



SCUOLA
NORMALE
SUPERIORE

RELAZIONE ATTIVITA' ANNUALE DEI PERFEZIONANDI/DOTTORANDI – SECONDO ANNO
REPORT ON THE PHD ACTIVITY – SECOND YEAR

NOME E COGNOME NAME AND SURNAME	Elisa Martino
DISCIPLINA PHD COURSE	Nanoscienze

CORSI FREQUENTATI CON SOSTENIMENTO DI ESAME FINALE ATTENDED COURSES (WITH FINAL EXAM)	VOTAZIONE RIPORTATA MARK	NUMERO DI ORE HOURS
Ciclo di seminari – Biophysical Sciences	Da sostenere entro 31 ott	45
CORSI FREQUENTATI SENZA SOSTENIMENTO DI ESAME FINALE ATTENDED COURSES (ATTENDANCE ONLY)		NUMERO DI ORE HOURS
Topics in Structural Biology		20

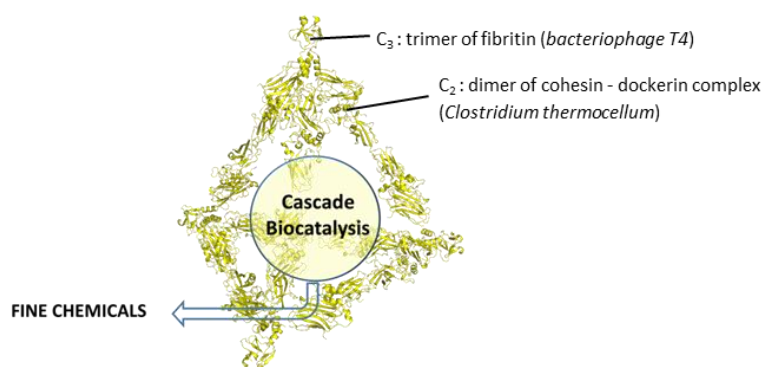
ALTRE ATTIVITÀ FORMATIVE (SEMINARI, WORKSHOP, SCUOLE ESTIVE, ECC.) – DESCRIZIONE OTHER PHD ORIENTED ACTIVITIES (SEMINARS, WORKSHOPS, SUMMER SCHOOLS, ETC) – DESCRIPTION	NUMERO DI ORE HOURS
15/11/2018 – Seminario (NANO Colloquia 2018) <i>Lucia Angella</i> “Autophagy in Krabbe disease: a double-edged sword?”	2
03/12/2018 – Seminario (Seminari Biologia SNS) <i>Mariagrazia Pizza</i> “New strategies in the design of vaccines”	2
31/01/2019 – Workshop (ERC Starting Grant SLaMM Workshop) “Advanced theranostic nanomedicine in oncology”	10
02/04/2019 – Seminario (Accademia dei Lincei e Normale per la scuola) <i>Elena Cattaneo</i> “Cellule staminali neurali”	2
08/05/2019 – Seminario (Colloquio della Classe di Scienze) <i>Arthur Mallay Lesk</i> “The architecture of proteins”	2
09/05/2019 – Seminario (Seminari di Neuroscienze) <i>Arthur Mallay Lesk</i> “Evolution of protein structure”	2
10/05/2019 – Seminario (Seminari di Neuroscienze) <i>Arthur Mallay Lesk</i> “Evolution of protein function”	2
27-31/05/2019 – Advanced Course (Rome) “Trends in Enzymology and Biocatalysis”	26



ATTIVITÀ DI RICERCA SVOLTA (MAX. 8.000 CARATTERI)*

RESEARCH ACTIVITY (MAX. 8000 CHARACTERS)

The goal of this PhD project is the **development of a novel protein scaffold for application in the field of biocatalytic cascades**. We have engineered and designed for this two specific building bio-blocks, which should spontaneously self-assemble into a tetrahedral architecture. Also, they show anchoring points for the further functionalization with desired enzymes and their compartmentalization.



We were able to successfully synthesize and characterize all building blocks we designed for the assembly of our novel protein cage. The production of the vertex-BBs was very straightforward, while the crosslinking reaction to obtain the edge-BBs needed careful optimization. Protein cage assembly tests showed some equilibrium forms, thus indicating the lack of a predominating nanostructure. Characterization of the observed forms is underway. In the next year, we expect to optimize the production of the protein cage and to fully characterize it. Furthermore, we would attempt to functionalize the cage with enzymes to assess the catalytic properties of our nanobioreactor.

See the complete report on the attached file.

*se si intende sottoporre una relazione di ricerca più estesa, utilizzare il campo per una descrizione sintetica e allegare il documento in formato .pdf

If you are going to submit a longer report, please fill the box with a synthetic abstract and attach a document in pdf format

**EVENTUALI PUBBLICAZIONI
PUBLICATIONS (IF AVAILABLE)**



SCUOLA
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NOME DEL RELATORE

THESIS ADVISOR

Dr. Gianpiero Garau (CNI@NEST)

Dr. Stefano Luin (SNS)

DATA

06/10/2019

FIRMA

Stefano Luin

DATE

SIGNATURE

Research Activity Report

Second Year Final Exam Nanosciences - Elisa Martino

1. Introduction

Biocatalytic cascades represent a recent attractive tool for the development of innovative syntheses of fine chemicals, respecting the principles of green chemistry. They combine a series of enzymatic reactions in a single compartment for circumventing the isolation of reaction intermediates, preventing their unproductive spreading, and maximising the efficiency of the overall process.¹ In these processes, the desired enzymatic properties can be optimised by mechanism-guided directed evolution,² while the immobilization of different biocatalysts and their compartmentalisation remain often a challenge for process implementation. The most frequently used strategies are physical adsorption of the enzymes, their covalent attachment or cross-linking, but the co-immobilization of multiple enzymes can be difficult to achieve and requires significant optimization.³ **Protein-based nanoscaffolds** can overcome these issues. By proper monomer design, they can spontaneously self-assemble into ordered 3D structures to obtain many different useful technological nanoarchitectures.⁴ By protein fusion, it might be possible to functionalize the nanoscaffolds with desired enzymes, to generate innovative “nano-bioreactors”, directly from the biocatalyst expression, and with a fine control over the spatial arrangement of the enzymes inside the nanoarchitecture.⁵

The goal of this PhD project is the **development of a novel protein scaffold for application in the field of biocatalytic cascades**. We have engineered and designed for this two specific building bio-blocks, which should spontaneously self-assemble into a tetrahedral architecture. Also, they show anchoring points for the further functionalization with desired enzymes and their compartmentalization.

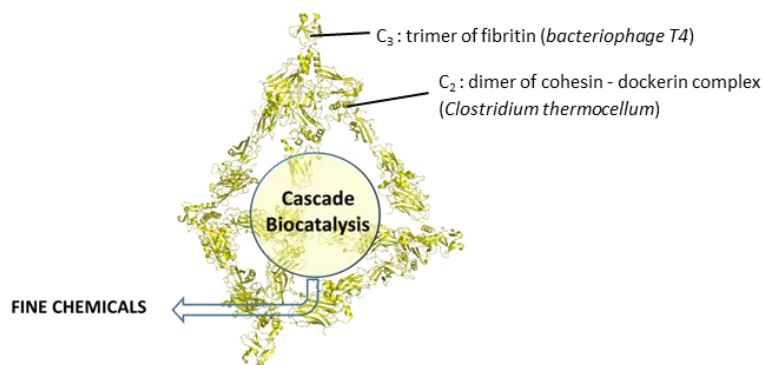


Figure 1 Nano-bioreactor for the production of fine chemicals.

2. Results and Discussion

In my second year of PhD, I mainly focused on the development of a novel protein cage to be used as scaffolding system for biocatalysts. We have used a symmetric fusion strategy for its design: two distinct oligomeric domains linked together to form the edge of the tetrahedral structure (*heterodimeric complex substructure*). The final 3D assembly is driven by interface-recognition at the level of the (six) vertices (*trimerization domain substructure*) of the triangular pyramid. Overall, we used three unique protein domains (fibrinin, cohesion and dockerin), whose association in C₂+C₃ symmetry axes provides a final tetrahedral nano-bioarchitecture (Figure 1).

After the successful production and purification of the units at high scale (several mgs), we started testing their ability to spontaneously self-assemble into higher order nanostructures. Optimization of the assembly-process and characterization of the final system are currently underway.

2.1 Synthesis of the nano-building blocks

At first, we started developing the two nano-building blocks of the final biocage:

- Vertex-building blocks (vertex-BB): cohesin-fibrinins (CF1, CF2 and CF3). This block was design by fusion proteins obtained from the trimeric fibrin domain and cohesin. This subunit goes to constitute the apex of the final pyramid (Figure 1), with the spontaneous formation of a vertex trimer.



- Edge-building blocks (edge-BBs): bis-dockerins (biD59 and biD7). This block was design by crosslinking between two engineered dockerin units obtained by cysteine single-point (in position 59 and 7). The resulting symmetrical dimer provides the edge of the final triangular pyramid. Each bis-dockerin is able to join two vertices of the pyramid through the recognition of the cohesin partner (Figure 1).



2.1.1 Vertex-BBs: Cohesin-Fibrinins

We designed and produced three different cohesin-fibrin complexes, with different α -helical linker lengths: 12 residues for CF1, 9 residues for CF2, and 2 residues for CF3. The different expression constructs were generated by PCR and cloned into the pET28c vector (NdeI and BamHI restriction sites). Protein over-expression (E. Coli BL21 strain) provided very high good yields (30-50 mg/L of culture) for all constructs (Figure 2).

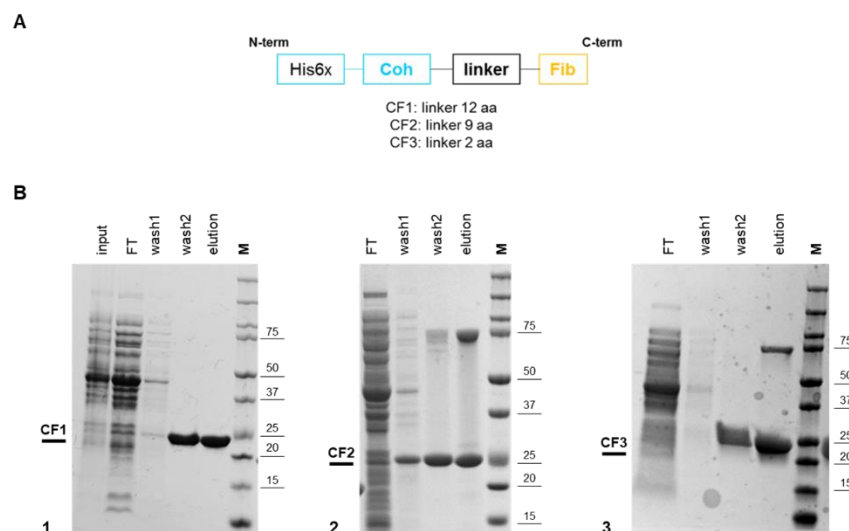


Figure 2 A Construct scheme of CFs; B SDS PAGE of 1 CF1, 2 CF2, 3 CF3.

After purification, we observed the formation of the trimer from the size-exclusion chromatography in solution (Figure 3). To validate this important result, we compared the retention volume observed for the

three constructs with a monomeric reference (Figure 3). We were also surprised to observe that the trimer is visible also by SDS-PAGE. This indicates a very high stability of the trimer, since the SDS-PAGE is conducted on samples pre-boiled at 95°C for 5-10 min. Also, it seems that the shorter the linker, the higher the thermal stability of the trimeric form: CF3 shows the higher percentage of trimer, while CF1 is present only exclusively as monomer.

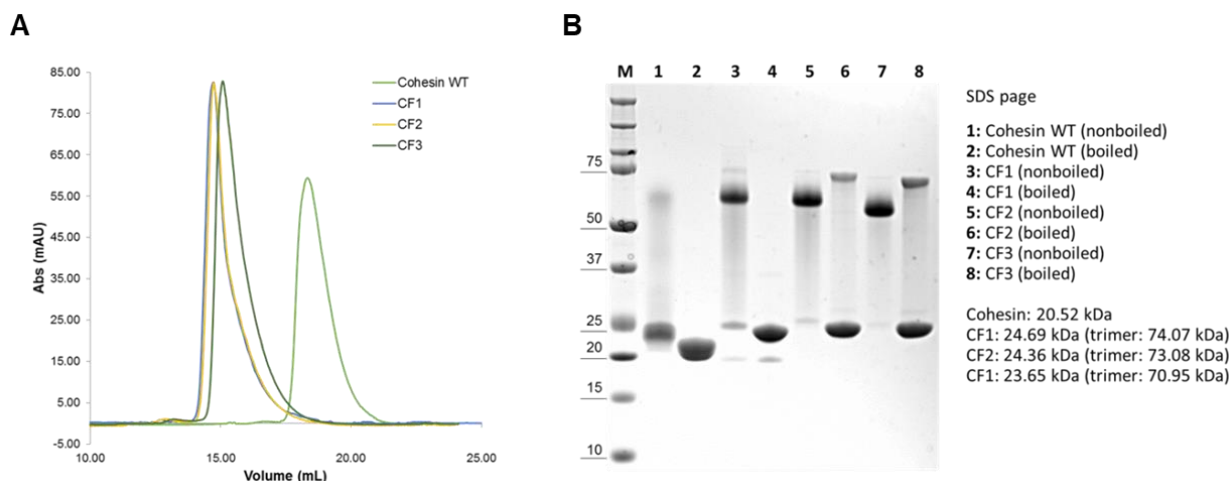


Figure 3 **A** Size exclusion chromatography and **B** SDS PAGE on CF1, CF2, CF3 and monomeric reference (Cohesin WT).

2.1.2 Edge-BBs: bis-Dockerin

To generate a rigid chemical linker and properly orient the two ends of the dockerin units, we modified the protein at the N-terminus by single-point mutagenesis (positions 59 and 7), thus preserving the recognition site of the cohesin partner (C-terminus). The resulting cysteine in position 59 is on a rigid protein loop, which guarantees an ideal orientation of the two linked dimer subunits. On the contrary, the cysteine in position 7 belongs to an unstructured protein region, which provides more flexibility and accessibility the two linked dimer subunits. Exploiting the specificity of the bond between maleimides and thiols,⁸ we used a bis-maleimide moiety (pPDM) to lock the junction between two dockerin units (Figure 4).

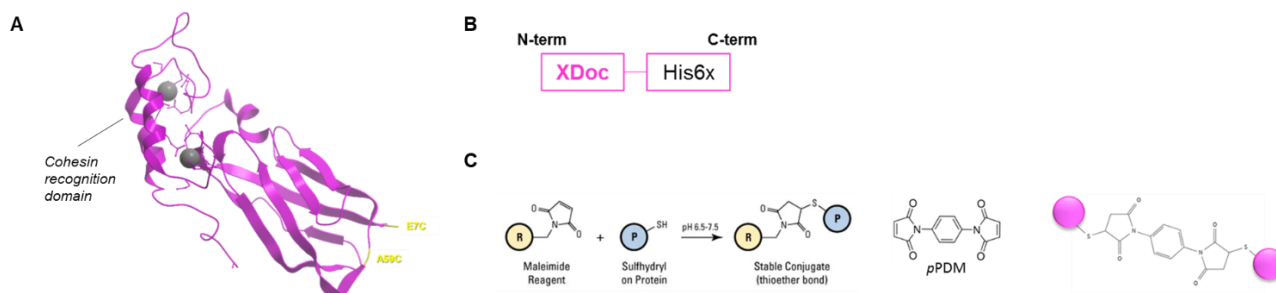


Figure 4 **A** Structure of XDoc, mutagenesis sites at positions 7 and 59 and Cohesin recognition domain at C-terminus; **B** construct of XDoc; **C** maleimide-thiol reaction.

The recombinant expression of the dockerin in soluble form was unexpectedly quite low (2.5 mg/L of culture). Despite several attempts of process optimization, we were not able to improve considerably the efficiency of protein recovery. In addition, the planned reaction of chemical crosslinking turned out to be efficient only partially (Figure 5). Among the reducing agents tested, TCEP-HCl provided the best performance and allowed higher conversions to the final desired products. Interesting, we observed that the cysteine at the position 59 showed higher reactivity compared to the 7 position).

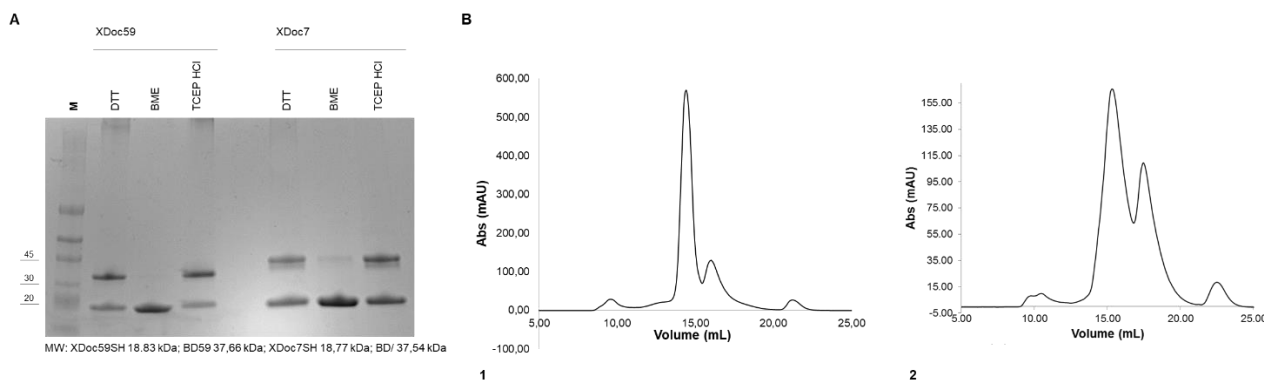


Figure 5 A SDS PAGE of crosslinking reaction on dockerins pre-incubated with different reducing agents; B Size exclusion chromatography of crosslinking reaction on 1 XDoc59SH and 2 XDoc7SH pre-incubated with TCEP HCl.

2.2 Test of protein cage assembly and preliminary crystallization attempts

Once obtained the designed building blocks, we tested their ability to spontaneously self-assemble into a higher order nanostructure. Our first attempts were conducted with the building blocks CF3 and 2-D59, due to the higher stability of the CF3 trimer (compared with CF1 and CF2), and higher D59SH-crosslinking conversion. From size exclusion chromatography, we observed several protein forms in solution, which suggested the absence of a predominant protein assembly (Figure 6). Also, we could see a rather defined and sharp peak at higher retention volume, which indicated the presence of a smaller form corresponding to the expected molecular weight of the tetrahedral assembly. Using the protein from this peak, we started preliminary crystallization screens for structural characterization, and successfully obtained microcrystals using PEG8000 as crystallization agent. We are currently optimizing the quality of the protein sample and its solid state for analysis by single particle cryo-EM and X-ray diffraction.

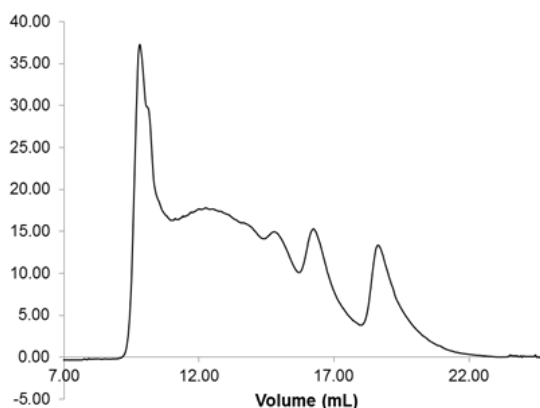


Figure 6 Size exclusion chromatography on 359 cage

3. Conclusions and Outlook

We were able to successfully synthesize and characterize all building blocks we designed for the assembly of our novel protein cage. The production of the vertex-BBs was very straightforward, while the crosslinking reaction to obtain the edge-BBs needed careful optimization.

Protein cage assembly tests showed some equilibrium forms, thus indicating the lack of a predominating nanostructure. Characterization of the observed forms is underway.

In the next year, we expect to optimize the production of the protein cage and to fully characterize it. Furthermore, we would attempt to functionalize the cage with enzymes to assess the catalytic properties of our nanobioreactor.

4. References

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