

AWARD NUMBER: W81XWH-20-1-0563

TITLE: Bioinspired Acellular Strategies to Optimize the Skin-Implant Interface of Osseointegrated Devices

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REPORT DATE: July 2021

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE July 2021		2. REPORT TYPE Annual		3. DATES COVERED 01Jul2020-30Jun2021	
4. TITLE AND SUBTITLE Bioinspired Acellular Strategies to Optimize the Skin-Implant Interface of Osseointegrated Devices				5a. CONTRACT NUMBER W81XWH-20-1-0563	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Conrado Aparicio, PhD E-Mail:				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) REGENTS OF THE UNIVERSITY OF MINNESOTA OFFICE SPONSORED PROJECTS ADMINISTRATION OFFICE OF SPONSORED PROJECTS 200 OAK ST SE # 224 MINNEAPOLIS MN 55455-2009				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution is unlimited.					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT This work directly addresses PRORP-Peer Reviewed Orthopaedic Research Program 2019, Applied Research Award; Focus Area: "Skin-implant interface-Identification of best practices to address infection at the skin-implant interface for osseointegrated prosthetic limbs." We also address residual limb skin integrity and durability. We address the general issue of prevention of infections and skin-to-implant healing and attachment for osseointegrated (OI) prostheses. Our reasoning centers on preventing OI prostheses infection by a simultaneous 2-tier protection system for improving skin healing and adhesion to the OI implant as it exits the skin and improving durability of this seal for implant longevity by controlling biofilm and microbiome challenge. We will engineer multifunctional, acellular materials deriving inspiration from (1) existing biological settings where a hard tissue or organ exits the body through the skin and the biomolecular/cellular structures that seal the attachment of the soft tissue on the hard tissue and (2) natural biomolecules that provide defense against infection.					
15. SUBJECT TERMS None listed.					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT Unclassified	18. NUMBER OF PAGES 48	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT Unclassified	b. ABSTRACT Unclassified	c. THIS PAGE Unclassified			19b. TELEPHONE NUMBER (include area code)

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1.INTRODUCTION:

The Research Idea and reasoning centers on preventing OI prostheses infection by a simultaneous 2-tier protection system for improving skin healing and adhesion to the OI implant as it exits the skin (first tier of protection) and improving durability of this seal for implant longevity by controlling biofilm and microbiome challenge (second tier of protection). We will engineer multifunctional, acellular materials deriving inspiration from (1) existing biological settings where a hard tissue or organ exits the body through the skin and the biomolecular/cellular structures that seal the attachment of the soft tissue on the hard tissue. This biological seal forms a natural barrier around teeth, nails, antlers, hairs, etc. that impedes bacterial invasion through the skin-organ interface and eventual bacteria colonization/infection; and (2) natural biomolecules from the host immune system that provide defense against infection. Building upon these premises, we utilize two different research strategies that rely on the development of two alternative types of bioinspired materials with strong, compliant and durable attachment as well as the ability to prevent infection at the skin-implant interface of OI implants. First strategy is based on the development of multifunctional coatings which can improve soft tissue attachment utilizing peptides derived from key proteins present in the extracellular matrix and prevent bacterial colonization and biofilm formation using antimicrobial peptide (AMP) We hypothesize that by coatings on titanium alloy made of cell adhesive peptide (CAP) in combination with AMP will simultaneously and specifically activate formation of hemidesmosomes by keratinocytes and prevent mono- and multi-species biofilm growth on the metallic substrate with no significant differences with respect to mono-peptide coatings, but with significant difference with respect to non-coated control substrates. The second strategy is based on designing a surgeon-friendly protein based biologic glue with antimicrobial peptides. This strategy is driven by the hypothesis that by modifying gelatin with metal-adhesive functional groups and combining it with AMP-loaded nanoparticles, significantly improved adhesion to metal and keratinocytes will be achieved in addition to reduction in biofilm formation when compared to unmodified gelatin or non AMP-charged hydrogels.

2.KEYWORDS:

Osseointegration, implants, titanium, multifunctional coatings, cell adhesive peptide, antimicrobial peptide, gelatin based adhesive glue, mesoporous nanostructures

3.ACCOMPLISHMENTS:

What were the major goals of the project?

Specific Aim 1	Timeline	Percent completed
Major Task 1: Selection of peptides for multifunctional coatings	1-18	70%
Subtask 1.1: Evaluate cell adhesion peptides (CAP) for improved keratinocyte attachment CAPs: LamLG3, Net1, AG86, AMBN CAPs coatings to titanium alloy (Ti): Water contact angles (WCA) Attenuated Total Reflectance Infrared Spectroscopy (ATR-FTIR) X-ray Photoelectron Spectroscopy (XPS) OKF6-TERT keratinocytes (KCs) Cytotoxicity: lactase dehydrogenase (LDH) Cell viability: mitochondrial activity (CCK-8) Hemidesmosomes (HD): immunofluorescence (IF) KCs vs fibroblasts Primary gingival fibroblasts (PGF) and KCs LDH, CCK-8 for PGF Co-culture: CellTracker	1-15	100%
Subtask 1.2: Evaluate antimicrobial peptides (AMP) for increased antimicrobial potency AMPs: GL13K, 1018, DJK2, DJK5 AMPs coatings to Ti WCA, ATR-FTIR, XPS Antimicrobial activity (quasi-static), 24 h Staphylococcus aureus Colony forming units (CFU) LIVE/DEAD fluorescence Adenosine triphosphate activity (ATP) Cytocompatibility KCs and PGF LDH, CCK-8	1-15	60%
Subtask 1.3: Determine best CAP and AMP for cell attachment and antimicrobial properties Milestone: Selection of two best CAP coatings: selective increase of proliferation and HDs in KCs CAP1, CAP2 Milestone: Selection of two best AMP coatings for increase of CFU and decrease cytotoxicity AMP1, AMP2	12-18	50%
Major Task 2: Manufacturing and optimization of multifunctional coatings	18-36	5%
Subtask 2.1: Evaluate combinations of co-immobilization of peptides (CAP-AMP) to improve keratinocyte attachment Peptides for CAP-AMP coatings: CAP1, CAP2, AMP1, AMP2, 2 peptide ratios per combination Design of experiments: DOE - 2 ³	18-32	0%

CAP-AMP coatings on Ti WCA, ATR-FTIR, XPS KCs on CAP-AMP LDH, CCK-8, IF for HDs KCs attachment strength: centrifugation		
Subtask 2.2: Evaluate combinations of co-immobilized peptides for antimicrobial potency Peptides for CAP-AMP coatings: CAP1, CAP2, AMP1, AMP2, 2 peptide ratios per combination Design of experiments: DOE - 2 ³ CAP-AMP coatings on Ti WCA, ATR-FTIR, XPS Antimicrobial activity (quasi-static), 24 h <i>S. aureus</i> CFU, LIVE/DEAD, ATP Antimicrobial activity (dynamic), 48 h Drip flow bioreactor (DFR) <i>S. aureus</i> CFU, LIVE/DEAD, ATP Milestone: Selection of two best CAP-AMP coatings for increase in HDs (cell attachment criterion) and decrease CFU (antimicrobial criterion) CAP-AMP1, CAP-AMP2	18-32	0%
Subtask 2.3: Evaluate manufacturing/non-biological properties for co-immobilization of CAP & AMP Chemical route for peptides immobilization Silanization vs click-chemistry Peptides: CAP1, CAP2, AMP1, AMP2 WCA, ATR-FTIR, XPS Amount of peptide: NanoOrange assay KCs on different coatings CCK-8, IF for HDs Antimicrobial activity (quasi-static), 24 h <i>S. aureus</i> CFU, LIVE/DEAD, ATP Type of amino acid linker KK-GGG vs KK-PAPAP Peptides: CAP1, CAP2 KCs on different coatings CCK-8, IF for HDs Resistance to degradation Peptides: CAP1, CAP2, AMP1, AMP2 pH challenge (6.0 and 7.5) – 21 days Enzyme challenge: matrix-metalloproteinase-8 Mechanical challenge: friction microtest – 90,000 cycles Fluorescence of fluorescent-labeled peptides, WCA, XPS	18-32	25%
Subtask 2.4: Determine manufacturing conditions for optimal bioactivity of co-immobilized peptides Coatings: with CAP1, CAP2, AMP1, AMP2, CAP-AMP1 or CAP-AMP2 with best manufacturing chemistry, spacer and resistance to degradation. WCA, ATR-FTIR, XPS KCs: CCK-8, IF for HDs KCs vs PGF (only CAP-AMPs): CellTracker <i>S. aureus</i> (quasi-static): CFU, LIVE/DEAD	24-36	0%

Major Task 3: Surgeon evaluation of developed multi-functional coatings Analysis of outcomes in Tasks 1 and 2 Feedback for lab researchers to make modifications in experimental design in Tasks 1 and 2 Feedback for lab researchers to make decisions on best coatings	6-36	10%
Major Task 4: Completion of full statistical analysis and submission of 1-2 manuscripts	3-36	50%
Milestone: Best coating of co-immobilized peptides for keratinocyte attachment and antimicrobial potency. Publication of 1-2 manuscripts ANOVA with post-hoc tests DOE selection and analysis Milestone: Publication of paper 1 Milestone: Publication of paper 2	1-36	25%
Specific Aim 2	Timeline	Site 1
Major Task 5: Selection of components for surgeon-friendly glue	3-18	50%
Subtask 5.1 and 5.2: Evaluate basic biopolymer properties and light-curing system for scaffolding of the glue Fractional Factorial DOE variables: Gelatin methacrylate (GelMA) - Macromer concentration: 5-15% w/v Phenyl1-2,4,6-trimethylbenzoylphosphinate (LAP) concentration: 0.05-0.5% w/v. Degree of Gel-MA methacrylation (DOM) Nuclear magnetic resonance spectroscopy (¹ H-NMR) Curing time: 60s-180s Fractional factorial DOE properties: Rheology Soluble fraction Swelling ratio Viscosity Mechanical properties Compressive strength Toughness Adhesion to Ti ASTM F225505 standard assessment/lap shear test Enzymatic degradation vs collagenase I Milestone: Selection of best GelMA-based hydrogel/glue for DOM/cross-linking efficacy >95%, soluble fraction <10% and best combination of adhesive strength to Ti+lowest curing time. Gbest	3-18	80%
Subtask 5.3: Evaluate modifiers for adhesion to titanium substrate and keratinocytes Gbest Peptides: CAP1, CAP2 4-dihydroxyphenethyl amine (DOPA) Derivatization of Gbest with peptides and DOPA ¹ N-NMR Adhesion to Ti ASTM F225505 standard assessment/lap shear test KCs LDH CCK-8 IF for HDs	8-18	0%

Subtask 5.4: Determine best combination of biopolymer, adhesive motifs and light-curing system for glue Milestone: Selection of two best Gel-MA based glues for increase in HDs in KCs (cell attachment criterion) and increase in adhesion to Ti (device attachment criterion) G1, G2	15-18	0%
Major Task 6: Development of antimicrobial nanoparticles	9-18	25%
Subtask 6.1: Evaluate combinations of mesoporous silica nanoparticles (MSN) with AMP Fractional Factorial DOE variables: Chemistry of MSN: Calcium-MSN (CaMSN), Strontium-MSN (SrMSN) Peptides: AMP1, AMP2 Ratio metal/silica Time of reaction Temperature of reaction Fractional factorial DOE properties: MSN size and charge Scanning electron microscopy (SEM) Transmission electron microscopy (TEM) Dynamic light scattering (DLS) MSN porosity Nitrogen Adsorption/Desorption Degradation in physiological buffer (Tris-HCL) TEM Inductively-coupled plasma (ICP) AMP loading capacity Fluorimetry of labeled AMPs AMP release (21 days) Fluorimetry of labeled AMPs Milestone: Selection of two best MSN for highest AMP capacity and targeted AMP release profile; that is initial burst (MSN size and charge) followed by sustain release up to 2 weeks (porosity, degradation) MSN1, MSN2	9-15	75%
Subtask 6.2: Evaluate cytocompatibility of antimicrobial nanoparticles MSN1, MSN2 Modified ISO 10993-5 Extracts with 50% release of loaded AMP KCs exposed to extracts: 48 h Viability: CCK-8 Proliferation; CyQuant	11-15	0%
Subtask 6.3: Determine best combination of MNP size with peptide load for optimal bioactivity MSN1, MSN2 Antimicrobial activity (quasi-static), 24 h S. aureus Extracts at different % of release of loaded AMP CFU, LIVE/DEAD, ATP	15-18	0%
Major Task 7: Manufacturing and optimization of glues	18-36	0%
Subtask 7.1: Evaluate combinations of glue components (gel, nanoparticles, peptides) to improve adhesion to metal & cells G1, G2 MSN1, MSN2	18-32	0%

AMP1, AMP2 DOE - 2 ³ Soluble fraction Swelling ratio Degradation: TEM, ICP Mechanical properties: compressive strength Adhesion to metal: lap test Adhesion to KCs: IF for HDs		
Subtask 7.2: Evaluate combinations of glue components (gel, nanoparticles, peptides) to improve antimicrobial potency G1, G2 MSN1, MSN2 AMP1, AMP2 DOE - 2 ³ Antimicrobial properties: CFU, LIVE/DEAD	18-32	0%
Major Task 8: Surgeon evaluation of developed surgeon-friendly glues Analysis of outcomes in Tasks 5, 6 and 7 Feedback for lab researchers to make modifications in experimental design in Tasks 5, 6, and 7 Feedback for lab researchers to make decisions on best MNPs and glues	9-36	10%
Major Task 9: Completion of full statistical analysis and submission of 1-2 manuscripts	3-36	40%
Milestone: Best glue for adhesion to Ti, keratinocyte attachment, and antimicrobial potency. Publication of 1-2 manuscripts ANOVA with post-hoc tests DOE selection and analysis Milestone: Publication of paper 1 Milestone: Publication of paper 2	3-36	20%
Reporting	Timeline	Site 1
Major Task 10: Year 1 Reporting	3-13	15%
Subtask 10.1: Technical progress reports Quarterly technical progress reports Y1Q1-Tech report Y1Q2-Tech report Y1Q3-Tech report Quarterly Quad Charts Annual technical progress report: Y1-Tech report	3-13	100%
Subtask 10.2: Other reports Semiannual recipient integrity and performance reports: Y1H1-Int report Y1H2-Int report Annual financial reporting – Y1-Fin report	6-13	100%
Major Task 11: Year 2 Reporting	14-25	0%
Subtask 11.1: Technical progress Reports Quarterly technical progress reports Y2Q1-Tech report Y2Q2-Tech report Y2Q3-Tech report Quarterly Quad Charts Annual technical progress report: Y2-Tech report	14-25	0%
Subtask 11.2: Other reports Semiannual recipient integrity and performance reports: Y2H1-Int report	18-25	0%

Y2H2-Int report Annual financial reporting – Y2-Fin report		
Major Task 12: Year 3 Reporting	24-39	0%
Subtask 12.1: Technical progress Reports Quarterly technical progress reports Y3Q1-Tech report Y3Q2-Tech report Y3Q3-Tech report Quarterly Quad Charts Final technical progress report: Final-Tech report	24-39	0%
Subtask 13.2: Other reports Semiannual recipient integrity and performance reports: Y3H1-Int report Y3H2-Int report Award Expiration Transition Plan Final financial reporting – Final-Fin report	30-39	0%

Major Task 1: Selection of peptides for multifunctional coatings

Subtask 1.1: Evaluate cell adhesion peptides (CAP) for improved keratinocyte attachment

Experimental:

Candidate peptides Net1 (K_{N3}-GGG-QWRDTWARRLRKFQQREKKGKCRKA)¹, LamLG3 (K_{N3}-GGG-PPFLMLLKSTRFC)², AG86 (K_{N3}-GGG-LGGLPSHYRARNI)³, and AMBN (K_{N3}-GGG-VPIMDFADPQFPT)⁴ were produced (>95% purity; AAPPTec; high-performance liquid chromatography and mass spectrometry characterization were performed (data not shown).

CAPs are summarized in Table 1. The N-terminus of each peptide was modified with three flexible glycine spacers (previously shown to be important to ensure biological activity)⁵⁻⁷ plus an azide-conjugated lysine for copper free, alkyne azide click chemistry immobilization.⁸⁻⁹

Table 1: Summary sequence and selection criteria for cell adhesion peptides (CAPs)

Peptide Abbreviation	Peptide Sequence	BM Glyco/protein	Selection Criteria
Net1	QWRDTWARRLRKFQQREKKGKCRKA	Netrin-1	Relatively exclusive binding affinity to $\alpha 6 \beta 4$
LamLG3	PPFLMLLKSTRFC	Laminin 332	Upregulates HDs in vivo; integrins involved
AG86	LGGLPSHYRARNI	Laminin 111	Binding affinity to $\alpha 6 \beta 4$
AMBN	VPIMDFADPQFPT	Ameloblastin	Upregulates HDs

Glass disks were used a model substrate to select the two best candidates for CAP which will be then immobilized on titanium alloy.

Borosilicate glass disks (Yuanbo Engineering Co. Ltd., China) were activated by plasma cleaning [O₂ for 20 minutes, PDC-32G, Harrick Plasma). Plasma activated disks (pGlass) were silanized as previously described.¹⁰ In short, disks were placed in an N₂-rich atmosphere and immersed in pentane containing 0.05M N,N-Diisopropylethylamine and 0.5M (3-Chloropropyl)-triethoxysilane (all obtained from Sigma-Aldrich) and thoroughly rinsed. Silanized, plasma activated glass (pGlass-sil) were immersed in 0.1 mM dibenzocyclooctyne-amine (DIBO, Sigma-Aldrich) in 0.1 M Na₂CO₃ buffer overnight and cleaned with solvents (pGlass-DIBO). These disks were then immersed in 0.1 mM peptide solutions in 0.1 M Na₂CO₃ buffer overnight and again cleaned with solvents (yielding Net1, LamLG3, AG86, and AMBN).

X-ray photoelectron spectroscopy (XPS) characterization

An X-ray photoelectron spectrometer (XPS; PHI 5000 VersaProbe III, ULVAC Inc.) was used to quantify the surface elemental composition with a monochromatic Al K α X-ray source (45°, 1486.6 eV, 50 W, sampling area; 200- μ m diameter). Survey spectra were collected using a pass energy of 280 eV and a step size of 1.0 eV. Charge compensation was used, and spectra were calibrated to the C 1s signal at 284.8 eV. Surface elemental composition [as atomic composition (At %)] was determined using MultiPak (ULVAC Inc) from background-subtracted (system optimized) peak areas.

Water contact angle (WCA) characterization

WCAs were measured using the sessile-drop method (>18 M Ω deionized water; 2 μ L) to assess wettability of the surfaces using a contact angle goniometer (DM-CE1, Kyowa, Japan). Dynamic contact angles were captured every 1 s for 60 s using the circle method.

Oral keratinocytes (OK) and gingival fibroblast (HGF) culture

Immortalized human OKF6/TERT-2 (OKs; BWH Cell Culture and Microscopy Core, Boston, MA, USA) OKs from normal tissue from the floor of the mouth were cultured in defined keratinocyte serum-free medium (Gibco) with 1% penicillin/streptomycin (Gibco) antibiotics under standard conditions.¹¹ Primary human gingival fibroblasts (HGFs; PCS-201-018, ATCC) were cultured in low-serum medium (ATCC) with 1% penicillin/streptomycin.

OK proliferation on coatings

OKs were seeded (6×10^4) and then washed in PBS and incubated for four hours in CCK8 solution (Dojindo; 9:1 CCK8:OK medium). Optical density (OD; $\lambda=450\text{nm}$) was obtained on a plate reader (Synergy HT, Biotek). OD values were blanked with virgin CCK8 solution similarly incubated ($n=5$). The number of nuclei (based on DAPI staining) were also quantified per field of view (FOV) to complement metabolic activity (five FOVs per sample, $n=5$).

HGF proliferation on coatings

HGFs were seeded (1×10^4) and cultured in a similar manner to OKs. Samples were stained with rhodamine-conjugated phalloidin (R415; Thermo-Fisher) for 10 minutes at room temperature and DAPI. Proliferation was measured using a CCK8 assay and the number of nuclei per FOV was performed, both as described.

OK and HGF cytotoxicity on coatings

Initial cell viability was determined through lactate dehydrogenase (LDH) release. Cells were seeded as previously described and allowed to adhere for four hours. Disks were then transferred to a new wellplate. A CyQUANT colorimetric assay (C20300, Thermo Fisher) was used twenty hours later to quantify the amount of LDH in solution, per the manufacturer's instructions.

Immunofluorescence and OK hemidesmosome (HD) formation

OKs were seeded and cultured for one or three days and then fixed for ten minutes in ice-cold methanol or 4% paraformaldehyde. Cells were immersed in 5% bovine serum albumin (BSA) in PBS and probed with a primary rabbit polyclonal antibody for collagen XVII [(critical later marker for HD assembly)¹² ab28440; Abcam; 1:500] for 1 h at room temperature or primary mouse monoclonal integrin $\beta 4$ [(critical early marker for HD assembly)¹³ NB10065599; Novus Biologicals; 1:500]. Samples were immersed in an anti-rabbit secondary (ab97037; abcam; 1:500) or anti-mouse (A-11005; Invitrogen; 1:500) after extensive washing for 3 hours. In some cases, samples were counterstained with DAPI (4',6-diamidino-2-phenylindole dihydrochloride; D1306; Sigma-Aldrich). Total fluorescent intensity in each field was quantified after accounting for secondary-only controls at constant microscope settings across all samples. Micrographs ($\times 10$) were obtained on an upright fluorescent microscope as previously described. Five FOVs were captured per sample ($n=5$).

OK and HGF Co-Culture

OKs and HGFs were pre-labeled with fluorescent probes (CellTracker, Thermo Fisher) to track proliferation during co-culture. OKs were labeled with DeepRed (700nm; red) and HGFs were labeled with CM-Dil (2.5 μM ; green) following the manufacturer's instructions. After this, 700 OKs and 1,300 HGFs (65:100) were simultaneously seeded and cultured in a 1:1 ratio of HGF:OK medium following a previous report.¹⁴ This ratio was chosen after extensive optimization based on differences in cell size and population doubling times to achieve an approximately 1:1 ratio on pGlass (control) at 1 day. Fluorescent micrographs were periodically captured to track proliferation; three FOVs were captured per sample ($n=3$).

Statistics

Mean values were compared with a one-way analysis of variance (ANOVA) followed by Tukey's HSD (honest significant difference) post hoc test. GraphPad Prism 8.2.0 (GraphPad Software) was utilized for calculations. A p-value of <0.05 was considered statistically significant; dissimilar letters indicate statistically significant differences. One standard deviation on the mean is reported. Figures were partially created with BioRender. All biological experiments were independently repeated twice.

Results

CAP coatings on glass were successfully synthesized based on changes in WCA (Figure 1A, Figure 1B, and Figure 1C) between CAP-coated substrates compared to controls pGlass, pGlass-sil, and pGlass-DIBO. XPS results further supported the presence of nitrogen atoms associated with CAPs on CAP substrates (Figure 1D).

No statistically significant difference in cytotoxicity towards OK (Figure 2A) or HGFs (Figure 2B) were seen between CAP coatings and controls.

As a first evaluation of the peptide coatings' activity and cytocompatibility, we cultured OKs and HGFs for 1 and 3 days and measured metabolic activity and cell proliferation (OK: Figure 3A and Figure 3B; HGF: Figure 3C and Figure 3D). OK metabolic activity and proliferation were significantly increased on Net1, LamLG3, and AMBN at 1 day compared to controls (pGlass and pGlass-DIBO), suggesting some cell-peptide signaling. By day 3, OK proliferation on Net1 and LamLG3 was significantly higher than on AG86 and AMBN, with Net1 higher than all other groups.

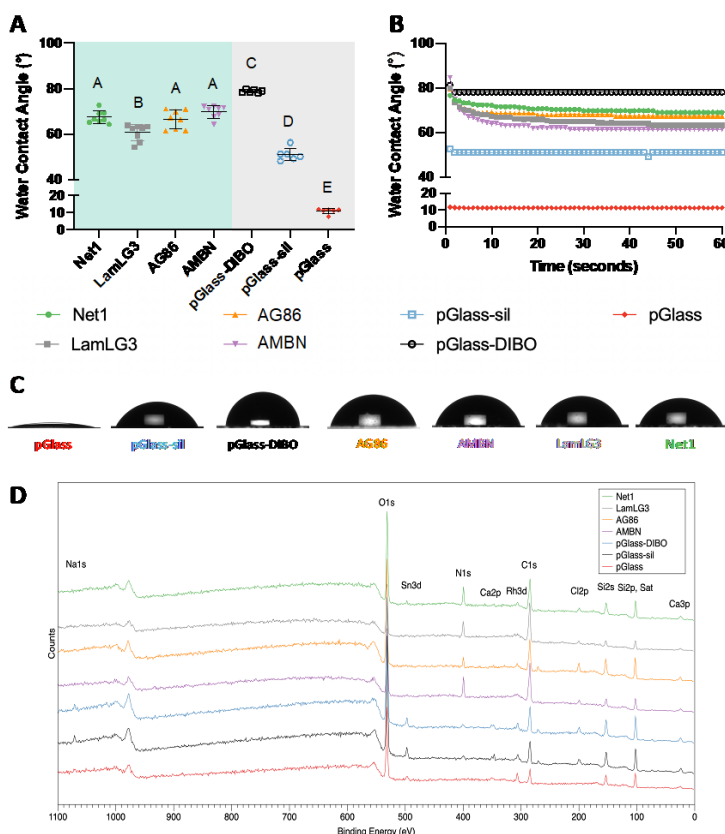


Figure 1: Surface characterization of peptide coatings and controls. A) Water contact angles at equilibrium. B) Representative dynamic water contact angles through 60 s. C) Photomicrographs of water contact angles at equilibrium. D) X-ray photoelectron spectroscopy (XPS) of pristine coatings.

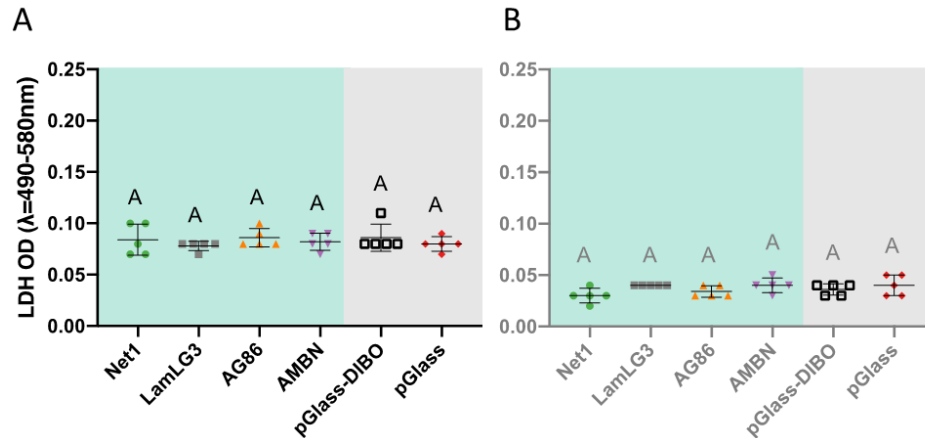


Figure 2: Lactate dehydrogenase (LDH) release of keratinocytes and fibroblasts on coatings and controls after 24 hours. A) Keratinocyte LDH release. B) Fibroblast LDH release.

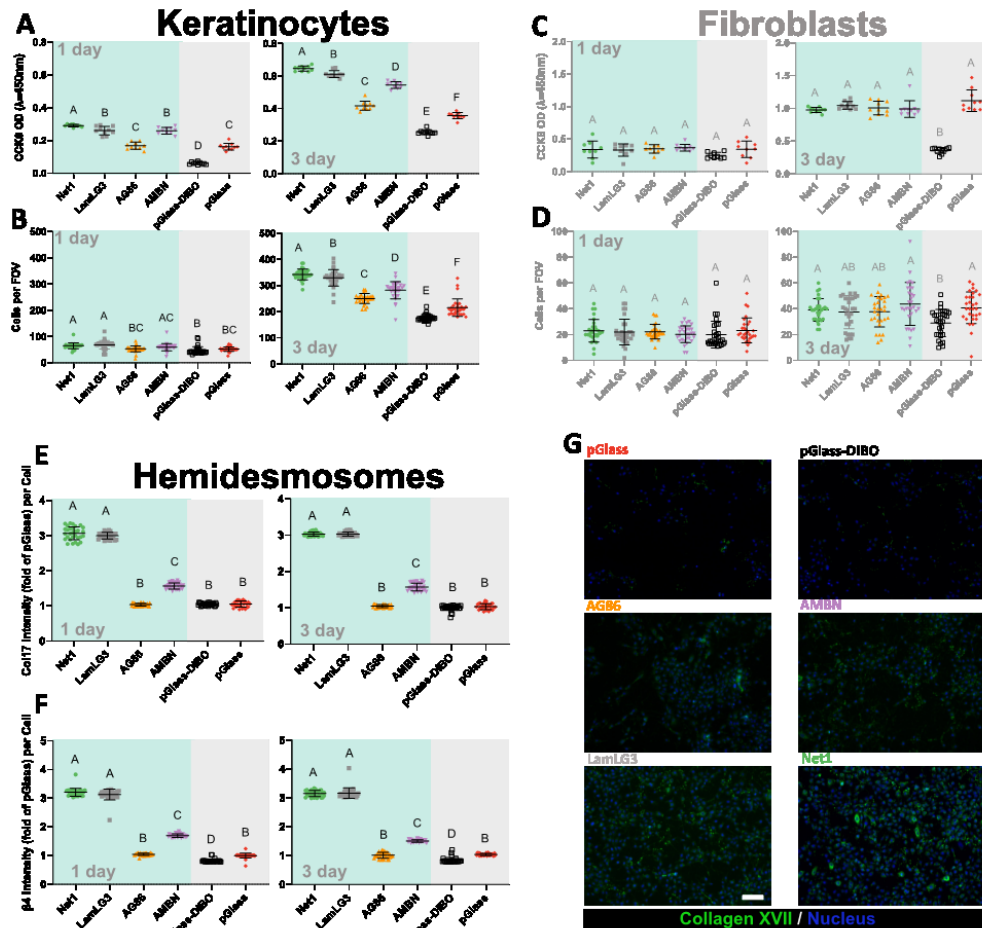


Figure 3: Keratinocyte and fibroblast proliferation and hemidesmosome formation (Collagen XVII and integrin β 4) of oral keratinocytes on peptide coatings and controls. A) Metabolic activity (CCK8) of keratinocytes at 1 and 3 days. B) Number of keratinocytes per field of view (FOV) at 1 and 3 days. C) Metabolic activity (CCK8) of fibroblasts at 1 and 3 days. D) Number of fibroblasts per FOV at 1 and 3 days. E) Immunofluorescent intensity (Collagen XVII) per keratinocyte relative to pGlass control at 1 and 3 days. F) Immunofluorescent intensity (Integrin subunit β 4) per keratinocyte relative to pGlass control at 1 and 3 days. G) Immunofluorescent micrographs (DAPI and Collagen XVII-stained) of keratinocytes at 3 days. Scale bar is 100 μ m.

Summary

CAP coatings were successfully synthesized on glass and leading candidates' ability to increase oral keratinocytes proliferation and hemidesmosomes formation, without increasing gingival fibroblast proliferation, was evaluated. Combined with co-culture studies, our results suggest that LamLG3 and Net1 are leading CAP coating candidates for further studies toward increasing soft tissue around osseointegrated devices.

Subtask 1.2: Evaluate antimicrobial peptides (AMP) for increased antimicrobial potency

Introduction/Rationale/Challenges: This is the first time we use our route of covalent immobilization of peptides on Ti-6Al-4V alloy, the metallic material is used to manufacture OI devices for amputees. We successfully transferred our peptide-immobilization protocols to this substrate.^{15,16} The selected AMPs (List in Table 2) were screened for their antimicrobial activity against methicillin resistant *S. Aureus* (MRSA), macromolecular configuration, and ability to form sound immobilized coatings on Ti alloy. We also report on appropriate experimental conditions to test antimicrobial properties of the peptide coatings against the bacterium of interest.

Experimental:

Materials: High strength grade-5 Ti alloy (Ti6Al4V) sheets were purchased from McMaster-Carr (Cat. No.9039K16). Table 2 provides a list of all peptides tested for antimicrobial potency and immobilization on Ti6Al4V samples. Antimicrobial peptide candidates (D-GL13K, 1018, DJK-2, DJK-5 and W10) were produced at >95% purity by AAPPTec (Louisville, KY) or purchased from Peptide Sciences (LL-37). The peptides were selected to provide a range of L-peptides and D-peptides with some having the ability to acquire beta-sheet secondary configurations and some having the ability to acquire alpha-helix configurations. The potency and mechanism of these peptides can be associated to these two properties, among others kept similar in the selected peptides.

Peptide secondary structure in solution with and without surfactants by circular dichroism (CD):

Peptide secondary structures were determined in two different solutions (1) 10 mM Tris buffer (pH = 7.4) and (2) 10 mM sodium dodecyl sulfate (SDS) in 10 mM Tris (pH = 7.4). The addition of SDS, a potent surfactant was used as surrogate of relevant components in bacteria membranes that can trigger macromolecular conformational changes in the peptides, which is indicative of their ability to specifically interact with bacteria membranes. For CD experiments (Jasco J-815 CD spectrometer), 200 μ L of peptide solution were dispensed into a quartz cuvette with a 1 mm path-length. CD spectra were obtained at room temperature. The CD signal was monitored over a range of wavelengths from 190 to 260 nm with a data pitch of 1.0 nm, a scanning speed of 50 nm min⁻¹ and a response time of 2 s. All measurements were averaged over 3 scans and were corrected by subtracting their corresponding buffer spectrum.

Antimicrobial screening of selected AMPs in solution against methicillin resistant *Staphylococcus aureus* (MRSA):

Peptides in Table 2 were dissolved in deionized water at stock concentrations of 1.0 mg/mL. Working concentrations of the peptides were prepared by diluting the stock in the necessary amount of THB media. Amoxicillin (AMX) was used as the positive control to ascertain bacterial growth inhibition. For the experiment, MRSA (USA 300) was revived on brain heart infusion (BHI) agar from cryopreserved glycerol stocks. A single colony was inoculated in 5 mL of Tryptone Soya Broth (TSB) and cultured for ~6 h to reach log growth phase. The bacterial optical density (OD) @600 nm was adjusted to 0.1 with TSB. In a 96 well plate, 100 μ L of media or AMP or AMX solution and 100 μ L of diluted bacterial suspension were added. Using a multimode plate reader (Biotek Synergy) in the kinetic mode pre-heated to 37 °C, the bacterial OD @630 nm was recorded at intervals of 1 h. For each experimental condition, 5-6 wells/replicates were used.

Peptide name	Peptide sequence	Secondary structure	Enantiomer	Molecular weight (g/ mol)	Charge (pH 7.0)	Reference
L-GL13K	GKIIKLASLKLL-NH ₂	β -sheet	L	1,424	+5	15
D-GL13K	GkiiKkaslkll-NH ₂	β -sheet	D	1,424	+5	16
1018	VRLIVAVRIWRRNH ₂	β -sheet	L	1,536	+5	16
DJK-2	vfwrriwvrvir-NH ₂	β -sheet	D	1,685	+5	16
DJK-5	vqwrairvrvir-NH ₂	β -sheet	D	1,551	+5	16
W10	WKKWWKWWK-NH ₂	α -helix	L	1,589	+5	17
LL-37	LLGDFFRKSK-EKIGKEFKRI-VQRIKDFLRN-LVPRTES	α -helix	L	4,493	+6	18

Table 2: Summary of the AMPs selected for immobilization on Ti6Al4V

Ti alloy sample preparation for peptide coating: Grade-5 Ti alloy sheets were punched into discs of 6 mm diameter and polished following the protocol recommended by Buehler. Briefly, the discs were polished using CarbiMet2 SiC sheets with an increasing gradient of grit sizes (P400-P1200), followed by 9 μ m MetaDi Supreme diamond suspension and finally with 0.02-0.06 μ m MasterMet Silica. The fine polished (pTi6Al4V) discs were cleaned in a series of solvents and dried.

Silanization and Peptide Immobilization: Selected antimicrobial peptides (AMPs) for their high potency against MRSA in solution were anchored onto Ti6Al4V using silanization chemistry as performed previously with commercially pure Grade-2 Ti.¹ Briefly, the pTi6Al4V were etched in 5 M NaOH overnight at 60 °C, rinsed in deionized water and dried with N₂ gas. The etched (eTi6Al4V) discs were silanized in anhydrous pentane using 1.2 mL of (3-(chloropropyl)-triethoxysilane (CPTES) and 0.6 mL of diisopropylethylamine (DIPEA) under N₂ atmosphere. The etched and silanized (eTi6Al4V_Si) discs were rinsed in a series of solvents and dried with N₂. The silanized discs were subsequently used for the immobilization of select AMPs.

For the immobilization of AMPs, 0.1 mM of the AMP (D-GL13K or LL-37) dissolved in Na₂CO₃ solution (0.5 mg/ mL, pH = 9.5) were reacted with the silanized discs overnight under vacuum with a prior purging of the reaction chamber with 5%O₂, 95% Ar. A S_N2 nucleophilic substitution of the chlorine (-Cl) with primary amines (-NH₂) is used to drive peptide tethering through covalent bonding.

The reacted discs were rinsed in deionized water and dried under N₂ to result in the AMP coated samples (eTi6Al4V_Si_AMP acronym).

Surface characterization of AMPs immobilized Ti6Al4V: To confirm surface modification and presence of AMPs, XPS analysis was performed following the same method as described in previous section. For each sample composition, 3 discs were analyzed at 2 spots each resulting in 6 replicates. Changes in surface wettability post silanization and peptide coating, WCAs were measured using the same method as described previously.

Characterization of test conditions for assessing antimicrobial properties of peptide coatings against MRSA.

Bacteria culture and inoculation: Biofilms of MRSA (USA 300) were grown on Ti discs coated with D-GL13K peptides; pTi6Al4V and eTi6Al4V discs were used as negative control. MRSA were cultured in Tryptone Soya Broth (TSB) at 37°C with shaking (100rpm) for 6 hours until reaching log growth phase (OD600=1.0). A dilution culture to OD600=0.1 was further diluted in TBS to 1:250. Biofilms were formed on peptide coated Ti alloy discs and controls by inoculating 0.5 mL of the obtained bacteria dilution (aprox. 10,000 CFU/ ml) in an incubator at 37°C for 18 hours and mild shaking (50rpm).

Antibiofilm activity: Antibiofilm activity was quantified by two complementary techniques, ATP activity (metabolic activity, cell vitality) and CFU counting (number of alive cells, cell viability).

The metabolic activity was measured by quantifying ATP using the BacTiter-Glo kit (Promega). To that end, after rinsing the coatings, 500 µL of the kit solution was mixed with 500 µL of PBS and added to a 24-well plate and the coatings were incubated for 5 min in the darkness. Then a 200 µL aliquot was transferred into a white 96-well plate and the luminescence measured using a SpectraMax L microplate reader (Molecular Devices).

For CFU counting, bacteria were detached from the surfaces using a Endoultra (VISTA) for 20 second, twice and the bacteria were collected in 300 ml 1xPBS. 100 ml of the obtained solution was serially diluted 10–1,000,000 fold. 0.1 ml of each dilution was plated on TSB plates for 24h at 37°C and the number of CFU was counted.

Results

Figure 4: CD spectra of all tested peptides in solutions with and without SDS showed that when peptides are in neutral buffer and absence of surfactant they all showed a mainly random-coil configuration; that is, absence of defined secondary structure. The only exception was W10 as this peptide had some alpha-helix signal, which was expected. Once exposed to the SDS surfactant, all peptides except for W10, transformed their secondary structure to show significant beta-sheet configuration. The self-assembly of the peptides provides this changes in the secondary conformation of these antimicrobial peptides, and proves their potential ability to interact with components of the bacterial membranes. This is, indeed, an important physical chemical characteristic of these peptides that not only validates their potential potency against bacteria but also provides an avenue for a quick quality control of these peptides. W10 slightly increased the portion of alpha-helix molecular configuration as it was exposed to SDS. Overall, these results were consistent with our hypothesis and validates our chose of peptide candidates.

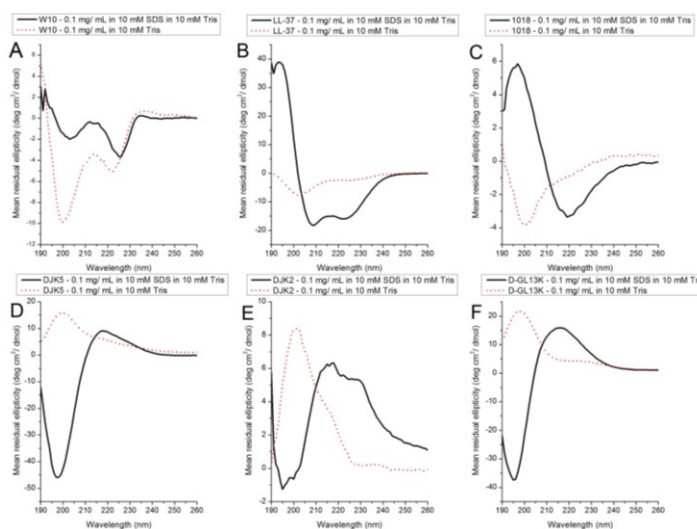


Figure 4: CD spectra for all tested peptides. First row L-peptides, Low row, D-peptide. Note the reverse shape of the D-peptides due to their chirality Same results for L-GL13K have been previously published by us.¹⁹

Figure 5: Growth curves of MRSA treated with different concentrations of all tested peptides showed that all D-peptides were potent against MRSA. The minimum inhibitory concentrations (MIC) for D-GL13K, DJK2, and DJK5 peptides against MRSA were 6.25 $\mu\text{g}/\text{mL}$, 6.25 $\mu\text{g}/\text{mL}$, and 25 $\mu\text{g}/\text{mL}$, respectively. On the contrary, only W10 peptides had activity against MRSA of all L-peptides tested. The MIC for W10 against MRSA was 25 $\mu\text{g}/\text{mL}$. The antibiotic positive control AMX inhibited MRSA growth at 5.0 $\mu\text{g}/\text{mL}$.

Taking into consideration these results, we decided to move forward in the development of the antimicrobial peptide coatings on Ti6Al4V surfaces by testing coatings that included only peptides that showed potency against MRSA in solution, that is, W10, D-GL13K, DJK5, DJK2. This enables us to also test peptide coatings that will be made of both D-peptides and L-peptides as well as coatings made of both alpha-helix forming and beta-sheet forming antimicrobial peptides.

The characterization of the coatings included assessment of the chemical and physical-chemical properties of the control and peptide coatings surfaces to demonstrate the presence of the peptides in these coatings. We also tested the initial biofilm growth conditions with MRSA on the coatings and proved the concept for effectiveness of the coatings to kill MRSA with one of our most studied peptides; that is, D-GL13K peptides.

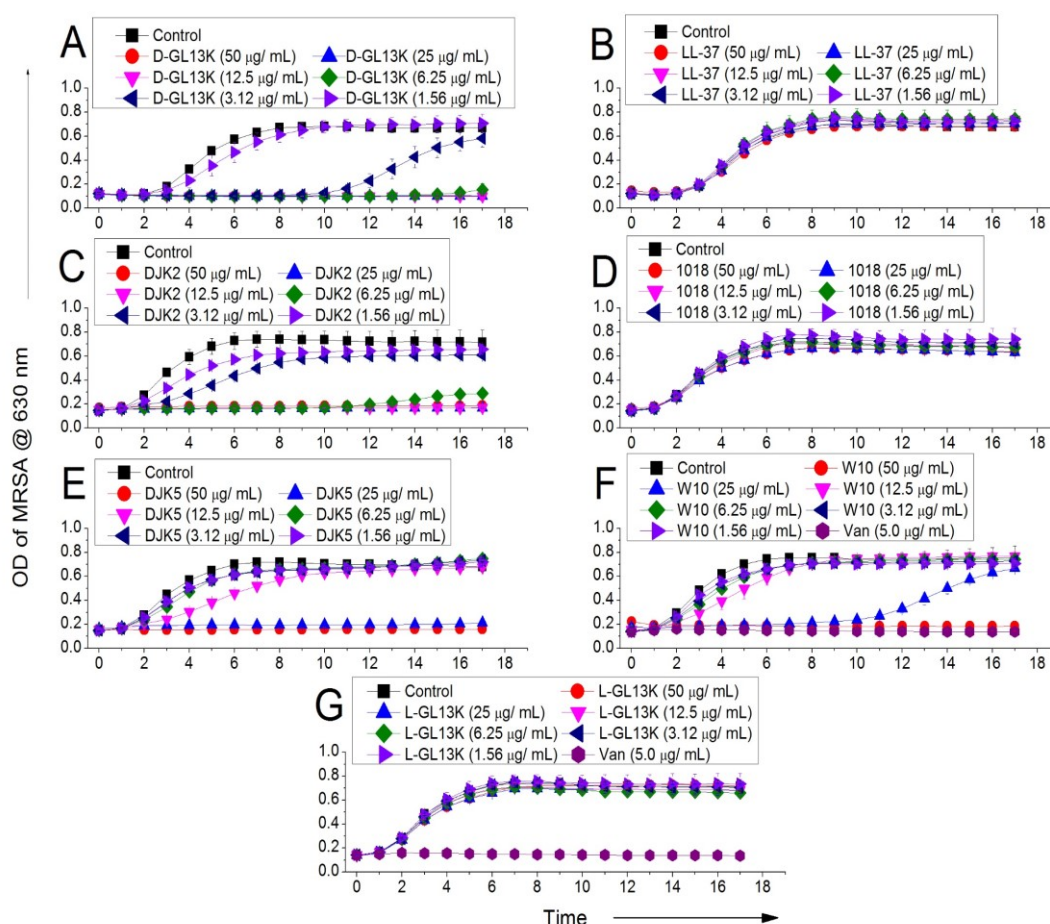


Figure 5. Growth curves of MRSA treated with all tested peptides. Amoxicillin (AMX at 5.0 $\mu\text{g}/\text{mL}$) was used as an antibiotic control. Data plotted are mean of $n = 5-6$ replicates.

Figure 6 presents the surface morphology and elemental composition of polished Grade-5 Ti alloy (pTi6Al4V). The elemental mapping and area scan patterns indicate the presence the Al and V in the Ti alloy and thus the adequacy of it to continue our experimentation.

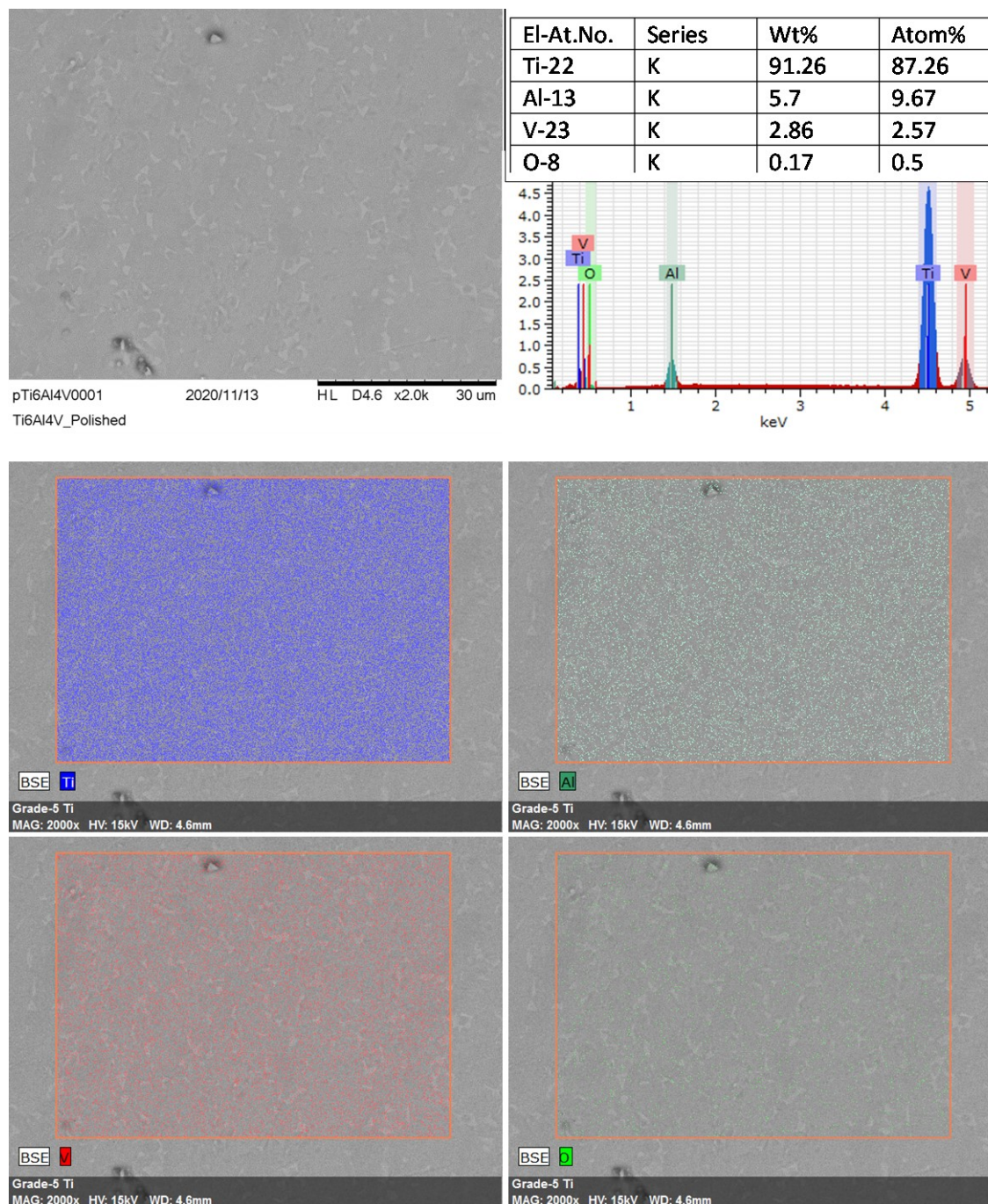


Figure 6: Surface morphology and elemental mapping of polished Grade-5 Ti alloy (Ti6Al4V)

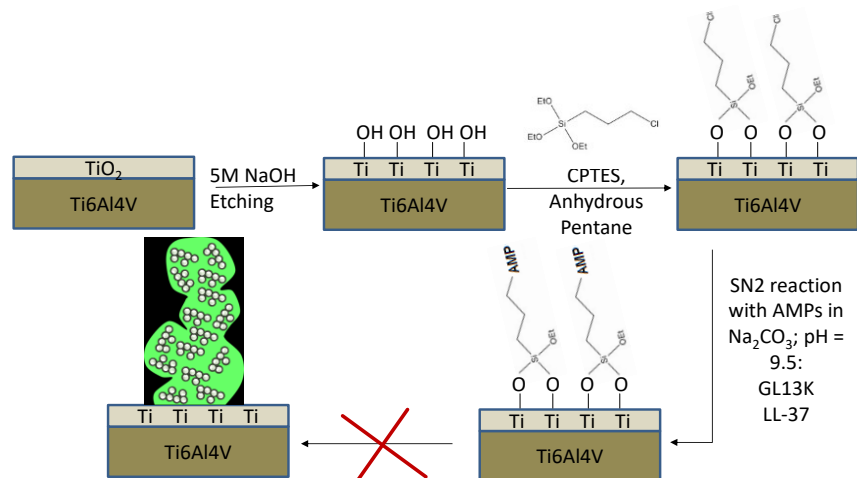


Figure 7: Reaction scheme for silanization and AMP immobilization on Ti6Al4V against MRSA infections

Figure 8, 9 and Table 3: Ti6Al4V alloy was successfully silanized and antimicrobial peptides were successfully immobilized on the alloy as indicated by the N1s signals in the XPS spectra (Figure 6). Table 2 shows that DJK2 and W10 have the maximum amount of N1s of ~13.5 atom%. DJK2 was partially soluble in the Na_2CO_3 buffer (pH=9.5) used for the peptide immobilization via $\text{S}_\text{N}2$ reaction. W10 formed cloudy micelles or secondary structures when it was dissolved in the Na_2CO_3 buffer, but did not precipitate unlike DJK2. It is probable that these two peptides are not only covalently anchored but also physically adsorbed on the surfaces; resulting in a higher N1s signal. The water contact angles in Fig. 4 show the expected hydrophobicity of L-GL13K and D-GL13K coatings (WCA: 90-110 °) as in our previous reports². While W10 coatings yielded WCA ~75 °, both DJK2 and DJK5 yielded highly hydrophilic surfaces with WCA of ~25-30 °. All coatings with peptides had a dynamic WCA response, which was not the case for the controls surfaces with no peptides, irrespective of the previous treatment to peptide immobilization. These results indicate that the peptides were present and able to change physical-chemical properties of the metal. Our ability to obtain sound coatings with all previously-selected peptides leads to full analysis of all of them for antimicrobial properties vs MRSA.

Table 3: Elemental surface composition of silanized and AMP immobilized Grade-5 Ti alloy. Data shown are mean $\pm$ standard deviation of n = 3-4 replicates

Surface/Coating	Atom%							
	C1s	O1s	Ti2p	N1s	Si2p	Cl2p	Ca2p	N/Ti
eTi6Al4V_Si	29.9 $\pm$ 9.1	53.8 $\pm$ 7.6	10.5 $\pm$ 1.7	1.1 $\pm$ 0.3	1.1 $\pm$ 1.0	0.8 $\pm$ 0.3	3.4 $\pm$ 1.3	0.13 $\pm$ 0.1
eTi6Al4V_Si_D-GL13K	61.5 $\pm$ 7.3	27.1 $\pm$ 5.1	2.9 $\pm$ 2.0	7.6 $\pm$ 0.8	0.2 $\pm$ 0.1	0.4 $\pm$ 0.2	0.6 $\pm$ 0.2	3.6 $\pm$ 2.0
eTi6Al4V_Si_DJK2	55.2 $\pm$ 1.0	25.8 $\pm$ 2.1	3.2 $\pm$ 0.8	13.4 $\pm$ 1.4	0.3 $\pm$ 0.1	0.5 $\pm$ 0.4	0.7 $\pm$ 0.2	4.4 $\pm$ 1.5
eTi6Al4V_Si_DJK5	44.1 $\pm$ 2.8	36.8 $\pm$ 2.7	7.0 $\pm$ 0.4	10.0 $\pm$ 1.8	0.3 $\pm$ 0.2	0.4 $\pm$ 0.3	1.5 $\pm$ 0.2	1.4 $\pm$ 0.3
eTi6Al4V_Si_W10	71.1 $\pm$ 3.2	14.4 $\pm$ 1.5	0.5 $\pm$ 0.5	13.4 $\pm$ 1.8	0.2 $\pm$ 0.1	0.4 $\pm$ 0.2	0.2 $\pm$ 0.1	38.4 $\pm$ 21.4
eTi6Al4V_Si_L-GL13K	49.2 $\pm$ 2.6	34.8 $\pm$ 3.0	5.3 $\pm$ 0.7	8.2 $\pm$ 1.0	0.9 $\pm$ 0.6	0.5 $\pm$ 0.2	0.9 $\pm$ 0.2	1.6 $\pm$ 0.3

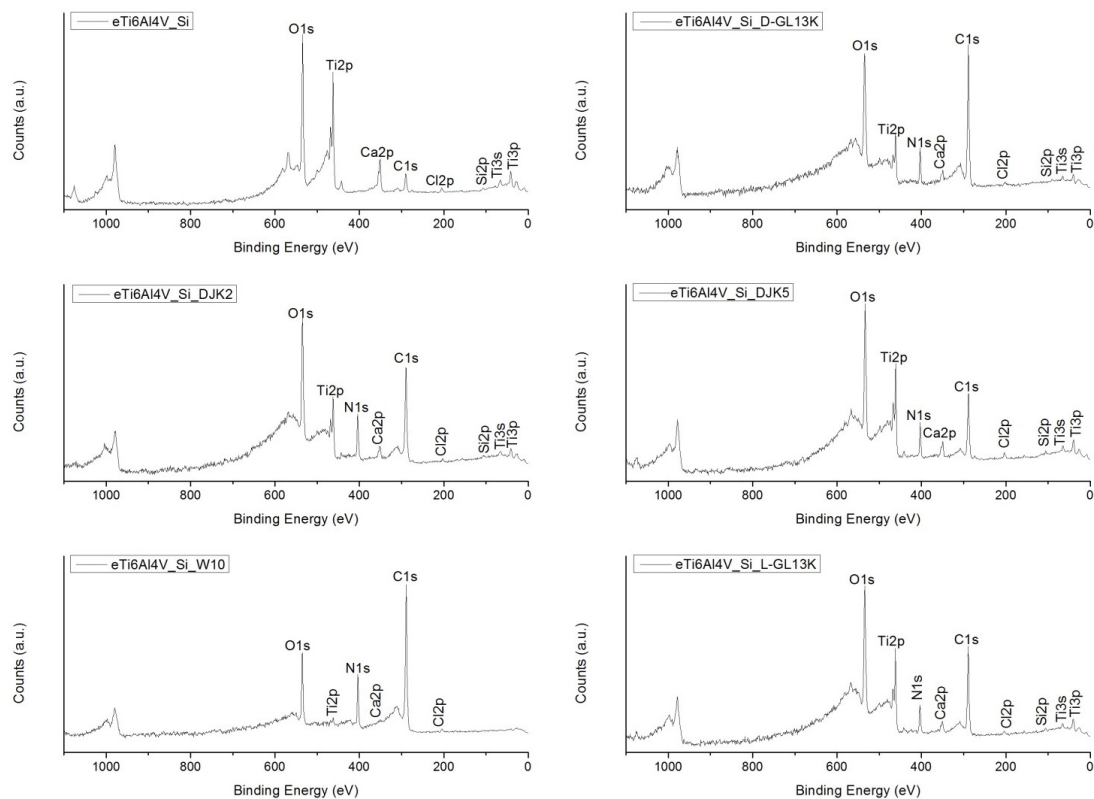


Figure 8: XPS wide survey scans spectra of Ti6Al4V surfaces after silanization and immobilization of the selected antimicrobial peptides.

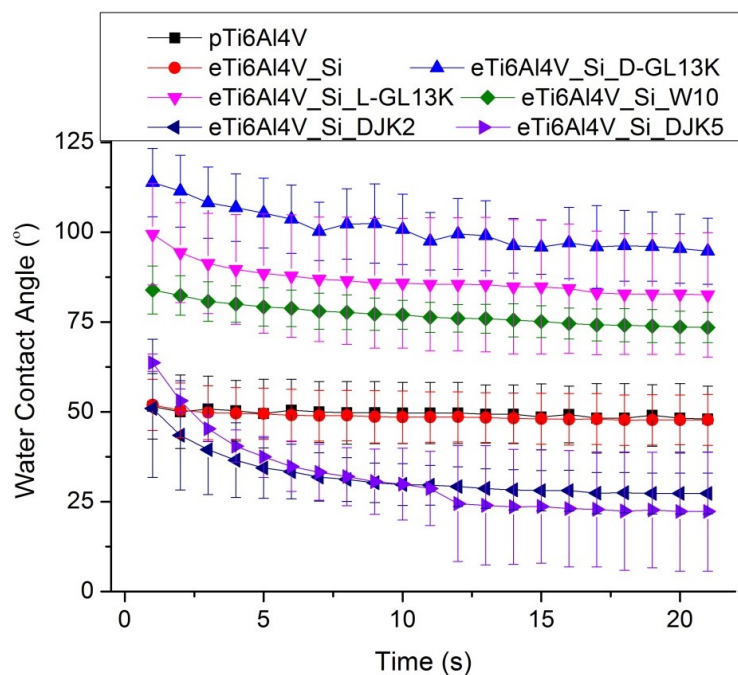


Figure 9: Dynamic water contact angle (WCA) measurements recorded for polished, etched + silanized and antimicrobial peptide immobilized Ti6Al4V. The eTi (etched Ti alloy) samples were superhydrophilic to the point that the WCA was not measurable.

Figure 10 and 11: The D-Gl13K coatings reduced the vitality (Figure 10, ATP metabolic activity) and viability (Figure 11, CFU counting) of MRSA biofilms with respect to both controls; i.e., polished and etched Ti6Al4V surfaces under the optimized experimental conditions. These are encouraging results, but the reduction in CFU counts was of one order of magnitude and can be improved by optimizing both, coating manufacturing conditions and MRSA experimental in vitro conditions. We will also performed biological repeats of these experiments to validate the appropriateness of robustness of our experimental conditions.

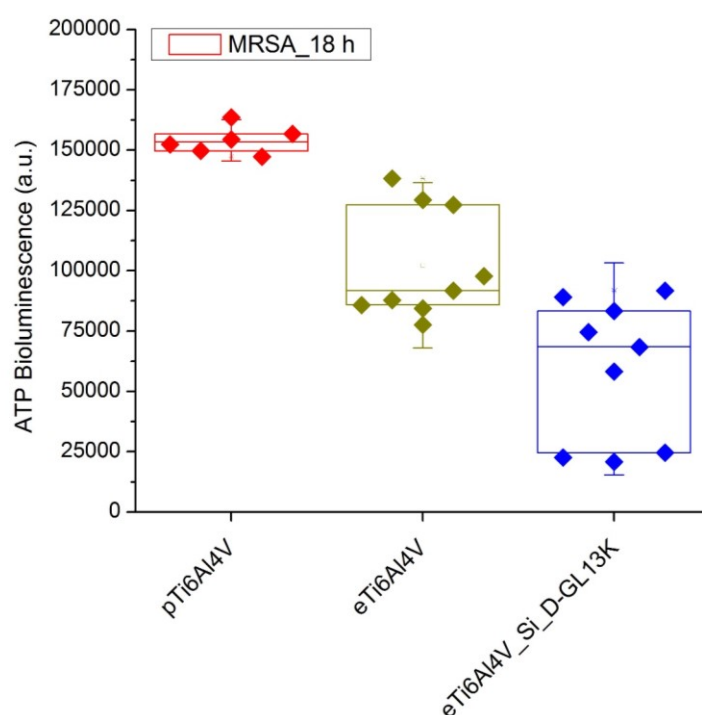


Figure 10: Metabolic activity (ATP) of MRSA biofilms on control (polished and etched Ti6Al4V) and D-Gl13K coated surfaces

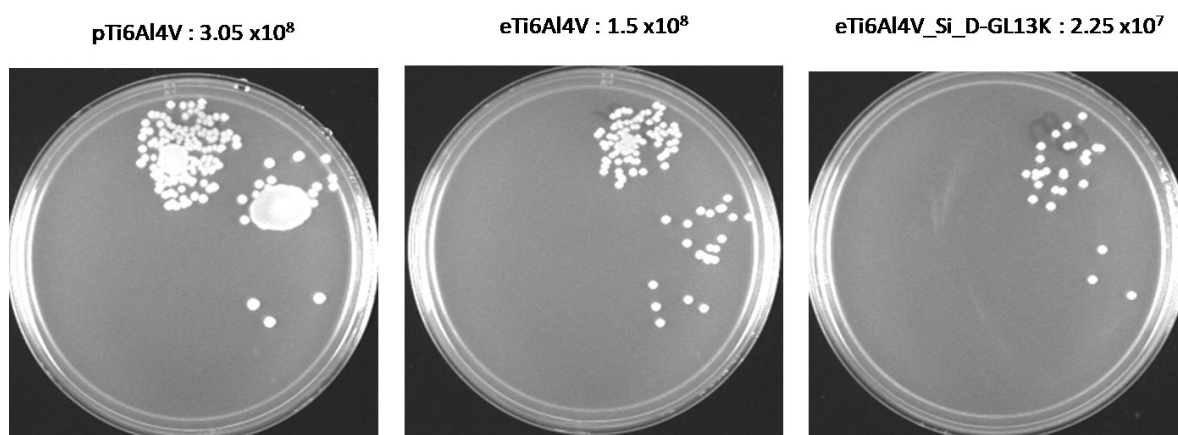


Figure 11: Images of MRSA colonies and corresponding CFU counting for bacteria grown on control (polished and etched Ti6Al4V) and D-Gl13K coated surfaces

Summary: We have selected the best antimicrobial peptides against MRSA to be tested as coatings of Ti6Al4V. We have successfully immobilized all the selected peptides on the clinically relevant Ti6Al4V alloy and preliminarily assessed that the coatings can be effective to prevent MRSA biofilm growth. The next step will be to finalize the biological evaluation of the peptide coatings against MRSA and of those peptide coatings with the highest potency assess their cytocompatibility.

Major Task 2: Manufacturing and optimization of multifunctional coatings

Subtask 2.3: Evaluate manufacturing/non-biological properties for co-immobilization of CAP & AMP

Introduction/Rationale: In the previous section, we presented conclusive data for selection of the best cell-adhesive peptides (CAP); that is Net-1 and LamLG3 peptides. We did this selection based on best keratinocyte proliferation and hemidesmosomes formation. However, we did the analysis with coatings on glass, as a model surface. Then, we optimized conditions for immobilizing the peptides on Ti6Al4V, our clinically relevant material, by translating knowledge from our previous work on peptide coatings on c.p. Ti and additional work previously presented in this report for immobilizing antimicrobial peptides (AMP) on the Ti alloy. So here we translated this knowledge to develop the candidate coatings on the Ti alloy. Further, we anticipated that the biggest challenge for these coatings durability on an in vivo setting would be the potential enzymatic degradation, so before we move forward in developing these coatings, we assessed the durability of them following challenge with a relevant enzyme in vitro.

Durability of CAP coatings on titanium alloy

Experimental Peptides: Candidate CAPs Net1 (KN3-GGG-QWRDTWARRLRKFQQREKKGKCRKA)¹ and LamLG3 (KN3-GGG-PPFLMLLKSTRFC)² were produced (>95% purity; AAPPTec; high-performance liquid chromatography and mass spectrometry characterization were performed (data not shown). The N-terminus of each peptide was modified with three flexible glycine spacers (previously shown to be important to ensure biological activity)⁵⁻⁷ plus an azide-conjugated lysine for copper free, alkyne azide click chemistry immobilization.⁸⁻⁹

Peptide coatings on Ti6Al4V: Titanium alloy Ti-6Al-4V disks (President Titanium) were activated by 5M NaOH basic etching. Etched disks were silanized as previously described.¹⁰ In short, disks were placed in an N₂-rich atmosphere and immersed in pentane containing 0.05M N,N-Diisopropylethylamine and 0.5M (3-Chloropropyl)-triethoxysilane (all obtained from Sigma-Aldrich) and thoroughly rinsed. Silanized disks were immersed in 0.1 mM dibenzocyclooctyne-amine (DIBO, Sigma-Aldrich) in 0.1 M Na₂CO₃ buffer overnight and cleaned with solvents. These disks were then immersed in 0.1 mM peptide solutions in tris-buffered saline buffer overnight and again cleaned with solvents (yielding Net1 and LamLG3). In some experiments, fluorescent Cyanine5 labelled peptides were used. Peptides were labeled in-house using a commercially available kit (43020, Lumiprobe) directed against free amino groups. Successful CAP coating synthesis was confirmed by water contact angle and X-ray photoelectron spectroscopy (XPS).

Enzyme-mediated degradation of peptide coatings: CAP coatings were then challenged by matrix metalloproteinase-8 (MMP8), a representative enzyme at the skin-implant interface²⁰ that may degrade CAP coatings, and thus inactivate potential biological activity. Coated disks (Net1 and LamLG3) were immersed in approximately 50 ng/mL²¹ recombinant MMP8 in an MMP activating buffer (including zinc, calcium, and 4-aminophenylmercuric acetate)^{22,23} and incubated at 37 °C for one hour and 24 hour. Controls were treated identically without the MMP8 added.

Previous work has shown that one hour incubation in buffers alone results in minimal CAP loss.²⁴ CAP retention was evaluated through XPS and retention of fluorescent label intensity following extensive washing tests.

X-ray photoelectron spectroscopy (XPS) characterization: Surfaces were characterized using the same technique as described in the earlier section (Subtask 1.1)

Fluorescent intensity quantification: Cyanine5 labelled CAPs were visualized with an upright fluorescent microscope (DM 6B, Leica). Background-subtracted pixel intensities were calculated and analyzed in ImageJ (NIH).

Cell culture

The keratinocyte response to CAP coatings that had been degraded for one and twenty-four hours was compared to freshly synthesized CAP coatings, as well as control eTi and eTi-DIBO following the same methodology as described in Subtask 1.1. Cell proliferation was measured using CCK8 assay and hemidesmosome formation using immunofluorescence following the same method as described in earlier section.

Results:

Ti6Al4V alloy was successfully silanized and surface modified with LamLG3 and Net1 as indicated by the N1s signals in the XPS spectra (Figure 12)

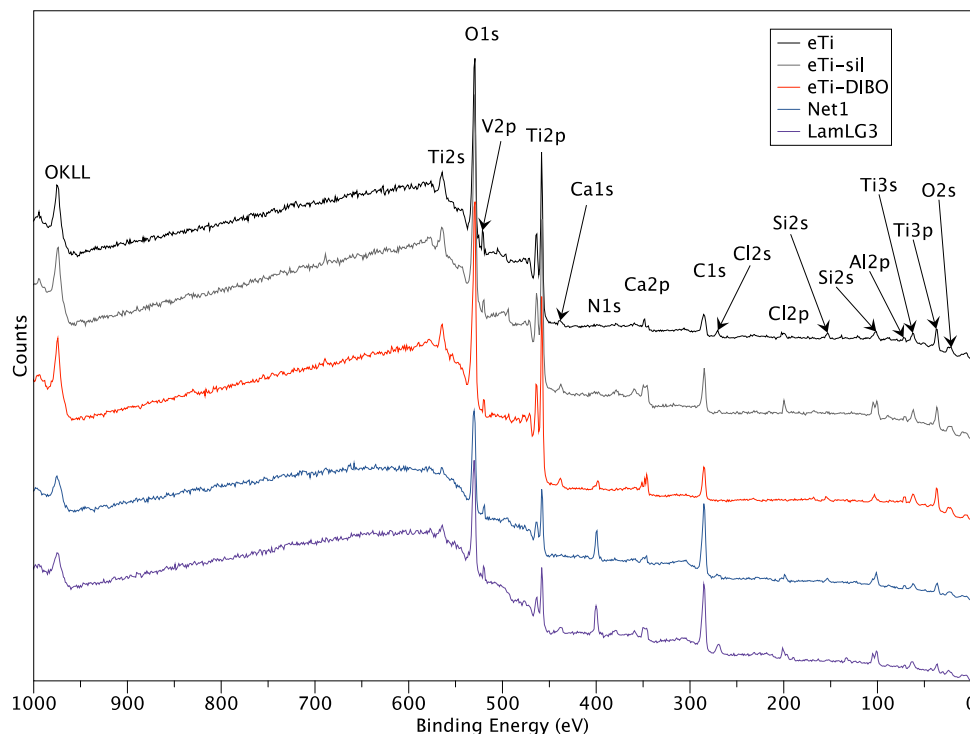


Figure 12: XPS wide survey scans spectra of Ti6Al4V surfaces after silanization and immobilization of the selected cell adhesive peptides

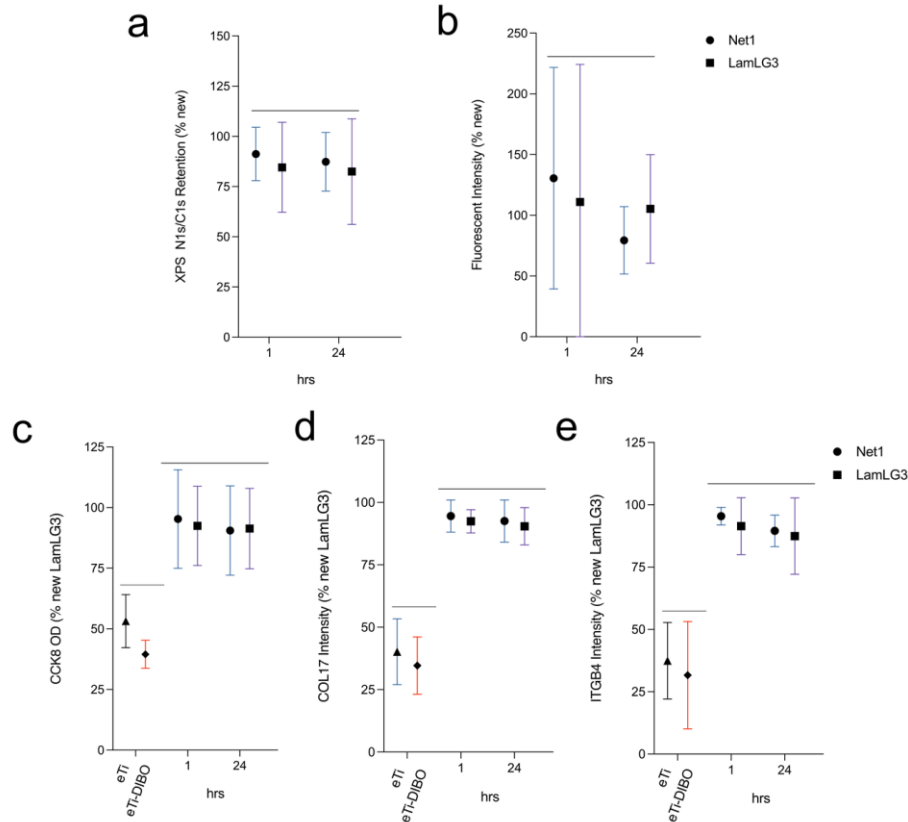


Figure 13: Peptide retention and biological activity on titanium alloy following 1 and 24 hour MMP8. a. Percentage of fluorescent intensity of MMP8-challenged surfaces compared to buffer-only controls. b. X-ray photoelectron spectroscopy (XPS) atomic percentage of N1s/C1s of freshly synthesized surfaces compared to MMP8-challenge. c. OK metabolic activity (CCK8 OD) at one day of culture on control eTi and eTi-DIBO and MMP8-challenged Net1 and LamLG3 surfaces compared to freshly synthesized LamLG3. d. OK collagen XVII expression (COL17 Intensity) at one day on control eTi and eTi-DIBO and MMP8-challenged surfaces compared to freshly synthesized LamLG3. e. OK integrin $\beta 4$ expression (ITGB4 Intensity) at one day on control eTi and eTi-DIBO and MMP8-challenged surfaces compared to freshly synthesized. Horizontal lines indicate no statistical differences ($P > 0.05$) between indicated groups for a - e. Data are mean $\pm$ s.d.

Both LamLG3 and Net1 peptides were retained following one and twenty-four hour incubation in recombinant MMP8 (**Figure 13a** and **13b**). No differences in resistance to enzymatic activity were seen. LamLG3 and Net1 retained their ability to upregulate OK metabolic activity (**Figure 13c**) and expression of hemidesmosome markers collagen XVII (**Figure 13d**) and integrin $\beta 4$ (**Figure 13e**) compared to controls.

Future work will extend the current work to longer time periods (three days) and verify MMP8 activity.

Major Task 5: Selection of components for surgeon-friendly glue

Subtask 5.1 and 5.2: Evaluate basic biopolymer properties and light-curing system for scaffolding of the glue

Introduction/Rationale: To identify the scaffold to be used for future biological evaluation, further characterization of scaffolds adhesive properties to Ti6Al4V alloy was the focus of this quarter using a half factorial DoE to fabricate 8 different scaffold formulations. Along with the adhesivity, statistical measurements were performed to assess the effect of different input variables on the output properties. These include: macromer (GelMA) concentration, macromer degree of methacrylation (DoM), photoinitiator concentration and time of curing as input variables. Swelling ratio, soluble fraction, compressive modulus, adhesive strength to metal and skin along with ability of these hydrogels to support cell attachment were measured as output properties.

Experimental

Gelatin methacryloyl (GelMA) was synthesized according to a previously published protocol.²⁵ Briefly, 10 g of porcine gelatin (bloom strength 300) was dissolved in phosphate buffer saline at 50 °C and once the gelatin was completely solubilized, 0.6 g/g of methacrylic anhydride (Sigma Aldrich, USA) was added and the reaction was carried out for one hour. After the completion of reaction, the reaction product was centrifuged at 3000g for 3 minutes at room temperature and the supernatant collected was diluted in pre-warmed deionized water and dialyzed against fresh deionized water using 13kDa cellulose membrane for 5 days. The dialysis was performed at 40°C to prevent thermal gelation of GelMA with water being refreshed twice a day. Post-dialysis, GelMA was frozen and lyophilized to form GelMA macromere, which was then used. To compare the effect of degree of methacrylation, GelMA was purchased from Advanced Biomatrix (USA) with degree of methacrylation (DOM) > 90%.

A 2⁴⁻¹ design of experiment (DoE) was used to determine the effect of four different variables at two levels. The low and high level of DoM, macromer concentration, PI concentration and cross-linking time were set at 60% (in-house synthesized GelMA) and 90% (GelMA purchased from Advanced Biomatrix), 5% and 12 weight%, 0.05% and 0.2 weight% and 30 and 180 seconds, respectively. A total of 8 combinations were tested which were then used to assess three different outputs, namely soluble fraction, swelling ratio and compressive modulus of the fabricated hydrogel glues.

Briefly, lyophilized GelMA was solubilized in PBS at either 5 wt% or 12 wt% which was then mixed with either 0.05 wt% or 0.2% LAP (Lithium phenyl-2,4,6-trimethylbenzoylphosphinate, Sigma, USA) and cross-linked in silicon molds (6 mm diameter, 1mm thickness) using light with λ_{max} 400 nm for either 30 seconds or 180 seconds. Fabricated hydrogels were then used to determine the soluble fraction (measure of cross-linking efficiency) swelling ratio and compressive moduli (MTS criterion 41). To determine the effect of input variables on soluble fraction and swelling ratio, six samples per group were weighed immediately post cross-linking to obtain initial wet mass (m_{initial}, t₀), after which three samples were lyophilized immediately to obtain their dry weights (m_{dry}, t=0). The actual macromer fraction was then calculated using equation (1).

$$\text{actual macromer fraction} = \frac{m_{dry;t0}}{m_{initial;t0}} \quad (1)$$

The remaining three samples were incubated in PBS at 37 °C to allow swelling and the un-crosslinked macromer to leach out of the hydrogel network. After 24 h, the samples were weighed again (mswollen). The samples were subsequently lyophilized and weighed again (mdry). The sol fraction was defined as the mass loss after 24 h and was calculated using equations (2) and (3). Mass swelling ratio (q) was calculated using equation (4).

$$m_{initial,dry} = m_{initial} (\text{actual macromer fraction}) \quad (2)$$

$$\text{sol fraction} = \frac{m_{initial,dry} - m_{dry}}{m_{initial,dry}} \times 100\% \quad (3)$$

$$q = \frac{m_{swollen}}{m_{dry}} \quad (4)$$

To determine the compressive moduli, fabricated hydrogels were allowed to equilibrate at 37 °C in PBS for 24 h after which the compressive test was performed using a MTS Criterion 41 equipped with a 5N load cell. Equilibrated hydrogels (6mm x 1mm) were compressed parallel to the long axis at a constant cross-head displacement rate of 0.01 mm/min. The compressive Young's modulus was calculated by the slope of stress-strain curve in the linear elastic range of 10-15% strain.

For adhesive strength measurements, a lap-shear test was performed. Briefly, different macromer concentrations (5 wt%, or 12 wt%) prepared by dissolving lyophilized GelMA in PBS were mixed with 0.05% or 0.2% LAP (prepared in PBS) and cross-linked between titanium coupon and gelatin coated glass slide using light with maximum at 400 nm for 30 or 180 seconds. Post cross-linking, the adhered glass-titanium sample was mounted between the fixtures and strained at a speed of 1.5 mm/min until failure using a MTS Criterion 41 equipped with a 100 N load cell. For determining glue adhesivity to porcine skin, similar setup was used as used for metal adhesivity. Small coupons of porcine skin were collected, fat tissue removed and different hydrogel formulations were used as glue between skin sample and gelatin coated glass slide using the conditions same as that used for measuring adhesivity to titanium. Lap shear test was performed under same test conditions as used for titanium.

Metabolic activity of keratinocytes and dermal fibroblast of different hydrogel groups

To assess the capability of different hydrogel groups to support attachment and proliferation of cells, immortalized epithelial keratinocytes (HaCaTs) and dermal fibroblasts (HDFs) were used. HaCaTs (2.5×10^4) and HDFs (1×10^4) were seeded on different hydrogel platforms and after 24 h of incubation, samples were gently washed in PBS and incubated for three hours in alamarBlue™ solution (ThermoFisher; 9:1 medium:alamarBlue™). Fluorescence intensity (excitation/emission = 530/590 nm) was obtained on a plate reader (Synergy HT, Biotek).

Collected data from different analysis was used as input in Design Expert 12.0 to assess the relationship between different input variables and measured output responses. The analysis was performed to determine the effect of independent input variables and different interactions between the variables and how they influence the output response. The positive effect on a response means that when the input variable is increased, that output increases and vice-versa.

Results:

Table 5 : Combination of different input variables and output responses of the corresponding DoE used for fabricating different hydrogel glues.

Run	Factor 1 A:DoM %	Factor 2 B:Macromer con... %	Factor 3 C:PI concentration %	Factor 4 D:Time of Crossl... seconds	Response 1 Swelling ratio	Response 2 Soluble fraction	Response 3 Compressive m... kPa	Response 4 Adhesive Strength kPa
1	90	5	0.2	30	11.7619	13.2127	11.25	78.709
2	60	5	0.05	30	22.5	21.0071	2.46	29.2269
3	90	12	0.2	180	5.97222	4.51423	76.8	47.1171
4	90	5	0.05	180	10.0667	16.0979	11.16	36.5933
5	90	12	0.05	30	5.92593	20.0276	13.61	49.8705
6	90	12	0.2	180	6.02703	2.31203	72.9	41.2225
7	60	12	0.2	30	9.1	14.2263	25.14	45.6089
8	60	5	0.05	30	25.5	31.8652	3.52	33.4631
9	90	5	0.2	30	11.2381	11.5437	10.55	76.3323
10	90	12	0.05	30	6.7619	24.904	14.38	48.3846
11	60	5	0.2	180	13.7059	0.737926	6.07	65.1825
12	60	12	0.05	180	6.82759	12.075	30.8	25.1938
13	60	12	0.2	30	9.24138	16.4299	26.82	41.3563
14	90	5	0.05	180	9.5625	12.8956	13.24	41.7928
15	60	5	0.2	180	14.125	1.94432	8.9	65.6117
16	60	12	0.05	180	6.65517	12.4475	32.67	27.8088
17	90	12	0.2	180	5.89744	2.40386	90.46	56.5647
18	60	5	0.2	180	13.7647	2.27687	5.24	50.119
19	90	12	0.05	30	17.3994	6.05	13.99	38.5604
20	60	12	0.05	180	6.18919	5.1714	30.67	33.317
21	90	5	0.2	30	10.6818	5.14248	10.59	70.8992
22	60	12	0.2	30	9.1	15.2236	22.4	42.5369
23	90	5	0.05	180	10.0667	16.0979	13.22	37.3538
24	60	5	0.05	30	23.7	27.8486	2.34	35.7551

Table 5, Figure 13 and 14: Time of crosslinking significantly reduced soluble fraction and swelling ratio of the hydrogel glues, but increased hydrogel stiffness. This implies, for a given degree of methacrylation, macromer concentration, and photo-initiator concentration, an increase in time of cross-linking produces a highly cross-linked matrix, as measured by reduced soluble fraction and

swelling ratio but also a matrix with improved material stiffness. For adhesion to titanium, the photo-initiator concentration seems to have the highest effect; but the overall effect is small, at least in the range of variable values studied. For adhesivity to skin, the three input variables that positively influenced the response include macromer concentration, DoM and time of crosslinking.

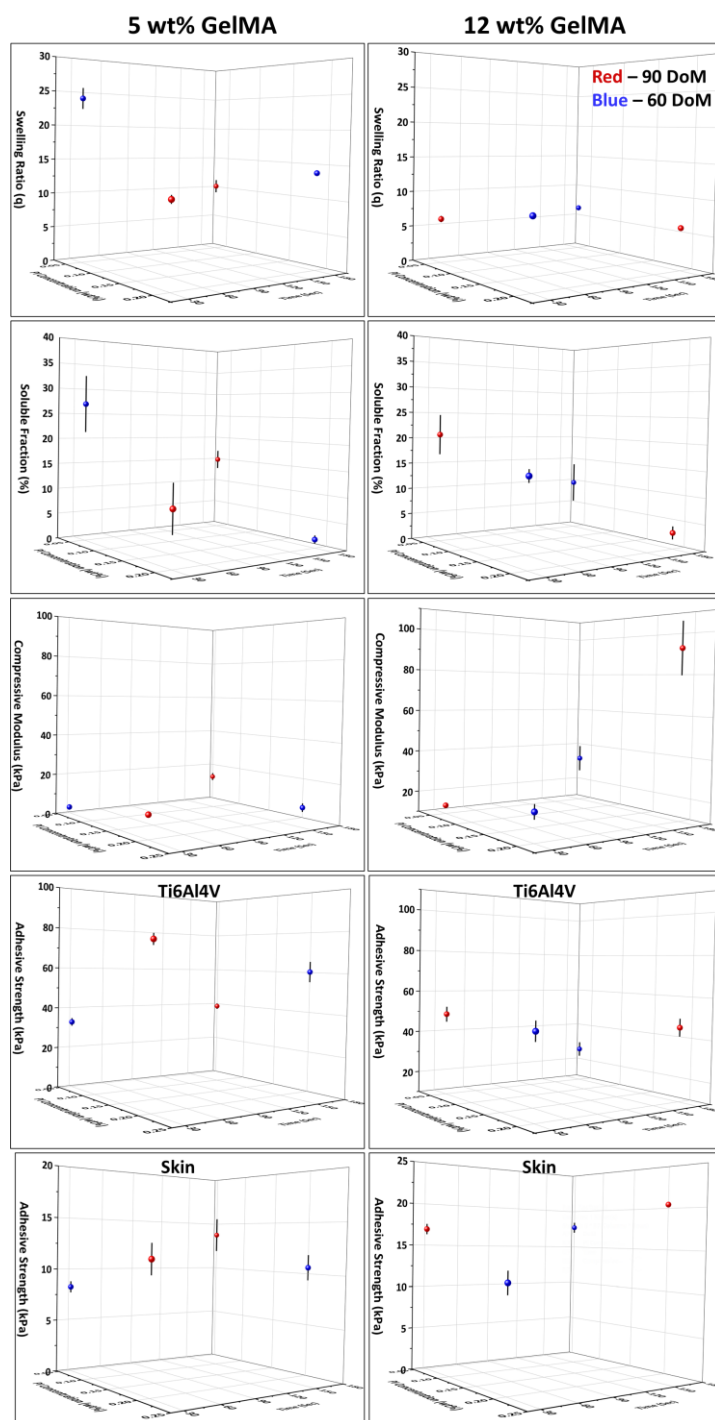
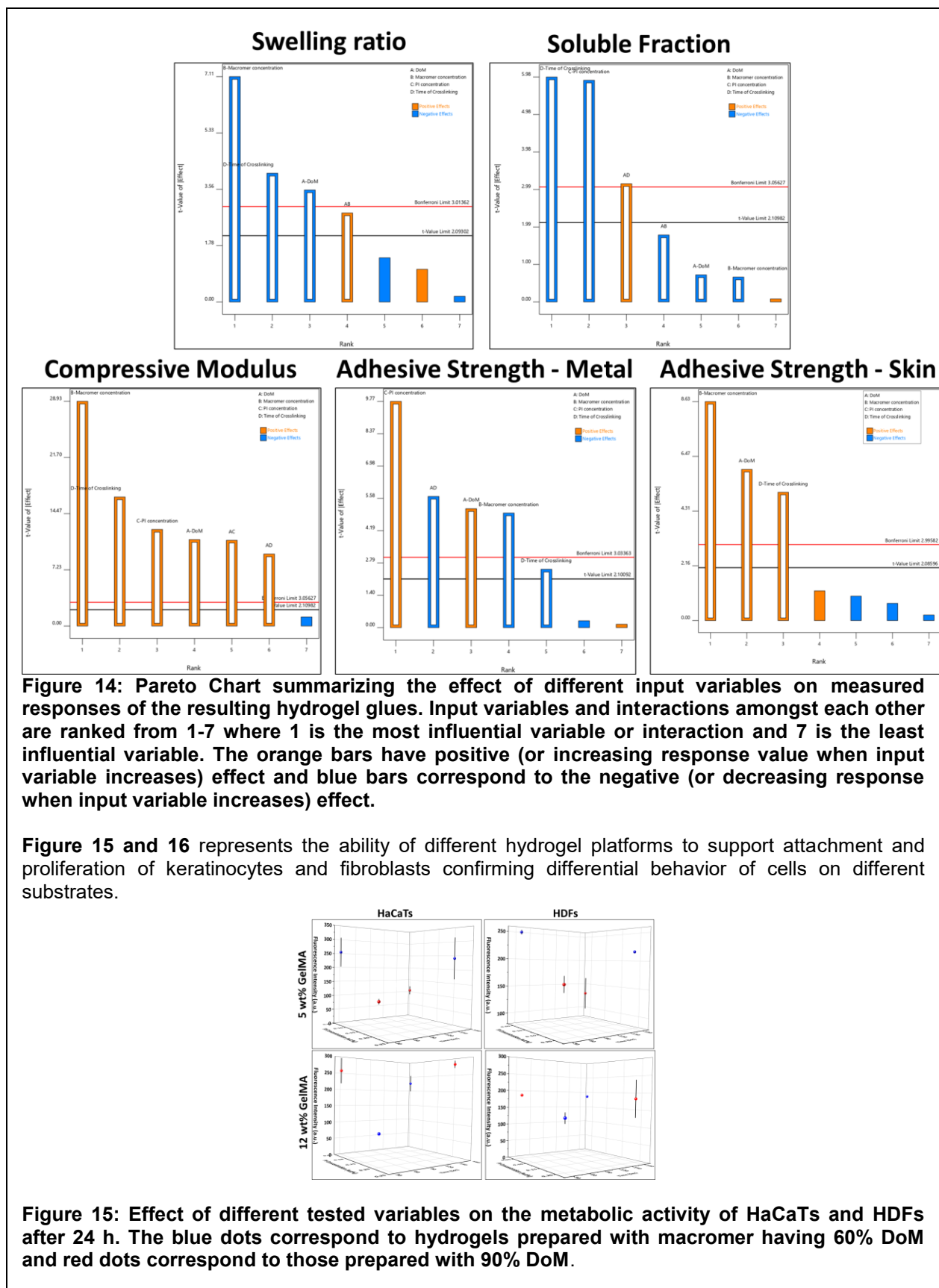


Figure 13: Effect of different tested variables on the soluble fraction, swelling ratio, compressive modulus and adhesive strength to Ti6Al4V alloy and porcine skin of the hydrogel. The blue dots correspond to hydrogels prepared with macromer having 60% DoM and red dots correspond to those prepared with 90% DoM.



One combinatorial factor that positively influences the metabolic activity of both HaCaTs and HDFs is the macromer concentration and degree of methacrylation. And for the negative influence, the combination of degree of methacrylation and time of cross linking decreases as the two factors are proportionately increased.

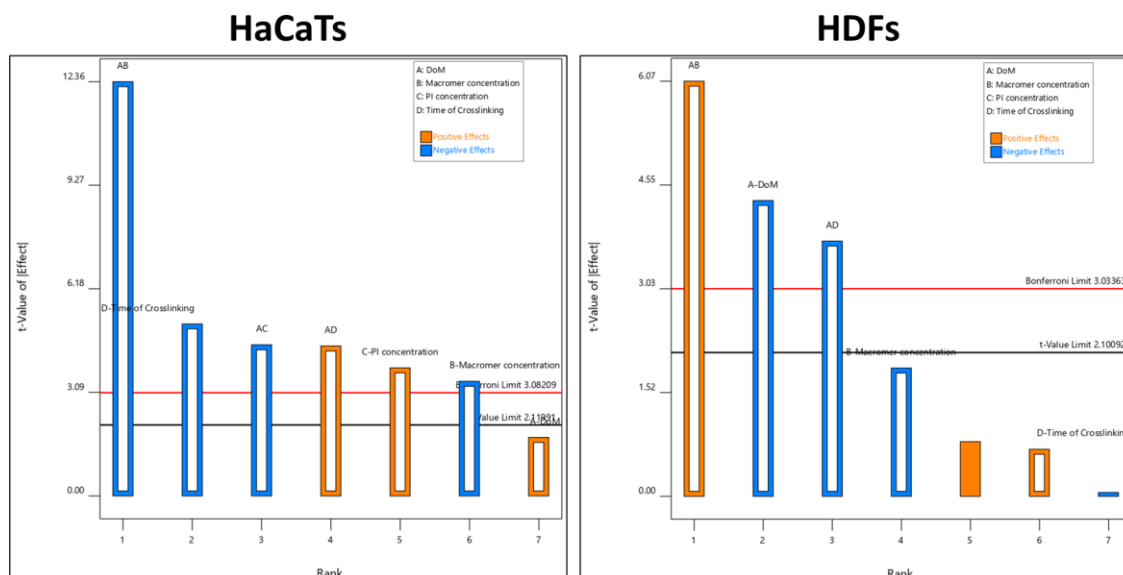


Figure 16: Pareto Chart summarizing the effect of different input variables on measured responses of the resulting hydrogel glues. For HaCaTs post inverse square root transformation was applied whereas for HDFs no transformation was used. Input variables and interactions amongst each other are ranked from 1-7 where 1 is the most influential variable or interaction and 7 is the least influential variable. The blue bars for HaCaTs and orange bars for HDFs have positive (or increasing response value when input variable increases) effect and orange bars for HaCaTs and blue bars for HDFs correspond to the negative (or decreasing response when input variable increases) effect.

Summary:

These results confirm that we can tune the properties of our hydrogel glues by purposely selecting the input values for the variables studied. An ideal glue will have low soluble fraction and swelling ratio, i.e., a highly-crosslinked matrix with strong adhesivity to metal and skin tissue.

In the next quarter, we will focus on assessing the ability of different scaffolds on hemidesmosome formation for HaCaTs and collagen I for HDFs. This will help us narrow down the hydrogel candidates which will be combined with cell adhesive peptide and assess its influence on different hydrogel properties and ability to support HaCaTs and HDFs proliferation and hemidesmosome and collagen I expression.

Task 6: Development of antimicrobial nanoparticles

Subtask 6.1: Optimization of mesoporous antimicrobial nanoparticles formulation using DoE

Introduction/Rationale: Overall, in this task we aim loading and releasing antimicrobial peptides (AMP) from synthesized mesoporous metal silicate nanostructures. To that end, we target the design of nanostructures with high surface area, small pore size and controlled degradation profile so that these nanoparticles enable high loading efficiency with prolonged release profile.^{26,27} Another important parameter which needs investigation and optimization is the shape of the synthesized nanostructures, to overcome shape-mediated nanostructure cytotoxicity. All these output variables can be controlled by concentration of the polymer template used for synthesis and the catalyst used along with the ratio of metal:silica used.^{26,27} To decipher the relationship between these variables and their effect on different output properties, the effect of these variables on synthesized strontium mesoporous silicate (SrMSN) were examined using a 2^{4-1} design of experiment (DoE). The variables studied were the polymeric template CTAB, liquid ammonia – basic catalyst to promote hydrolysis and condensation of silica, TEOS – source of silica and strontium nitrate, metal source. The experiments were done in triplicate, which is critical to soundly establish the correlation between the effect of different input variables on measured outcomes.

Experimental: *Synthesis of SrMSN*

Mesoporous metal silicate nanostructures were synthesized using a self-assembly route which relies on a sacrificial micellar template formed by cetyl trimethyl ammonium bromide (CTAB, Sigma, St Louis, MO) dissolved in basic water solution. After thirty minutes, triethyl orthosilicate (TEOS, Sigma, St Louis, MO) and strontium nitrate is added and the reaction is vigorously stirred for another 2h after which the product is collected by centrifugation, washed with deionized water and absolute ethanol and heat-dried at 60°C overnight. The heat-dried powder was then calcined at 550°C for 5 h to remove CTAB.

DoE for SrMSN

A 2^{4-1} DoE was used to determine the effect of four different variables at two levels. The low and high level of amount of CTAB, volume of liquid ammonia used, amount of TEOS, and strontium nitrate were set at 1.1 g and 2.2 g, 2 ml and 6 ml, 5 ml and 10 ml and 4.75 g and 9.5 g, respectively. A total of 8 experimental conditions were tested, which were then used to assess shape and size, surface area, pore size, pore volume and surface charge of the calcined SrMSN.

The effect of different variables and their interactions on the measured outcome was determined using Design Expert 12 software. Necessary transformations were applied based on the recommendation dependent on Box-Cox plots.

Characterization of SrMSN

FEI Tecnai Spirit BioTwin TEM was used for collecting TEM micrographs of ethanol solubilized metal silicates which were drop dried on 400 mesh carbon coated copper grid (TedPella, USA) at an acceleration voltage of 120 kV. The nanostructure morphology of the SrMSN was confirmed with SEM. For this, ethanol solubilized SrMSN were drop dried on aluminium stubs (Ted Pella, USA). Then the samples were coated with 2nm Iridium (Leica, EM ACE600, Vienna, Austria) and imaged on a high resolution SEM, SU8230 (Hitachi, Monato, Tokyo, Japan) at an acceleration voltage of 1 kV. N₂ adsorption desorption isotherm were collected on Quantachrome Autosorb-1 instrument. Samples were first degassed by heating the samples at 300 °C for 4 h (ramp rate of 5°C/minute) under vacuum after which the data was acquired and processed on ASiQwin software to determine pore size distribution, pore volume and total surface area of different metal silicate nanoparticles. Surface charge of PBS solubilized nanostructures was measured using Malvern Nano ZetaSizer ZS in a U-shaped cuvette with gold electrode.

Results:

Table 6, Figure 17-20: TEM and SEM micrographs confirmed that the range of input variables used in our DoE affected notably the size and shape of the SrMNs obtained. TEM micrographs were used to measure D1 and D2 (diameter along long axis and short axis) and the aspect ratio (D1/D2) was determined. An aspect ratio of more than 1.2 indicates rod-shaped particles whereas values lower or equal to 1.2 are indicative of a (quasi)spherical shape of the nanoparticles. The statistical analysis of the DoE confirmed that amount of liquid ammonia and CTAB and their interaction positively influence

the aspect ratio of the SrMNs (note inverse transformation in results shown in Figure 12), which indicates that as we increase the concentration of liquid ammonia and CTAB, the shape of synthesized nanostructures transition from spherical or quasi-spherical to rod-like. Controlling the shape of the SrMNPs is an additional variable to those originally proposed in our project that can be used to tune the degradability and/or release of the antibacterial agents.

Run	Factor 1 A:DoM %	Factor 2 B:Macromer con... %	Factor 3 C:PI concentration %	Factor 4 D:Time of Cross... seconds	Response 1 Swelling ratio	Response 2 Soluble fraction	Response 3 Compressive m... kPa	Response 4 Adhesive Strength kPa
1	90	5	0.2	30	11.7619	13.2127	11.25	78.709
2	60	5	0.05	30	22.5	21.0071	2.46	29.2269
3	90	12	0.2	180	5.97222	4.51423	76.8	47.1171
4	90	5	0.05	180	10.0667	16.0979	11.16	36.5933
5	90	12	0.05	30	5.92593	20.0276	13.61	49.8705
6	90	12	0.2	180	6.02703	2.31203	72.9	41.2225
7	60	12	0.2	30	9.1	14.2263	25.14	45.6089
8	60	5	0.05	30	25.5	31.8652	3.52	33.4631
9	90	5	0.2	30	11.2381	11.5437	10.55	76.3323
10	90	12	0.05	30	6.7619	24.904	14.38	48.3846
11	60	5	0.2	180	13.7059	0.737926	6.07	65.1825
12	60	12	0.05	180	6.82759	12.075	30.8	25.1938
13	60	12	0.2	30	9.24138	16.4299	26.82	41.3563
14	90	5	0.05	180	9.5625	12.8956	13.24	41.7928
15	60	5	0.2	180	14.125	1.94432	8.9	65.6117
16	60	12	0.05	180	6.65517	12.4475	32.67	27.8088
17	90	12	0.2	180	5.89744	2.40386	90.46	56.5647
18	60	5	0.2	180	13.7647	2.27687	5.24	50.119
19	90	12	0.05	30	17.3994	6.05	13.99	38.5604
20	60	12	0.05	180	6.18919	5.1714	30.67	33.317
21	90	5	0.2	30	10.6818	5.14248	10.59	70.8992
22	60	12	0.2	30	9.1	15.2236	22.4	42.5369
23	90	5	0.05	180	10.0667	16.0979	13.22	37.3538
24	60	5	0.05	30	23.7	27.8486	2.34	35.7551

Table 6: Combination of different input variables and output responses of the corresponding DoE used for fabricating differentSrMSN

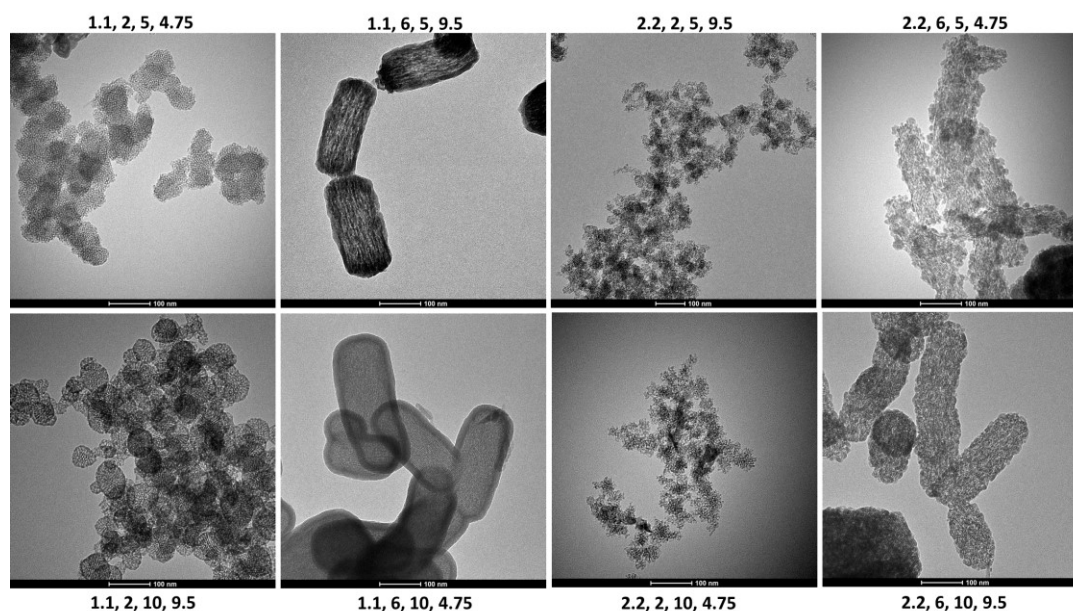


Figure 17: TEM micrographs of different SrMSNs synthesized using 8 different experimental conditions as per a 2^{4-1} DoE. The numbers depicted in the images correspond to the concentration of CTAB, volume of liquid NH_3 , volume of TEOS and amount of $\text{Sr}(\text{NO}_3)_2$ used, respectively.

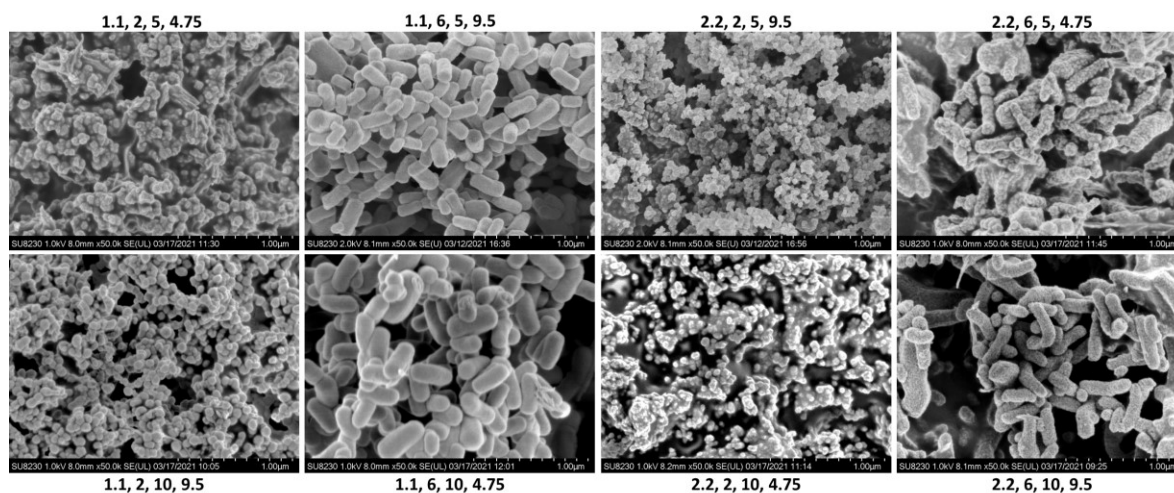


Figure 18: SEM micrographs of different SrMSNs synthesized using 8 different experimental conditions as per a 2^{4-1} DoE. The numbers depicted in the images correspond to the concentration of CTAB, volume of liquid NH_3 , volume of TEOS and amount of $\text{Sr}(\text{NO}_3)_2$ used, respectively.

On the one hand, similar inverse transformation was applied for the total pore volume values, which revealed that as the concentration of liquid ammonia is increased, the total pore volume of the SrMSNs increased. The concentration of strontium salt also had positive correlation with SrMSN pore volume. On the other hand, the positive correlation between pore volume and the amount of CTAB is only obtained when 2ml of liq ammonia is used, irrespective of the level of other variables. Finally, for liquid ammonia, a negative correlation with SrMN pore volume was determined when 2.2g of CTAB was used. TEOS had no effect on the particles total pore volume. The total pore volume can be highly correlated to the total amount of antimicrobial agent that can be loaded for therapeutic effects.

For all other output responses, mathematical transformations were not necessary for proper analysis, and a strong negative correlation was determined between the charge of the SrMSNs. Charge is also a relevant property as it can enable or hinder proper load and/or release of payload of the antimicrobial agent from the mesoporous nanoparticles.

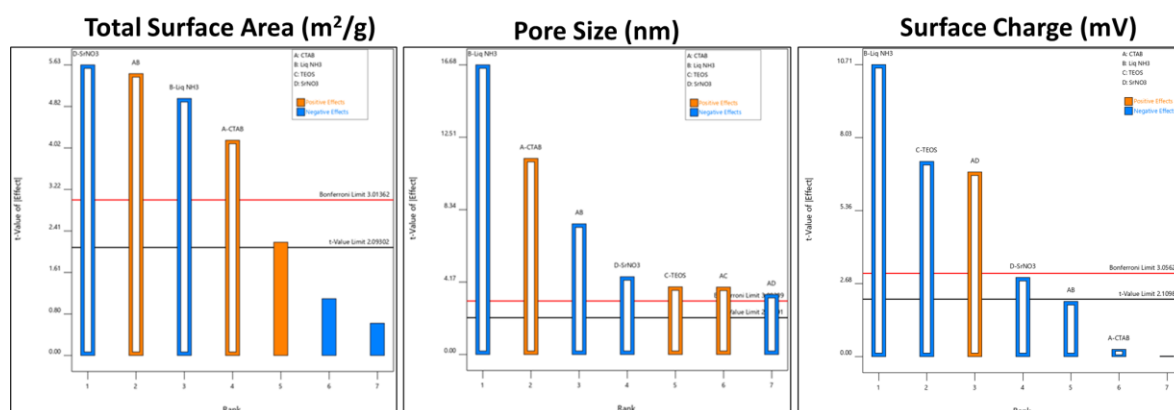


Figure 19: Pareto Chart summarizing the effect of different input variables on measured responses of the resulting SrMSNs. Input variables and interactions amongst each other are ranked from 1-7 where 1 is the most influential variable or interaction and 7 is the least influential variable. The orange bars have positive (or increasing response value when input variable increases) effect and blue bars correspond to the negative (or decreasing response when input variable increases) effect.

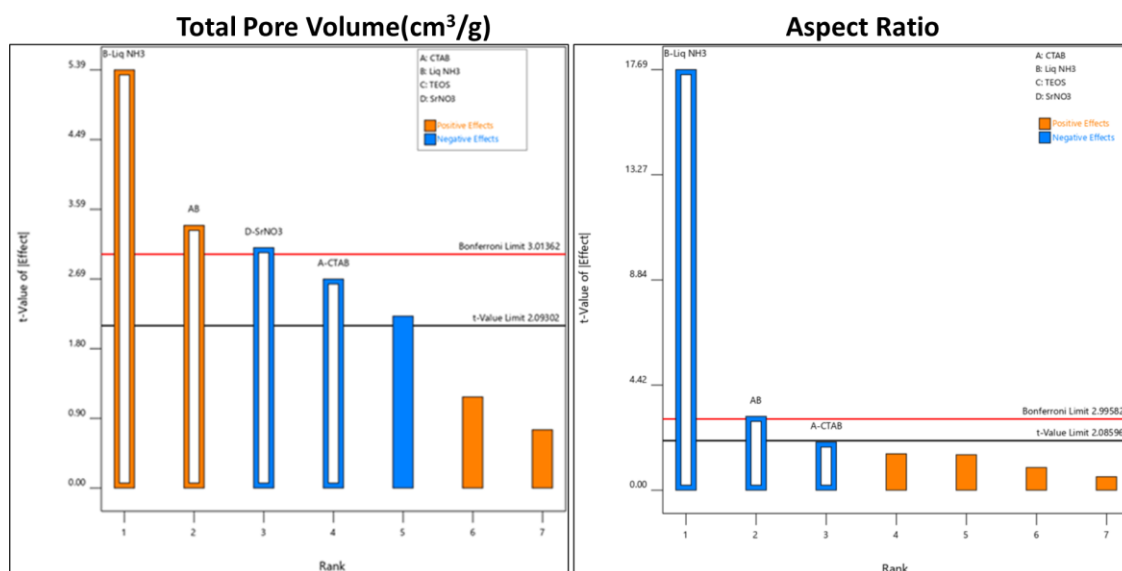


Figure 20: Pareto Chart summarizing the effect of different input variables on measured responses post inverse square root transformation for total pore volume and aspect ratio of the SrMSNs. Input variables and interactions amongst each other are ranked from 1-7 where 1 is the most influential variable or interaction and 7 is the least influential variable. The orange bars have positive (or increasing response value when input variable increases) effect and blue bars correspond to the negative (or decreasing response when input variable increases) effect.

Objective: Antibiotic loading and release

To exploit the high surface area and pore volume of pore size, mesoporous nanostructures have been used for drug delivery application. Under the premise of or project, gentamicin was used as a model antibiotic for assessing the influence of MSN shape, pore size and surface area on antibiotic loading efficiency and time required for 100% release. This was done as we optimize the pore size of MSNs to allow efficient antimicrobial peptide loading.

Methodology: For loading gentamicin, different MSNs were incubated in antibiotic solution (20 mg of MSN in 1 ml of 10 mg/ml of gentamicin in PBS) at 37°C for 24h.²⁸ Post incubation, the samples were centrifuged and the supernatant was collected. MSN pellet was resuspended in 1 ml PBS, centrifuged and supernatant was removed. This was done to remove superficially adsorbed gentamicin.

Gentamicin – loaded MSNs were then resuspended in 1 ml PBS and supernatants were collected at different time points to determine the release profile. For this complete PBS was removed and fresh PBS was added. Gentamicin loading and release was quantified via UV-Vis spectrophotometric assay at 247 nm.

Results:

MSNs synthesized under different conditions showed different loading efficiencies and release profile as shown in **Figure 21**. The statistical analysis, as shown in **Figure 22** confirmed that by carefully selecting the experimental conditions, the loading efficiency and release profile can be pre-determined atleast for the small molecules like gentamicin.

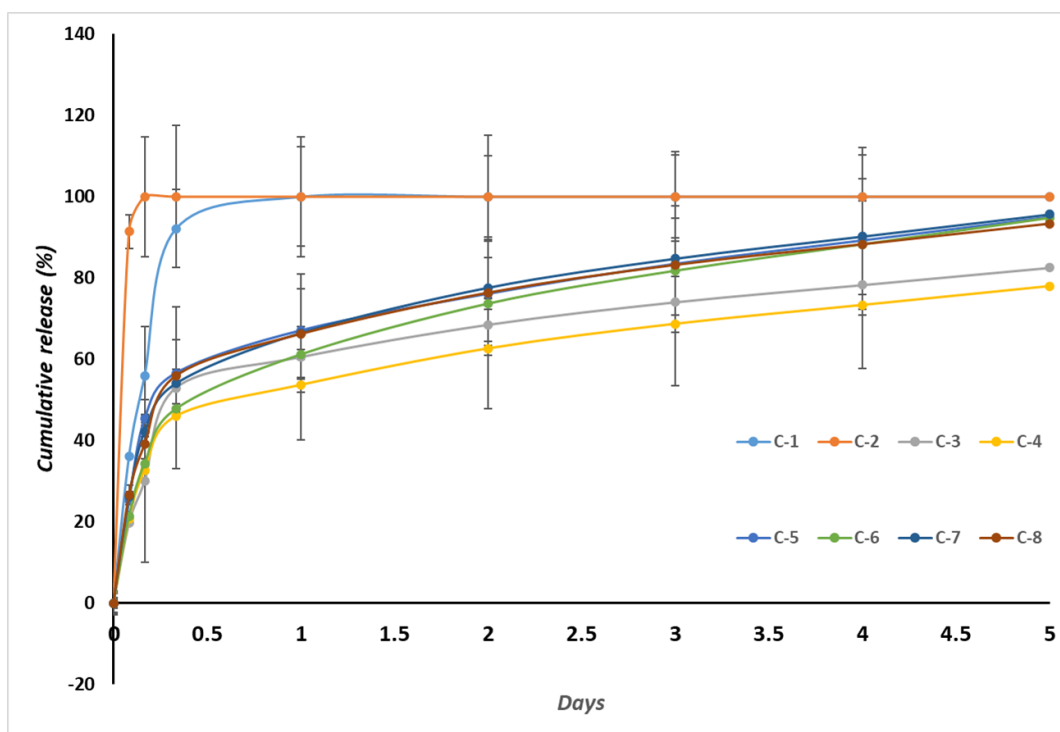


Figure 21: Cumulative release profile of gentamicin from different MSNs over the period of 5 days.

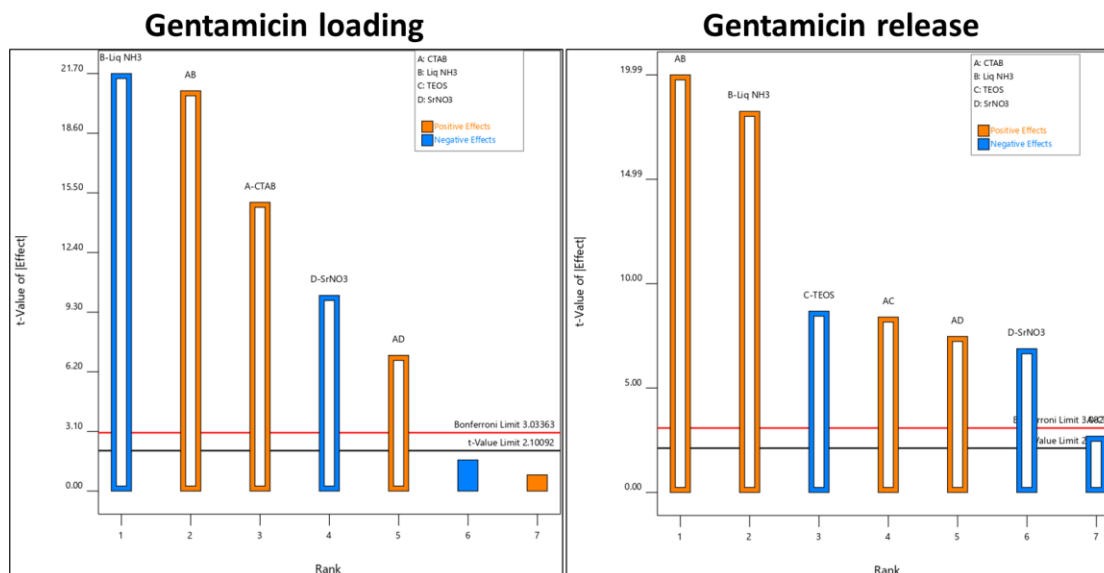


Figure 22: Pareto Chart summarizing the effect of different input variables on measured responses post inverse square root transformation for total pore volume and aspect ratio of the SrMSNs. Input variables and interactions amongst each other are ranked from 1-7 where 1 is the most influential variable or interaction and 7 is the least influential variable. The orange bars have positive (or increasing response value when input variable increases) effect and blue bars correspond to the negative (or decreasing response when input variable increases) effect.

Summary: The physico-chemical characterization of synthesized MSNs confirmed that as amount of reactants, the shape, size and mesoporous nature (total surface area, pore size and pore volume) can be tuned precisely. These properties play an important role in their capability of acting as a cargo carrier but also the release profile of the payload.

Objective: Pore Widening approach to facilitate antimicrobial peptide loading and release over prolonged periods. This was done due to small average pore size of synthesized MSNs under different conditions. This was further validated with vancomycin (antibiotic with molecular size of 3.2×2.2 nm) which has an the molecular size larger than the average pore size achieved under our different synthesis conditions. To overcome these challenges, we are utilizing secondary polymers like ethyl acetate to achieve nanostructures with larger pore size which will allow us to efficiently load GL13K as our selected AMP. The molecular weight of this AMP is 1423.99 g/mole which is comparable with the molecular weight of vancomycin (1449.2 g/mole).

Methodology: The protocol relies on a previously published article utilizing ethyl ether as a co-solvent which is highly volatile with boiling point of 34°C .²⁹ Briefly, 1g of CTAB is dissolved in 150 ml of deionized water containing 2ml of liquid ammonia, 40 ml ethyl ether, 20 ml ethanol along with 0.63 g of strontium nitrate. The reaction mixture was allowed to stir for 30 minutes to allow complete dissolution after which 4 ml of tetraethyl orthosilicate was added and the reaction mixture was vigorously stirred for another 4 h. The white precipitate obtained was centrifuged, dried overnight after which the white precipitate was calcined at 550°C for 5h. The calcined nanostructures were characterized using SEM, TEM and N₂ adsorption-desorption as described in previous section.

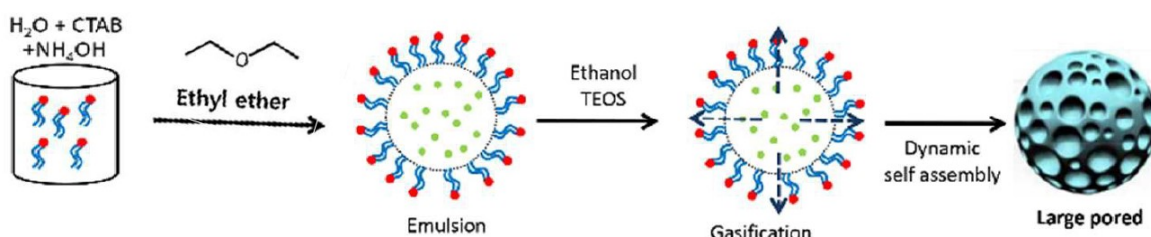


Figure 26: Schematic illustrating synthesis of SrMSN nanostructures utilizing CTAB and ethyl ether for generating mesoporous nanostructures with large pore size.²⁹

Results: The SEM and TEM analysis (Figure 27) confirmed the presence of large pores in calcined SrMSN. The N₂ adsorption-desorption isotherm confirmed formation of nanostructures with significantly improved surface area, average pore size and total pore volume. The surface area of calcined nanostructures was $839.42 \text{ m}^2/\text{g}$, average pore size being 35.98 nm and $1.224 \text{ cm}^3/\text{g}$.

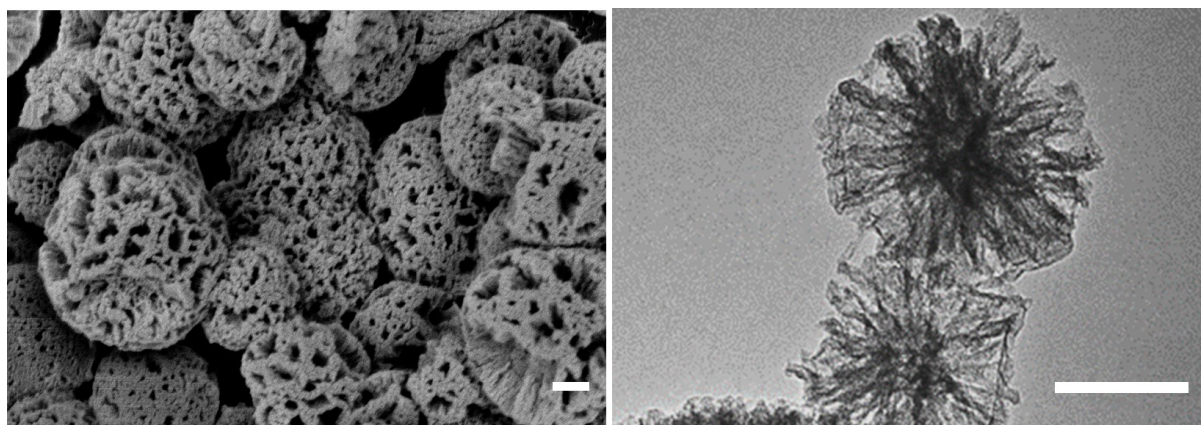


Figure 27: SEM and TEM micrograph of calcined SrMSN nanostructures utilizing CTAB as the primary scaffolding system and ethyl acetate as pore widening agent. Scale bar – 200 nm.

Summary: This is the first time that we have synthesized strontium silicate nanostructures with wide pores making the system feasible for loading antimicrobial peptide and studying its release profile.

The next quarter will focus on optimizing the reaction conditions to control different properties of synthesized MSNs following which fluorescently-labelled AMP will be loaded and release profile determined in addition to use of vancomycin as a model drug for loading and release profile.

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What opportunities for training and professional development has the project provided?

One PhD student, Nick Fischer, and two undergrad students, Jiahe He and Dina Monsour that have contributed to Aim 1 of this project have had regular one-on-one meetings with the PI, Conrado Aparicio, to track progress of work, design experiments, and prepare presentations. Dina Mansour has had also the opportunity to discuss in depth parts of her work related to this project with senior personnel of this project; Dr. Mutreja and Dr. Rudney. This has given the opportunity to all these students to learn specific experimental approaches from the different disciplines of this project.

Over this period, the postdoctoral associates, Dr. Isha Mutreja and Dr. Sunil Boda, had regular one-on-one meetings with the PI, Conrado Aparicio, to track progress of work, design experiments, and prepare presentations. Dr. Mutreja had also the opportunity to discuss in depth parts of his work related to this project with Dr. Rudney and Dr. Bechtold. Dr. Boda had also the opportunity to discuss in depth parts of his work related to this project with Dr. Rudney.

In 2018, the PI, Dr. Aparicio incorporated the use of Individual Development Plans (IDP) for all his trainees pursuing an MS or a PhD degree as well as his postdoctoral fellows. For the latter, Dr. Aparicio gave the option to each postdoc to decide if s/he would like to use the tool to support their professional development. Because of that, Nick Fischer and Dr. Mutreja worked with Dr. Aparicio using IDP. Regular quarter meetings were scheduled and taken, so that the IDP would be guiding the activities and decisions they would take as they progressed and evolved their skills, including soft skills, in Dr. Aparicio's lab.

How were the results disseminated to communities of interest?

Dr. Aparicio gave an invited lecture at the Science and Society Lecture Series at Eckerd College, St. Petersburg, FL on December 2020. This is a seminar series, organized by professionals in different technological and medical disciplines who are retired, that covers technological topics of general interest with a divulgative presentation. The title of the lecture was 'Percutaneous devices at the intersection of standard and innovation'. In this talk, Dr. Aparicio covered his and others' research on preventing infection around percutaneous devices, including those used for amputees (the subject of this project) and dental implants.

What do you plan to do during the next reporting period to accomplish the goals?

Aim 1

Task 1:

- (a) Sign UIC subcontract
- (b) Recruit postdoctoral associate at UIC

Subtask 1.2:

- (a) Evaluate cytocompatibility of AMP coatings against keratinocytes and fibroblasts

Subtask 1.3:

- (a) Select two best AMP coatings on Ti alloy

Subtask 2.1:

- (a) Initiate evaluation of CAP-AMP coatings on Ti alloy

Subtask 2.3:

- (a) Evaluate and compare silanization vs click-chemistry to immobilize peptides on Ti alloy

Task 4:

- (a) Re-submitting manuscript for selection of two best CAP coatings

Aim 2

Task 5:

Subtask 5.1 and 5.2

- (a) DoE: Assessment of viscosity
- (b) DoE: Assessment of enzymatic degradation
- (c) DoE: Functional assessment of HaCaTs and HDFs cultured on different hydrogel scaffolds
- (d) Based on the assessment of cell function, candidate scaffolds will be selected to combine with CAP and assess cell function.

Task 6:

Subtask 6.1

- (a) DoE: Degradation of synthesized SrMSN under physiological conditions and analyzing the degraded products using ICP.
- (b) Optimization of synthesis of SrMSN nanostructures with wide pore and use fluorescently-tagged AMP to assess the loading and release profile.

Task 4:

- (a) Submitting manuscript with characterization and biological evaluation of MNPs

4.IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Nothing to Report

What was the impact on other disciplines?

Nothing to Report

What was the impact on technology transfer?

Nothing to Report

What was the impact on society beyond science and technology?

Nothing to Report

5.CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them

Dr. Aparicio, who has been the PI for this project until June 30, 2021 has changed institutions, from the University of Minnesota to the International University of Catalonia, Spain. He started his new appointment in Spain in June 1, 2021. Previous to that, we submitted to DoD officers a plan for transitioning the PI role to Dr. Mutreja, one of the original key personnel in the project, who stays at the University of Minnesota. The proposed plan also included a subcontract with the new University of Dr. Aparicio, so that he can take over Aim 1 of the project, as co-PI with Dr. Mutreja, who will take over Aim 2. This plan was approved by DoD officers in May 2021 and since then worked has progressed slowly but steadily towards setting up the International University of Catalonia as an Institution able to receive US Federal funds and drafting the subcontract, which is now in progress and close to be completed.

Thus, we are accumulating some delay in the progress of Aim 1 as funds are not yet available to implement the tasks in Spain and recruiting new personnel, a postdoctoral associate, for starting the associated tasks, as approved in the transitioning plan. Dr. Aparicio is expecting to be able to initiate the process of recruitment of the postdoctoral associate in August 2021 and initiate experimental tasks in September 2021. He will prioritize tasks of this project so that the work can be resumed with the slightest delay. In any case, we have finished activities for Aim 1 with some advancement over the initial timeline, as presented in the statement of work, so we are not anticipating that this delay will not compromise the completion of the tasks for this Aim as originally planned.

Changes that had a significant impact on expenditures

Nothing to Report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Not applicable

Significant changes in use or care of vertebrate animals

Nothing to Report

Significant changes in use of biohazards and/or select agents

Nothing to Report

6.PRODUCTS:

•Publications, conference papers, and presentations

Journal publications.

Nothing to Report

Books or other non-periodical, one-time publications.

Nothing to Report

Other publications, conference papers and presentations.

Nothing to report.

•Website(s) or other Internet site(s)

Nothing to Report

•Technologies or techniques

Nothing to Report

•Inventions, patent applications, and/or licenses

Provisional patent application: "Adhesive for osseointegrated percutaneous devices".C. Aparicio, I. Mutreja. Provisional Application #63/213,571. Filed on October 7, 2020.

•Other Products

7.PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Conrado Aparicio, PhD.
Project Role: Principal Investigator (UMN)
Researcher Identifier (e.g. ORCID ID): 0000-0003-2969-6067
Nearest person month worked: 1
Contribution to Project: Responsible for overall project coordination.

Name: Isha Mutreja, PhD.
Project Role: Associate Investigator (UMN)
Researcher Identifier (e.g. ORCID ID): 0000-0002-8998-7563
Nearest person month worked: 5
Contribution to Project: Oversight of research tasks in Aim 2 and performance of tasks in Aim 2.

Name: *Ruoquiong Chen*
Project Role: Researcher(UMN)
Researcher Identifier (e.g. ORCID ID): 7406313818 (Scopus Author Id)
Nearest person month worked: 1
Contribution to Project: Performance of research tasks in Aims 1 and 2.

Name: *Sunil K. Boda*
Project Role: Postdoctoral Associate (UMN)
Researcher Identifier (e.g. ORCID ID): 0000-0003-3565-1914 (ORCID ID)
Nearest person month worked: 1
Contribution to Project: Performance of research tasks in Aim 1.

Name: *Nichola G. Fischer*
Project Role: *PhD student (UMN)*
Researcher Identifier (e.g. ORCID ID): 0000-0003-2230-5158
Nearest person month worked: 1
Contribution to Project: Performance of research tasks in Aim 1

Name: *Jiahe (Dylan) He*
Project Role: *Undergraduate student (UMN)*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 1
Contribution to Project: Performance of research tasks in Aim 1

Name: *Dina Mansour*
Project Role: *Undergraduate student (UMN)*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 1
Contribution to Project: Performance of research tasks in Aim 1

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Our request for changing the PI role from Prof. Conrado Aparicio to Dr. Isha Mutreja, as Prof. Aparicio changed institutions from the University of Minnesota to the International University of Catalonia, Spain, was approved in May 2021 and effective July 1, 2021. The new subcontract for Dr. Aparicio to take over supervision and completion of tasks under Aim 1 in his new University in Spain is being drafted at this time. Once the subcontract is signed, a new post-doctoral associate will be hired to assist Prof. Aparicio in completing tasks associated with Aim 1.

What other organizations were involved as partners?

Nothing to Report

8.SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

9.APPENDICES: