D,L-cyclic peptides (DLCPs) are peptides composed of alternating D and L amino acids that self-assemble into nanotubular stacks and microcrystalline aggregates. The goal of this proposal was to determine the suitability of these self-assembled structures for the mechanical reinforcement of polymeric materials used in the fabrication of implantable medical devices. Our results show that the high aspect ratio microcrystalline aggregates (a.k.a. "microneedles") that come from the assembly process are capable of increasing the average stiffness of biodegradable polymers like poly-D,L-lactic acid (PDLLA), a common polymer used in resorbable load bearing scaffolds.
ABSTRACT

D,L-cyclic peptides (DLCPs) are peptides composed of alternating D and L amino acids that self-assemble into nanotubular stacks and microcrystalline aggregates. The goal of this proposal was to determine the suitability of these self-assembled structures for the mechanical reinforcement of polymeric materials used in the fabrication of implantable medical devices. Our results show that the high aspect ratio microcrystalline aggregates (a.k.a. “microneedles”) that come from the assembly process are capable of increasing the average stiffness of biodegradable polymers like poly-D,L-lactic acid (PDLLA), a common polymer used in resorbable load bearing implants. Preliminary experiments also demonstrated that, for a particular sequence of DLCP (cyclo-[Gln-Leu]4) the assembled microneedles do not exhibit any cytotoxicity toward sheep fibroblasts. Finally, nanomechanical characterization of the microneedles revealed that they were among the stiffest known proteinaceous substances in existence, suggesting their utility as mechanical reinforcers. However, significant challenges remain in rationally controlling the size and surface chemistry features of DLCP-derived microneedles, limiting the ease of their incorporation into biomedical implant devices.

Enter List of papers submitted or published that acknowledge ARO support from the start of the project to the date of this printing. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

Received Paper


TOTAL:  2

Number of Papers published in peer-reviewed journals:

(b) Papers published in non-peer-reviewed journals (N/A for none)

Received Paper

TOTAL:
Number of Papers published in non peer-reviewed journals:

(c) Presentations

ACS National Meeting New Orleans (2013)
MIT Synthetic Biology Group
MRS National Meeting (Boston 2013)

Number of Presentations: 0.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received Paper

TOTAL:

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received Paper

TOTAL:

Number of Peer-Reviewed Conference Proceeding publications (other than abstracts):

(d) Manuscripts

Received Paper

TOTAL:
Books

Received

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Patents Submitted
D, L-cyclic Peptide Nanotubes as Reinforcing Agents

Patents Awarded
D, L-cyclic Peptide Nanotubes as Reinforcing Agents

Awards

Graduate Students

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Names of Post Doctorates

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### Names of Faculty Supported

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<th>NAME</th>
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<td>Neel Joshi</td>
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FTE Equivalent: 0.08

Total Number: 1

### Names of Under Graduate students supported

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<tr>
<td>Brian Borsiquot</td>
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<td>Chemistry</td>
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FTE Equivalent: 0.00

Total Number: 1

### Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period: ...... 0.00

The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields: ...... 1.00

The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields: ...... 1.00

Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale): ...... 1.00

Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering: ...... 0.00

The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense: ...... 0.00

The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields: ...... 1.00

### Names of Personnel receiving masters degrees

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### Names of personnel receiving PHDs

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### Names of other research staff

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Sub Contractors (DD882)
D, L-cyclic peptide nanotube reinforcing agents

Patent Filed in US? (5d-1)  Y
Patent Filed in Foreign Countries? (5d-2)  N
Was the assignment forwarded to the contracting officer? (5e)  N

Foreign Countries of application (5g-2):

5a: Daniel Rubin
5f-1a: Harvard University
5f-c: 29 Oxford St.
    Cambridge  MA  02143

5a: Neel Joshi
5f-1a: Harvard University
5f-c: 29 Oxford St.
    Cambridge  MA  02138
Scientific Progress
This is the final report for an STIR grant awarded in September 2012 and ended in May 2013. The award provided important seed funding for a project that formed the basis for one PhD thesis in my lab and helped to forge two important collaborations. Below is provided a summary of the project during and after the award period. More technical details can be found in the two publications referenced in this report.

2) Statement of the problem to be studied
The purpose of this project was to investigate the use of a unique class of self-assembling crystalline nanostructures as reinforcing agents when incorporated as a minor component into polymeric composites. In particular, we were interested in self-assembling units comprised of cyclic peptides (8-mers) with alternating D- and L-amino acids. This scaffold had previously been shown to form nanotubular structures after solution-phase assembly, mediated by beta-sheet-like hydrogen bonding interactions that promoted stacking of the individual units. The rationale was that the chemical synthesis of these cyclic peptides could potentially yield a huge diversity in nanostructures by drawing upon the availability of Fmoc-protected amino acids with diverse side chains. These diverse structures could then be tailored to specific applications. At the time, a similar class of self-assembling nanostructured materials, based on the diphenylalanine scaffold, were mechanically characterized and declared to be the stiffest known organic material. This, in part, inspired our investigation into the mechanical properties of D-, L-cyclic peptides (DLCPs) and their potential application in reinforcing polymeric materials. The proposal was framed in the context of stabilizing load-bearing resorbable biomedical implants, like spinal fusion cages and bone screws, which are currently made from poly lactic acid and similar synthetic polymers, and sometimes fail prematurely and lead to poor healing outcomes for patients. However, if the surface chemistry of the DLCP nanotubes could be customized, there might be many applications where customizing polymer-filler interactions would be of utmost importance.

3) Summary of most important results
The actual term of the award lasted almost 12 months, during which time we were able to complete a preliminary investigation into the primary proposal goal. First, we synthesized a particular DLCP composed of alternating glutamine and leucine amino acids, QL4. This particular peptide was known to assemble from previous literature reports, but its micro-scale structure was ill-defined. We determined that the peptides formed polydispersed crystalline needle-like aggregates, with dimensions of ~1 micron (length) and ~100 nm (diameter), composed of longitudinally aligned nanotubes. These "microcrystals" could be isolated and co-dissolved with poly(D,L-lactic acid) (PDLLA) in organic solvents and then spun into microfibers using electrospinning. The resulting fibrous meshes contained the peptide microcrystals embedded in a polymer matrix. We tested the fibers by nanoindentation and found that the microcrystals increased the average stiffness of the fibers 5-fold. This work was published in Biomacromolecules.2

During the course of this investigation, we were intrigued by the mechanical properties of the microcrystals, given their ability to serve as filler materials in polymer composites. Therefore, after the award term concluded, we continued to build on our previous work to perform a more detailed study on the mechanical properties of the microcrystals themselves. This led to nanoindentation experiments and three-point bending experiments on the microcrystals, done with specialized instrumentation found in our collaborators’ lab in Singapore. The results of these experiments demonstrated that the DLCP microcrystals were comparable to the most mechanically robust proteinaceous materials known. This work was published in ACS Nano, and was made possible by the initial seed funding from ARO.3

Overall, the DLCP self-assembling system remains intriguing for their mechanical properties. However, we found that their assembly properties were much more adversely influenced by the peptide sequence than we originally hypothesized. For example, the DLCP composed of alternating leucine and glutamic acid forms interesting nano-scale tubular structures, but they were not robust enough to be harvested and incorporated into other fabrication protocols. Other DLCP sequences either did not assemble at all, despite exploring a wide variety of assembly conditions, or formed large aggregates that could not be resuspended in any solvents. Future work may explore conjugation of polymers to the amino acid side chains to increase processability of the nanotubes and further investigations of their biocompatibility.

4) Bibliography

Please refer to our publications for relevant references


**Technology Transfer**

The PI traveled to give invited talks at The ARL lab in Aberdeen, MD and the AFOSR lab in Dayton, OH. Slides from the talks are included as an attachment.
Building functional materials from proteins: Assembly and dynamism

Neel Joshi
6.18.2014
Living systems use proteins to build

SILKS

VERTEBRATE TISSUES
Synthetic Polymers

Limited sequence control

Proteins

Complete sequence control
Self-assembly  Structural Dynamism
peptides ----> proteins ----> networks of proteins
D,L-cyclic peptides (DLCPs)
DLCPs are remarkably stiff

Elastic Modulus = 10.2 ± 0.5 GPa

Hardness = 343 ± 12 MPa

Dan Rubin
DLCPs are remarkably stiff

Elastic Modulus = 10.2 ± 0.5 GPa

Hardness = 343 ± 12 MPa

DLCPs can reinforce polymeric fibers

QL4

Microcrystal

Electrospinning

Composite Fiber

AFM probe with glass colloid tip diameter ~ 40 µm

PDLLA-QL4 composite fiber diameter ~ 0.5 µm

Silicon Substrate

peptides ----> proteins ----> networks of proteins
Mechano-sensitive protein-polymer hybrids

A

Selective sequestration

Mechanically-stimulated release

B

Random crosslinking of linear polymers

Branched network

- More homogeneous structure
- Systematic protein incorporation
- Tunable flexible regions

= 4-arm PEG

= peptide

= target molecule

= unrelated molecule
Mechano-sensitive protein-polymer hybrids

Components for gelation reaction

\[ \text{Components for gelation reaction} \]

\[ = \]

Thiol-ene reaction

\[ \text{irradiation} \]

\[ \text{photoinitiator, aqueous buffer} \]

Trp-zip
Mechano-sensitive protein-polymer hybrids

**A**

- Variable binding domains
- Linker sequence
- Trp-zip

**B**

<table>
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<tr>
<th>Name</th>
<th>Sequence</th>
<th>Forms β-turn</th>
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<th>Binds VEGF</th>
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**C**

- Graph showing changes in wavelength (nm) against degree cm²/dmol.

**D**

- Graph showing changes in wavelength (nm) against degree cm²/dmol.

**E**

- Bar chart showing % folded at different temperatures (deg. C).
Mechano-sensitive protein-polymer hybrids

A

B

C

Eq. Swelling Ratio

Modulus (kPa)

 TZ03  TZ05

Ellipticity (deg*cm²/dmol)

Wavelength (nm)
Biosensing Platforms Based on Protein Conformational Dynamics

peptides  ---->  proteins  ---->  networks of proteins
Engineered protein manufacturing

Compared to biological materials:
• Difficult to obtain pure and in large quantities
• Time consuming, less cost effective
• Not appropriate for large-scale materials
Material design parameters

1) Must be able to control sequence using conventional genetic engineering

2) Must be able to produce material on large scales by harnessing biosynthetic potential of a living organism

3) No protein purification
Biofilms

Self-standing, macroscopic, biosynthetic materials

Epstein, et al. PNAS 2010

http://www.biofilm.montana.edu/node/2390

weitzlab.seas.harvard.edu

Biofilm Nano-architecture

**Domesticating the Microbe**

**Bacteria are dangerous!!!**
*(Germ Theory of Disease)*
Pastor and Koch, early 19th century

**We understand how they work...**
*(Antibiotics, Microbiology, Molecular Biology)*
Fleming, Watson, Crick, Lederberg, Brenner...et al.

**We can exploit them.**
*(Recombinant DNA Technology)*
Cohen, Boyer, and Lobban

**Biofilms are bad!!!**

**We are starting to understand how they work...**

**Can we exploit them?**

---

Industrial Biofilm Usage

Wastewater treatment

Chemical processing

Microbial Fuel Cells

Microbial circuits

Bacterial ECM Proteins: Functional Amyloids

- Mediate adhesion to surfaces

- Can be up to 60% of biofilm biomass

http://labs.mcdb.lsa.umich.edu/labs/chapman/
E. Coli: Curli Biosynthesis
The BIND Concept

*Biofilm-Integrated Nanofiber Display*

- Nanofibers have diameters from 4-7nm and are tens of microns in length.
- Nanofiber network is robust
- Amyloids: strength comparable to steel and stiffnesses comparable to silk
CsgA Protein Structure

- Assembled from the secreted CsgB protein (17.5kDa) which is membrane-anchored.

- Easily detected using Congo Red, which stains amyloid fibers.

- Assembly kinetics can be monitored *in vitro* by Thioflavin T.

*Annu. Rev. Microbiol. 2006, 60:131-47*

CsgA Insertion Library

Periplasmic localization sequence

CsgG-mediated secretion sequence

Sec N22 R1 R2 R3 R4 R5

Sec N22 MBD R1 R2 R3 R4 R5

Sec N22 R1 R2 R3 R4 R5 MBD

Linker Region = no linker / GS / GSGGSG

MBD Metal Binding Domain = KCTSDQDEQFIPKGCSK

Peter Nguyen
CsgA Insertion Library

Congo Red assay
Red = amyloid formation

Periplasmic localization sequence
CsgG-mediated secretion sequence

Sec N22 R1 R2 R3 R4 R5
Sec N22 MBD R1 R2 R3 R4 R5
Sec N22 MBD R1 R2 R3 R4 R5

Linker Region = no linker / GS / GSGGSG

MBD Metal Binding Domain = KCTSDQDEQFIPKGCSTK

Peter Nguyen
CsgA Insertion Library

- no CsgA (−)
- wt-CsgA (+CsgA)
- CsgA-MBD (C3)
# CsgA-peptide Insertion Library

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<th>Length (aa)</th>
<th>Type</th>
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<td>GBP</td>
<td>EPLQLKM</td>
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<td>Substrate Binding</td>
<td>Graphene edge binding</td>
<td>JACS 2011, 133: 14480.</td>
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<td>Quartz/Glass binding</td>
<td>Bioinformatics 2007, 23: 2816.</td>
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<td>Protein Display</td>
<td>General covalent capture/display of proteins</td>
<td>PNAS 2012, 109(12): E690.</td>
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<td>Prot. Sci. 1999, 8: 921.</td>
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<td>JACS 2010, 132: 4731.</td>
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<td>NP templating</td>
<td>Magnetite NP templating</td>
<td>JBC 2003, 278(10): 8745.</td>
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Quantifying CsgA-peptide production

Most peptides <50 amino acids do not hinder protein secretion or assembly

Peter Nguyen and Zsofia Botyanszki
Most CsgA-peptide mutants form amyloids

Scale bar = 1 µm

Peter Nguyen
CsgA-MBD enhances adhesion to steel

Growth of cells in suspension culture → Induction of CsgA-MBD production → Cultures spotted on coupon → Vortexing wash → Imaging by SEM

|------|--------------------|----|-------------------|-------------------------------------|--------------------------------------|

Peter Nguyen
CsgA-A3 templates AgNP growth

Grow of cells in suspension culture --> Induce CsgA-A3 production --> Form biofilm on TEM grid --> Incubate with AgNO₃ --> Stain and image

wt-CsgA

CsgA-A3

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<td>70</td>
<td>DQGSCALMGW</td>
<td>12</td>
<td>NP templating</td>
<td>Gold surface binding</td>
<td>Water Chem. 2003, 12: 2414</td>
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CsgA-SpyT enable protein immobilization

1. Grow cells on glass substrate
2. Induce CsgA-SpyT during growth
3. Incubate with GFP-SpyC
4. Wash unbound protein
5. Stain and image
BIND as a catalytic substrate

- Hydrolyzes alpha glycosidic bonds of sugars.
- Used for:
  - Ethanol production
  - HFCS production
  - Laundry detergents
- Accounts for \( \sim 30\% \) of world-wide industrial enzyme production
BIND as a catalytic substrate

Grow cells in suspension culture

Induce CsgA-SpyT in culture

Filter through 0.2 μm filter

Incubate with Amylase-SC

Monitor catalytic activity

Zsofia Botyanszki
BIND as a catalytic substrate

[Graph showing the relationship between [BIND] and [AmylaseSC] with data points for CsgA WT and CsgA-ST.]
BIND as a catalytic substrate
Co-assembly of Two CsgA Variants

Combine BIND functions:
- Surface adhesion
- Catalysis
- Binding to soluble species

Curli co-polymer
Bifunctional Curli Networks

Two CsgA::peptide variants can be displayed simultaneously to create multifunctional materials

Function 1: SpyTag
(Venus::SC-immobilization)

Function 2: FLAG tag
(anti-FLAG 1°, 633nm Dylight 2°)

GFP detection

DyLight 633 detection

DIC

Peter Nguyen
Amyloid network survives decellularization

Amyloid material remains intact after harsh treatments (solvents, pH)
Curli operon optimization

- Amyloid structure
- Amyloid nucleation
- Export
- Chaperone
- Transcriptional regulation
Curli operon optimization

Library of mutants with varying production levels for each music

Screen library for amyloid formation

Identify mutants with optimal curli production

Peter Nguyen
BIND: what is it good for?

Combining the versatile functions of recombinant proteins with scalable materials fabrication

CAPABILITIES

• Specific surface adhesion

• Nanoparticle templating

• Enzymatic catalysis

• Specific binding and capture of soluble entities (metals, small molecules, proteins, viruses, cells)

• Large scale material production (biofilm paint, spray-on coatings, self-standing 3D materials)

• Environmental responsiveness (sensing, programmed formation/breakdown, dynamic properties)

• Programmed biological interactions (antimicrobial coatings, live biotherapeutic)
BIND for biocatalysis
Solution-phase catalyst

- Activity affected by immobilization
- Limited catalyst stability

Existing biocatalysis strategies

- Limited substrate diversity
- Contaminants complicate product purification
- Activity affected by immobilization
- Cost of substrate and processing

Whole-cell catalyst

Surface-immobilized catalyst
Solution-phase catalyst

Surface-immobilized catalyst

Whole-cell catalyst

BIND for biocatalysis

- Modular immobilization strategy
- No enzyme purification or processing
- Enhanced enzyme stability
- Diverse substrate tolerance
- Compatible w/ continuous flow processes
Enzymatic BIND for water decontamination

- EPA Maximum Contaminant Level (MCL) = 3ppb
- Frequently found to be above 5ppb in the mid-east and mid-west
- 0.1ppb = endocrine disruption
Enzymatic BIND for water decontamination

Enzymatic water decontamination is inefficient and expensive:

• Naturally occurring strains do not eliminate atrazine to acceptable levels

• Cost of enzyme purification is too high

• Limited substrate diffusion across cell membrane inhibits breakdown with whole cells

➔ Can BIND facilitate an efficient continuous flow atrazine decontamination system by displaying enzymes?

Zsofia Botyanszki
BIND for specific metal removal/recovery
The need for rare earth metals
SEPARATING RARE EARTHS at MOUNTAIN PASS

A
Dissolve RE minerals

Contains 15 rare earth elements

Bastnasite
$\text{REFCO}_3$

→

Acid solution with dissolved rare earth ions

Heavies

Separate RE elements

Using immiscible solvents for counter-current solvent extraction can separate the combination of REE into heavies and lights (simplified schematic).
Rare earth isolation and separation

Lanthanide binding peptides
Other possible BIND applications

**BIOFUEL PRODUCTION**

**WATER PURIFICATION**

**CELL CAPTURE/AFFINITY SEPARATIONS**

**PROTECTIVE COATINGS**

**BIO-LECTRODE INTERFACE**

**BIOSENSING DECONTAMINATION**
Benefits of Biofilm Technology

• Self-generated and self-renewing scaffold; the bacterium as a nanomaterial factory.

• Vast surface area for immobilization.

• Robust – stable under conditions normally considered harsh for biology

- Easily scalable – could lead to cost effective large scale solutions.

- Living material – may allow for dynamic temporal control over material properties

- A green technology for nanomaterials.
Acknowledgements

Joshi Group
Glenna Meister
Peter Nguyen
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Pichet Praveschotininunt
Anna Duraj-Thatte

Collaborators
Christine Ortiz (MIT)
Michael Gevelber (BU)
Ali Miserez (NTU, Singapore)

Harvard Milton Fund