, even ferrying attached molecules through like cargo(40). While the mechanism of penetratin movement is unknown, it depends upon short arginine- and tryptophan-rich sequences. The antibacterial activity of arginine and tryptophan may thus be more related to allowing peptides into cell interiors to act upon intracellular targets, rather than or in addition to assisting in membrane disruption.

By reducing the cationic charge of the peptide and introducing a compensatory increase in antibacterial activity via the inclusion of the tryptophan and arginine residues, I aimed to produce a peptide that could both fold and self-assemble quickly at physiological conditions and effectively kill bacteria.

Chapter 2

MATERIALS AND METHODS

2.1 Peptide synthesis and purification

MAX1 and RWMAX1 were synthesized via Fmoc-based, HCTU-activated solid phase peptide synthesis techniques on an ABI 433A automated synthesizer. The newly-synthesized, resin-bound peptide was cleaved and deprotected using a cleavage cocktail of trifluoroacetic acid/thioanisole/ethanediol/anisole at a 90:5:3:2 ratio for 2 hours under a nitrogen atmosphere, then precipitated using cold diethyl ether. The free peptide was purified via RP-HPLC using a linear gradient between solvent STDA (.1% TFA in water) and solvent STDB (.1% TFA, 90% acetonitrile, 10% water) at .5%STDB/min (table 1). Purified peptide solutions were lyophilized to yield the TFA peptide salt. Peptide purity was verified using ESI-Mass spectroscopy and RP-HPLC.

Table 1 HPLC purification gradient.

Time (min)	%STDA	%STDB	%STDB/min
0	100	0	
2	100	0	0
10	81	19	2.375
172	0	100	0.5

2.2 Oscillatory shear rheology

Dynamic time, frequency, and strain sweeps were performed using a rheometer with a 25mm parallel plate geometry at a 500μm gap height. 2wt% hydrogels were prepared by combining equal volumes of 4wt% peptide in cold purified water and stock buffer solution. Formation of the gel network was monitored for 1 hour immediately after preparation of the 2wt% hydrogels by tracking storage modulus G' and loss modulus G'' at a fixed strain amplitude of .2% and fixed frequency of 6 rad/s. Dynamic frequency sweeps were performed at a strain amplitude of .2% and frequencies ranging from .1 to 100 rad/s. Dynamic strain sweeps were performed at a frequency of 6 rad/s and strain amplitudes ranging from .01 to 1000% strain. All experiments were performed at 37°C.

2.3 Circular dichroism

Experiments were performed on a J-810 spectropolarimeter with 1mm quartz cells. 150mM peptide solutions were prepared by combination of equal volumes of 300mM peptide in cold purified water and various stock buffer solutions. Wavelength sweeps were performed from 190nm to 260nm every 5° C from 5°C to 80°C, followed by a final scan after the cells had been allowed to cool back to 5°C.

MAX1 and RWMAX1 stock solution concentrations were determined by absorbance at 200nm (with MAX1's γ_{200}=15750cm$^{-1}\cdot$M^{-1}) or 278nm (RWMAX1's γ_{278}=5579cm$^{-1}\cdot$M^{-1}), respectively. Mean residue ellipticity was calculated with the equation $[\theta]=(\theta_{obs}/10\cdot l\cdot c)/r$, where θ_{obs} is measured ellipticity in mdeg, *l* is cell length in cm, *c* is concentration in M, and r is the number of residues in the peptide.

2.4 Cell culture

Bacterial strains were prepared from powdered bacteria (*S. aureus* ATCC 25923, *E. coli* ATCC 26922) suspended in 1mL Tryptic Soy Broth (TSB; Bacto 211824) and quadrant streaked onto Trypticase Soy Agar plates with 5% sheep blood (BBL221239). Bacteria were maintained in incubators at 37°C. Approximately every 24 hours bacteria from the fourth quadrant were transferred to a fresh plate. To ensure consistency, new bacterial strains were not used for antibacterial assays before 72 hours (3 transfers) had passed, nor were strains used past the age of approximately four weeks.

2.5 Antibacterial assays

MAX1 or RWMAX1 hydrogels were assayed for antibacterial activity in 96-well tissue culture-treated polystyrene plates (Costar 3595). 24 wells were prepared by introducing a 35 μL aliquot of 4 wt% peptide stock solution (containing 35mg MAX1 or RWMAX1 in 875 μL sterile H_2O), followed by 35 μL of serum-free Dulbeccos Modified Eagles Medium (DMEM; Sigma D6546) to induce gelation. The resulting hydrogels (2 wt%) were incubated at 37°C for 2 hours. 200 μL DMEM was

then added above each well and the gels were allowed to equilibrate at 37°C overnight. This excess media was removed prior to each assay. Empty TCTP wells in the same plates were left untreated to act as controls.

Stock solutions of bacteria were prepared by suspending one bacterial colony from the fourth quadrant of the agar plate in 1mL TSB. This suspension was diluted with additional TSB until its optical density reached .1AU at 625nm, corresponding to a stock solution of 10^8 colony forming units/mL.

100μL of sterile TSB was added atop the hydrogel or TCTP surface of 42 wells (3x7 hydrogel-containing wells and 3x7 TCTP controls). 111μL of the 10^8 CFU/mL stock solution was then added to the eighth and final column of wells and serially diluted 1:10 down the row, producing final inoculation densities of $2x10^3$, $2x10^4$, $2x10^5$, $2x10^6$, $2x10^7$, $2x10^8$, $2x10^9$, and $2x10^{10}$ CFU's/dm^2.

Bacteria were allowed to incubate at 37°C for 48 hours. Each well was then diluted with the addition of 100μL TSB and its optical density measured at 625nm. OD was corrected for dilution and scattering associated with each individual strain (see appendix).

Chapter 3

RESULTS

3.1 Synthesis and purification

Peptides were synthesized using Fmoc-based, HCTU-activated solid phase peptide synthesis techniques on an automated ABI synthesizer. Synthesis progress was gauged via detection of UV-active products of the deprotection reaction at 304nm. The resulting deprotection profile reveals that the vast majority of peptides completed each cycle correctly. Some loss is expected with each cycle due to terminal residues becoming buried and shielded from potential reactants.

Peptide was purified on HPLC using a linear gradient of STDA (.1% TFA in water) to STDB (.1% TFA, 10% water, 89.9% acetonitrile) at .25% STDB/min. RWMAX1 eluted at 39% STDB compared to MAX1's previously determined value of 34%, consistent with its increased hydrophobicity (figure 5). Peptide identity was also confirmed using ESI mass spectrometry (figure 6).

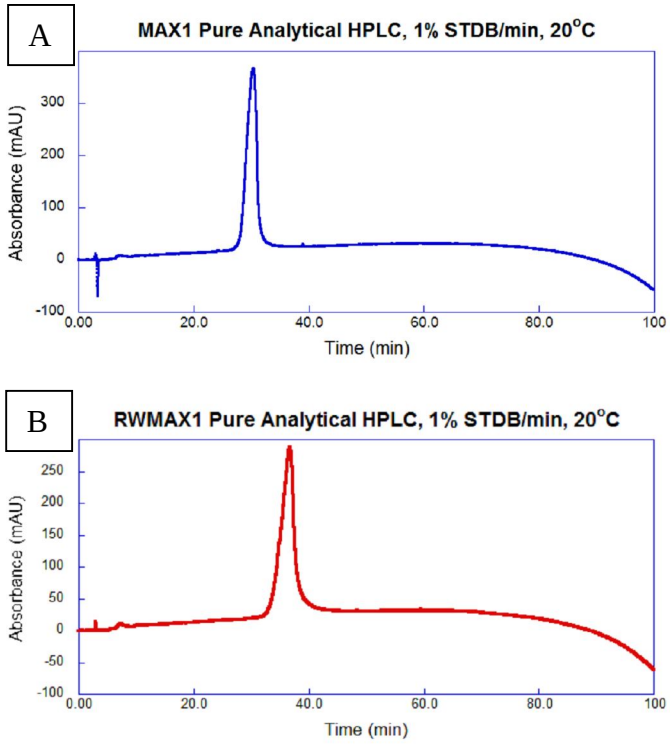

Figure 5 Analytical HPLC of 2µL purified MAX1 (A) and RWMAX1 (B) from STDA (.1% TFA in water) to STDB (.1%TFA in 10% water, 89.9% acetonitrile) at 1%STDB/min.

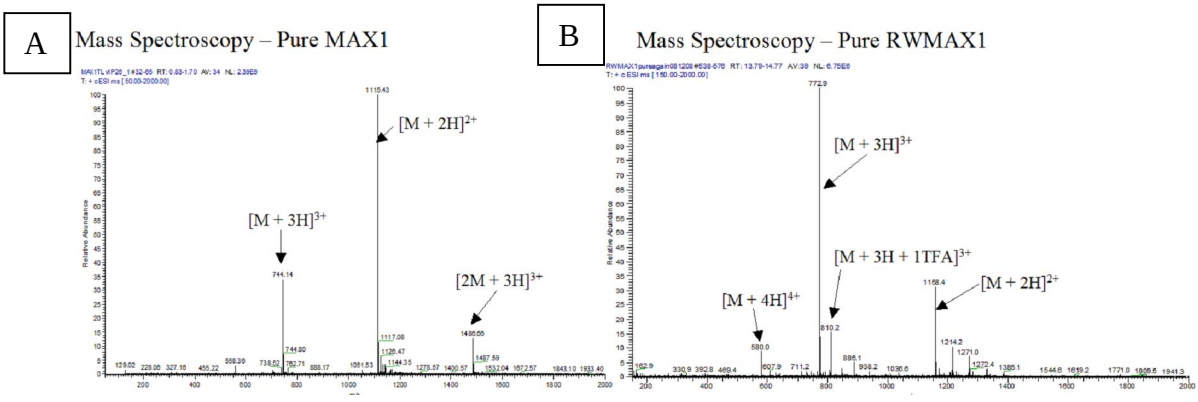

Figure 6 ESI mass spectroscopy of purified MAX1 (A) and RWMAX1 (B).

3.2 Circular dichroism

Onset of β-sheet formation (and thus, presumably, self-assembly) was measured using circular dichroism, measuring the mean residue ellipticity at wavelengths between 200 and 260nm (figure 7). Like MAX1, RWMAX1 shows a distinct minimum at 216nm at higher pH's and higher temperatures, indicating more extensive β-sheet, and thus presumably self-assembly, in these conditions. As expected by its design, RWMAX1 showed strong minimums even at lower temperatures and pH values, suggesting more extensive folding and self-assembly.

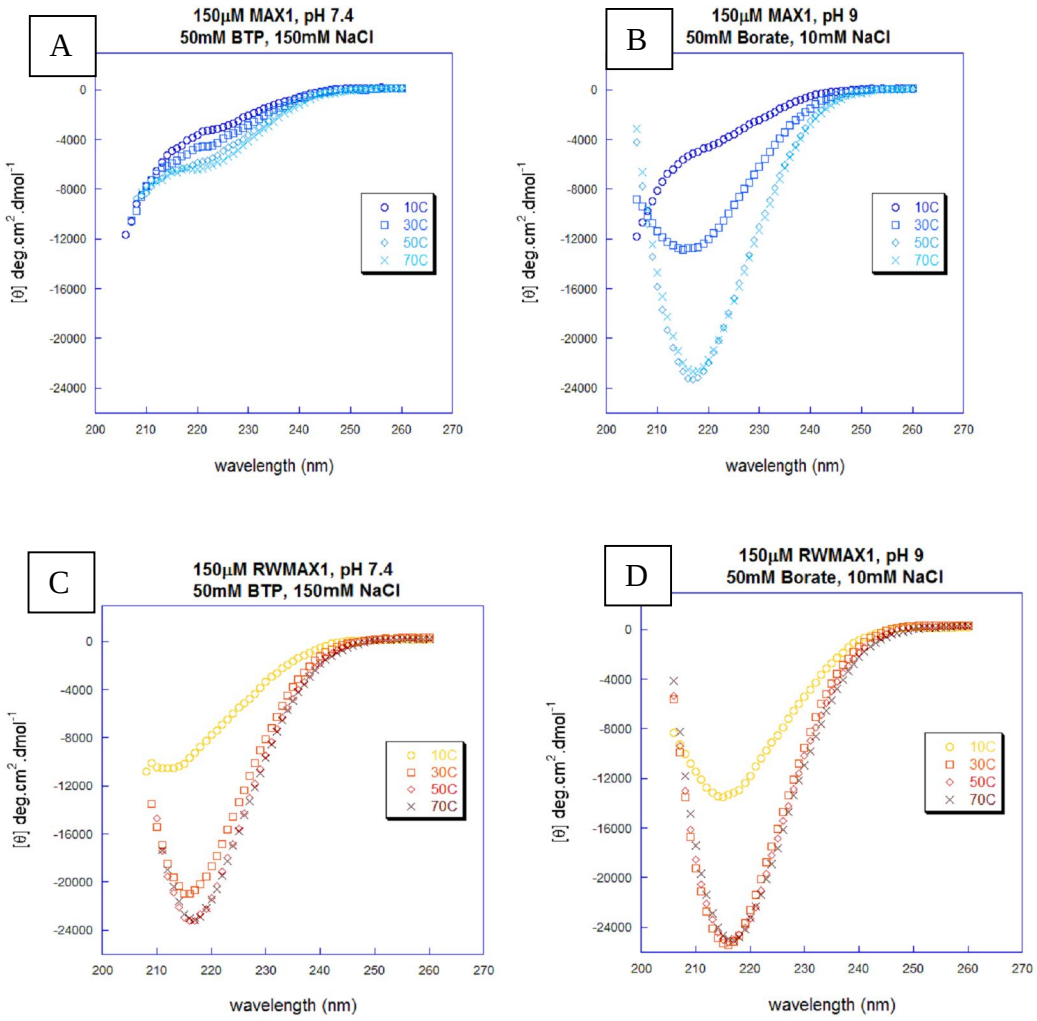

Figure 7 Circular dichroism measuring mean residue ellipticity as a function of wavelength and temperature of 150µM solutions of MAX1 at pH 7.4 (A) and pH 9 (B) and RWMAX1 at pH 7.4 (C) and pH 9 (D).

3.3 Oscillatory shear rheology

Gelation of peptide solutions was measured using oscillatory shear rheology. Samples were measured over time (figure 8) and with varying frequencies

and strains (see appendix). Both gels display increasing storage modulus (G'), and thus rigidity, over time. Both gels retain their rigidity across all tested frequencies. Both gels yielded at approximately 10-12% strain. Both gels exhibit higher final storage moduli at higher pH values, consistent with the circular dichroism data. As expected, RWMAX1 reached a much higher maximum storage modulus than MAX1 gels of the same concentration, again suggesting more extensive folding and self-assembly behavior.

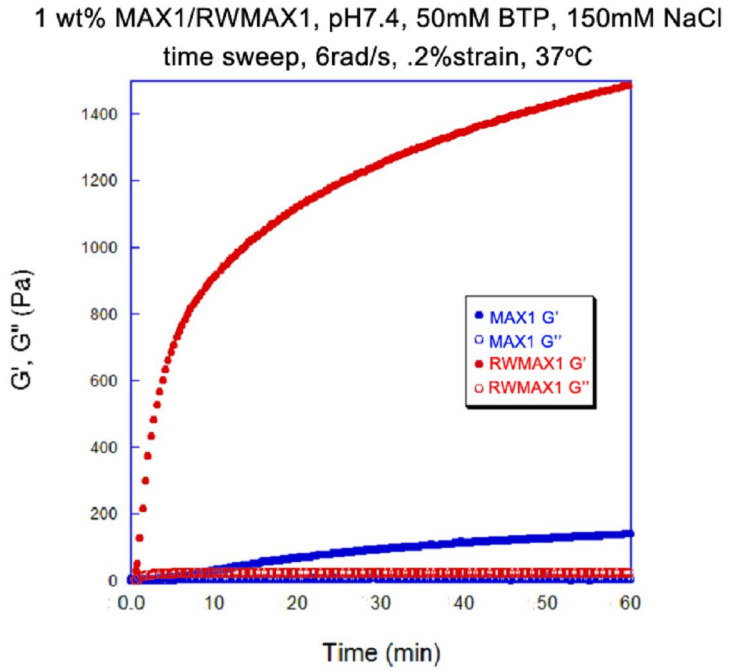

Figure 8 Oscillatory shear rheology measuring the evolution of storage modulus (G') and loss modulus (G") of 1wt% MAX1 (blue) and RWMAX1 (red) hydrogels.

3.4 Antibacterial assays

The antibacterial activity of MAX1 and RWMAX1 gels was tested against *Staphylococcus aureus* and *Escherichia coli* as representative gram-positive and gram-negative bacteria, respectively (figure 9). Both gels exhibited very potent activity against *S. aureus*, preventing growth even up to loading densities of $2x10^9$ CFU's/dm^2. Both gels exhibited reduced activity against *E. coli*, with MAX1 gels capable of preventing growth up to approximately $2x10^7$ CFU's/dm^2 and RWMAX1 up to $2x10^6$ CFU's/dm^2. Control experiments were performed on untreated TCTP surfaces and represented unchecked bacterial growth, which plateaued at a relatively constant level regardless of inoculation density. Growth curves demonstrated that both *S. aureus* and *E. coli* entered a stationary growth phase on TCTP by the time the experiments were performed (data not shown).

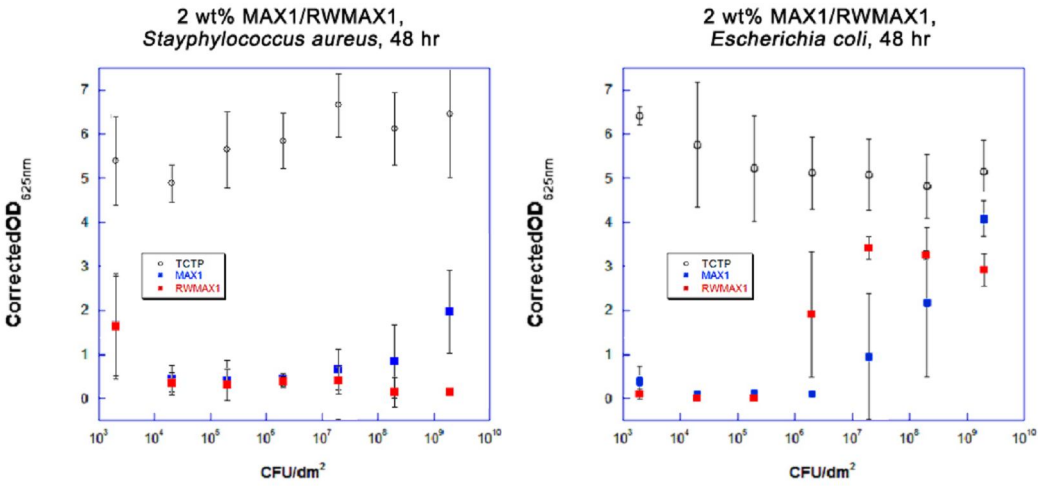

Figure 9 Antibacterial assays measuring the corrected (see appendix) OD at 625nm of supernatants above TCTP, 2wt% MAX1 (blue), or 2wt% RWMAX1 (red) hydrogel surfaces when inoculated with varying densities of either gram-positive *Staphylococcus aureus* (A) or gram-negative *Escherichia coli* (B).

Chapter 4

DISCUSSION

4.1 RWMAX1 exhibits favorable folding and self-assembly kinetics

The self-assembly kinetics of RWMAX1 were studied using two different approaches. Circular dichroism allows the direct measurement of β-sheet secondary structure (and thus, presumably, hairpin folding and self-assembly) in a peptide solution via the appearance of a characteristic β-sheet signal. Alternately, oscillatory shear rheology can be used as a more direct way of measuring hydrogelation itself by measuring the increasing rigidity of a gel as it evolves. Data from both of these methods indicated that RMWAX1 gels fold much more rapidly than do MAX1 gels under the same conditions. Even under physiological pH and ionic strength (conditions at which MAX1 folds only very slowly), RWMAX1 rapidly self assembles into a gel some 10 times more rigid than MAX1. This rapid folding was further corroborated by qualitative observation while handling the gels – RWMAX1 peptide solutions thickened visibly in a matter of minutes. Faster formation may also account for some of the increase in rigidity – faster-folding gels are more likely to develop packing errors, leading to non-covalent crosslinks within the gel network.

RWMAX1's improved folding and self-assembly kinetics are likely – like those of MAX8's – related to its reduced cationic charge compared to MAX1. We believe repulsion between protonated lysines is the primary force acting against these peptides' self-assembly, and the chief reason they fold faster at higher pH (due to

deprotonation) or ionic strength (due to charge screening). By replacing a lysine with a neutrally-charged Tryptophan, the RWMAX1 design presumably reduces these repulsive forces and stabilizes its folded form, leading to faster hydrogelation. The improved kinetics may also relate to the presence of a cross-strand tryptophan/arginine cation-π interaction, which might stabilize the folded hairpin along the order of 0-2.4kcal/mol (33).

While I have not yet completed cellular encapsulation studies with RWMAX1 to demonstrate that it self-assembles rapidly enough to homogenously encapsulate cells, the obtained circular dichroism and rheology data suggests similar behavior to MAX8, and thus it seems very likely that homogenous encapsulation is possible. RMWAX1 has arguably satisfied the first of its two design criteria.

4.2 RWMAX1 exhibits broad spectrum antibacterial activity

RWMAX1's antibacterial activity was studied using bacterial proliferation assays in which bacteria were inoculated at various densities atop a 2wt% hydrogel surface, and their proliferation subsequently measured via spectrophotometry. Like MAX1, RWMAX1 exhibited very strong antibacterial activity against gram-positive *Staphylococcus aureus*, preventing bacterial proliferation at every inoculation density tested, up to $2x10^9$ CFU's/dm^2.

Also like MAX1, RWMAX1's success against gram-negative *Escherichia coli* was somewhat reduced. It is not known why our hydrogels kill gram-positive bacteria more effectively than gram-negative, though the behavior is common in many Tryptophan-containing antibacterial peptides (37). It is perhaps related to the more complicated gram-negative bacterial surface, or in the relatively fast replication rates of

the gram-negative strains used, which would allow them to proliferate more in the supernatant before coming in contact with the gel, thus increasing the total number of bacteria the gel must overcome(41). It is perhaps most likely, however, to relate to the more negatively-charged plasma membranes of gram-negative bacteria (43). RWMAX1 showed reduced activity compared to MAX1, effectively preventing *E. coli* proliferation up to inoculation densities of $2x10^6$ CFU's/dm^2 as opposed to MAX1's $2x10^7$ CFU's/dm^2.

RWMAX1's reduced antibacterial activity can likely be attributed to the peptide's reduced cationic charge. Cationic charge is known to be associated with antibacterial activity in many naturally-occurring antibacterial peptides, where it may play a role in initial, nonspecific interaction with negatively-charged bacterial surfaces. The presence of the tryptophan/arginine cation-π interaction may also play a role. As in other antibacterial peptides in the literature, the exact nature of this role is not known, but may involve anchoring to bacterial lipid bilayers.

While RWMAX1's activity is, at least against *E. coli*, somewhat less than MAX1's, it is worth pointing out that the inoculation densities used in these experiments are very high, far and away higher than what would be expected in a real world situation. Based on sterility guidelines proposed by the Savino group, even against *E. coli* RWMAX1 remains active against almost twenty thousand times the threshold bacterial density of for unacceptable active operating theatre conditions (24) (table 2). RWMAX1 may represent something of a compromise between MAX1 and MAX8, but its antibacterial activity is likely still high enough to be valuable. With this in mind, RWMAX1 has arguably satisfied the second of its design criteria as well.

Table 2 Acceptable levels of total air microbial counts in different environments as determined by CFU on 9cm blood-agar petri dishes left open to air. (24)

	Total Microbial Count ($CFU/dm^2/h$)		
Location	Optimal	Acceptable	Not Acceptable
Medical wards	0-450	451-750	>751
Pharmacy	0-100	101-180	>181
Aseptic room	0-50	51-90	>91
Operating theatre (at rest)	0-4	5-8	>9
Operating theatre (in activity)	0-60	61-90	>91

4.3 Conclusions and future work

RWMAX1 satisfied both of its intended design criteria. By incorporating a tryptophan/arginine cation-π interaction into the MAX1 sequence, RWMAX1 manages to fold and self-assemble very rapidly like MAX8 while still maintaining much of MAX1's antibacterial activity, and thus may represent a superior candidate for wound-healing therapies than either of its predecessors. However, further studies remain.

Of particular interest is verifying that a cation-π interaction is actually present and, if so, exploring exactly how much it contributes to folding kinetics and antibacterial activity as compared to the change in cationic charge. It is conceivable that, as RWMAX1's +8 charge is intermediate between MAX1 (+9) and MAX8 (+7), its somewhat intermediate antibacterial activity may solely be a result of the reduced charge and not of any special activity of the tryptophan or arginine. To this end, it would be helpful to study similar peptides that included a single tryptophan or arginine substitution, along with peptides containing RW pairs at different locations or multiple RW pairs. While tryptophan and arginine substitutions have been shown many times to improve antibacterial activity, the improvement is sometimes very sensitive to specific (and somewhat unpredictable) positioning of the residues (36) – it is likely that by

tweaking the position of these residues in the RWMAX1 sequence, a peptide with superior antibacterial activity could be developed.

Further studies would also be necessary on RWMAX1 itself before it could truly be considered a candidate for therapeutic use. Most especially, while MAX1 has been demonstrated not to harm eukaryotic cells (8), this may no longer be the case for RWMAX1 – indeed, tryptophan and arginine substitutions have been known to increase peptide cytotoxic activity against T-cells and erythrocytes (21,37), so cytocompatability studies would be of critical importance.

These concerns aside, RWMAX1 did successfully incorporate both rapid folding and self-assembly kinetics and potent antibacterial activity into a single peptide, and thus, whether it represents an optimal design or not, suggests Tryptophan and Arginine substitution may be a valuable strategy for the design of future antibacterial peptides.

APPENDIX

A1 Oscillatory rheology frequency and strain sweeps

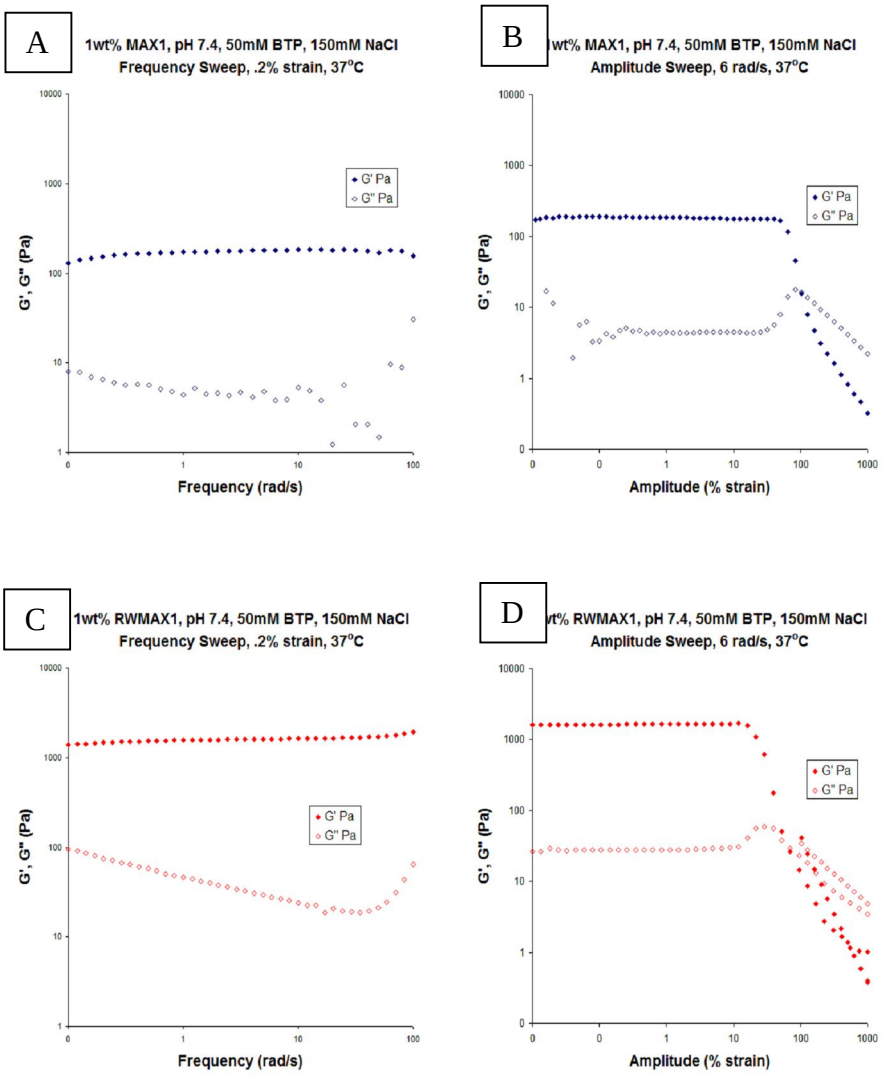

Figure 11 Oscillatory shear rheology data measuring storage (G') and loss (G") moduli as a function of frequency and strain amplitude for 1wt% MAX1 (A, B) and RWMAX1 (C, D) hydrogels.

A2 Correction for bacterial OD measurements

All bacterial data is presented as a corrected optical density at 625nm. Fluctuations are normalized for by using measurements taken at 1100nm in the following calculations:

$$\text{Corrected OD}_{625nm} = 2\left(\text{Observed OD}_{625nm} - \Delta\text{OD}_{1100nm}\right)$$

$$\Delta\text{OD}_{1100nm} = \text{Observed OD}_{1100nm} - \text{Expected OD}_{1100nm}.$$

$$\text{The Expected OD}_{1100nm} = \frac{\text{Observed OD}_{625nm}}{\left(\frac{\text{OD}_{625nm_{\text{Control}}}}{\text{OD}_{1100nm_{\text{Control}}}}\right)_{avg}}$$

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