Combining protein and peptide biomaterials with laser technologies towards tissue engineering scaffolds

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Can we exploit the self-assembly of natural biological materials towards the design of novel biomaterials from the nano- to the macro-scale?
Protein and peptide fragments can self-assemble into "amyloid-type" fibrils

TEM Analysis (bar: 100 nm)

Cross-beta model for amyloid fibrils

X-ray fiber diffraction pattern for amyloid fibrils

Fibril axis

~10 Å

4.8 Å
Amyloid fibrous assemblies

- Amyloid assemblies can also be formed by proteins and peptides that are not associated with disease.

They are also found in natural fibers / materials (insect chorions, curli fibers, egg stalks, etc).

**Chrysopa (Green lacewing fly)**

*egg stalks (Geddes et al. 1968)*
Fibrous nanostructured objects:

- promising for potential integration in future generations of nano-micro devices

- carbon nanotubes most investigated

  - however, limitations:
    (high production costs, extreme manufacturing conditions)

Advantages of biological counterparts (DNA, peptides, proteins):

- Spontaneous self-assembly in mild conditions

- ease of functionalization
  (possibility of modifications at the sequence level)
Self-Assembling peptides as templates for technological applications

A fragment from the yeast *Saccharomyces cerevisiae* Sup35 protein that forms amyloid fibrils was used for nanowire fabrication.

Susan Lindquist group

*Scheibel et al., PNAS 2003, 100: 4527*
Natural assemblies as a source of inspiration for technological applications

Natural assemblies can inspire the specific design of biological building blocks in order to fabricate supramolecular structures with advanced functionalities.
Adenovirus fiber as a self-assembly system

Properties of adenovirus fiber:

1. Long and thin shaft (30 nm) gives the virus ‘reach’
2. Trimeric, beta-structured
3. Extremely stable proteins: resistant to SDS, temperature, denaturants
4. Fibrous shaft built from sequence repeats (blue squares)

Model for the full-length fiber based on the crystal structure of a stable fragment with 4 repeats

Each fiber monomer contains 582aa

1. The fibrous shaft is built from a repeating sequence motif with a hydrophobic amino acids (ORANGE and GREEN residues) alternating with hydrophilic ones and glycine or proline at conserved positions (PURPLE residues)

2. The basic repeat fold contains a beta-strand almost parallel to the axis of the fiber followed by a beta turn and another beta strand which runs at an angle of $45^\circ$ relative to the fiber axis. The repeats are joined by a solvent exposed loop

Peptide sequences as a self-assembly system

Identification of minimal peptides corresponding to natural building blocks (blue squares, octapeptide NSGAITIG) that can self-assemble into amyloid-type fibrils

Can we rationally modify these fibrous building blocks towards specific functions?

eg. templating of inorganic materials

targeting sequences for metallization, Calcium binding, cell attachment etc
• The N-S-G-A-I-T-I-G peptide self-assembles into amyloid fibrils with cross-beta structure

• The beta strands are perpendicular to the fibril axis

• The peptide arrangement can be parallel or anti-parallel

• Molecular Dynamics simulations suggest that the N-S residues stay exposed out of the amyloid fibril core

Rational modification of these building blocks towards specific functions

e.g. templating of inorganic materials (metal nanoparticles, silica, Qdots, calcium) cell attachment etc

1) Kasotakis et al., 2009, Biopolymers 92:164-72
2) Kasotakis, Mitraki, 2012, Biopolymers, 98:501
Can we control the positioning of fibers accurately, in 2- or 3-dimensions at the micro / nano scale???

Directed positioning is a major challenge

For integration of self-assembling fibrous materials in devices
Development of inter-disciplinary approaches
towards controlled positioning on surfaces
and integration in devices

Example: peptide fibrils positioned on a hybrid
organic-inorganic material
using femtosecond Laser Technologies

V. Dinca et al., NanoLetters 2008, 8:538

(collaboration with Dr. M. Farsari et al., IESL, FORTH)
Hybrid Photostructurable Material

- Highly attractive and promising 3D microfabrication technology
- Real 3D Writing (no mask, mold or stamp)
- Complex Micro- & Nano- structures

Sakellari et. al, 2012, ACSNano, 3: 2302
Positioning of metal-binding peptides on 3D structures fabricated using a composite sol-gel with metal binding sites

**Metallization – Peptide deposition process:**

- 3D structure fabrication using 2PP
- HAuCl4 seeding + reduction (sodium citrate or NaBH4)
- formation of bridges

Hybrid organic-inorganic materials designed by Prof. Maria Vamvakaki
Positioning of Cysteine-containing peptides on 3D nanostructures fabricated using a composite sol-gel with metal binding sites

Cysteine containing peptide

control peptide
Can we develop « scaffold-on-scaffold » approaches?

*ie.*

Combine the advantages of top-down fabrication (eg. control of scaffold geometry and micro-nanotopography) With

Bottom-up design possibilities of Self-assembling peptides?
Design of bifunctional aspartate-containing self-assembled peptides

Peptide DDSGAITIG forms fibrils that:

a) Bind to metal nanoparticles
b) Nucleate calcium phosphates on their surface (important for bone and teeth regeneration)

L-Aspartic acid
(Asp / D)

a) DDSGAITIG peptide fibrils after incubation with gold tetrachloroaurate solution and reduction with 1% citric acid
b) DDSGAITIG peptide fibrils after incubation with CaCl₂ and Na₂HPO₄ on a holey grid
Development of a “Scaffold-on-Scaffold” strategy

- 3D structure fabrication using MPP

**STEP 1**

- HAuCl$_4$ seeding

**STEP 2**

- Peptide fibril deposition

controlled evaporation & washing

- Bridge formation

**STEP 3**

- CaCl$_2$ 1 M (pH 7.4)

- Na$_2$HPO$_4$ 0.6 M (pH 9)

Terzaki et al., Biofabrication 2013, 5:045002
Peptide bridges positioned on the hybrid scaffold, covered with calcium phosphates
And EDX pattern on the bridge
SEM images of pre-osteoblastic cells cultured onto DDSDGAITIG peptide-functionalized and Calcium mineralized 3D woodpile-shaped scaffolds

Terzaki et al., Biofabrication 2013, 5:045002 (with M. Chatzinikolaidou)
Designer peptides containing cell attachment (RGD) motifs as scaffolds for tissue engineering: theoretical and experimental studies

**RGD-SGAILTIG-C self-assembling peptides**

Loo et al., Adv. Healthcare Mat., 2015, 16: 1557


Can we produce self-assembling fibrous materials in the macro-scale, using microbial cell factories (e.g. recombinant production in bacteria)?

Concatamers, based on the minimal self-assembling building blocks production in quantities will allow to use electrospinning and other methods used mainly for polymers.

“RECOMBINAMER”

Fig. 5 Spider silk fiber spun from DP-1B protein produced in E. coli. Fiber was spun from HFIP solution by J. P. O’Brien (DuPont) essentially according to Lock (1992).
Next important challenge: recombinant production of self-assembling fibrous materials

1) Proteins composed of various lengths of the natural shaft

2) Concatamers based on the minimal self-assembling building blocks
Expression of the full-length shaft in *E.coli* (residues 61-392)

Lane *M*: molecular mass marker, Lane *C-*: non induced bacteria, Lane *C<sub>1h</sub>*: bacteria after 1h of induction, Lane *C<sub>2h</sub>*: bacteria after 2h of induction, Lane *C<sub>3h</sub>*: bacteria after 3h of induction, Lane *C<sub>4h</sub>*: bacteria after 4h of induction, Lane *S*: Supernatant of lysates, Lane *P*: pellet of lysates.
Protein is produced in form of “Inclusion bodies” consisting of amyloid-type fibrils

Processing of fibers from the purified proteins that self-assemble into amyloid-type fibrils

Fiber rod drawn from purified protein

Protein fiber drawn between two glass rods, viewed under crossed polars

Ariadni Prigipaki, PhD work
Can we photostructure protein and peptide-only materials and use them as scaffolds?

Peptide and protein Building blocks → self-assembled matrices → 3D photofabrication → cell attachment on scaffolds

(collaboration with Dr. A. Selimis and Dr. A. Ranella, IESL, FORTH)
Cuvette Preparation

Film Preparation

Protein → O.N. → KrF Excimer Laser → Cross-linked protein
Irradiated area 300 mJ/cm²

protein (2.5 mg/ml)
Protein Scaffolds with Fibroblasts

SEM image of the irradiated area of the protein film with one pulse (30ns) of the KrF excimer laser (248nm) at a fluence of 300 mJ/cm² (2.5mg/ml of protein - 40μl deposited, o/n drying at RT), covered with fibroblast cells (NIH/3T3 cells, 10⁵ cells/ml, 72h of culture).
Protein Scaffolds with Fibroblasts
Cells attach selectively to the irradiated areas

Protein Scaffolds with Fibroblasts - UV Microscope DAPI DAY 5

irradiated area

Non - irradiated area (red arrow)
Protein Scaffolds with Fibroblasts
Selective Cell Patterning

Fluorescence microscopy images of fibroblasts on laser patterned surfaces after 3 days of culture-DAPI staining.

*Prigipaki et al., Biofabrication, 2017: 9, 045004*
Summary:

Rational design of bio-nanomaterials from the nano- to the macroscopic scale using building blocks inspired from Natural folds and hard-won biochemical knowledge

and

Development of inter-disciplinary approaches

Towards combining these bottom-up design possibilities With top-down biofabrication technologies
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