The Role of SAPs and hybrid SAP-PNAs in the Fabrication of a Synthetic Erythrocyte

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ABSTRACT

This work is part of a bigger project, SYNERY (Figure 1), funded by Horizon Europe-EIC-PATHFINDEROPEN-01 and focused on the development of a synthetic carrier that mimics erythrocytes features. To advance the ambitious goal of **fabricating a synthetic erythrocyte** from the bottom-up, it is necessary to sculpt a biomimetic membrane at the nanoscale, to build a biomimetic erythrocyte cytoskeleton and to obtain evidence of biocompatibility and *in vivo* functionalities. In order to confer membrane complexity and nanostructure, the formation of a well-ordered biomimetic cytoskeleton is necessary. Therefore, we are investigating the possibility to build and anchor such nanostructures from **self-assembling peptides (SAPs)** and hybrid SAP-peptide nucleic acids (SAP-PNA). To mimic the mechanical properties of the erythrocyte cytoskeleton we synthesized biotinylated-Bone Marrow Homing Peptide 1 (BMHP1)-derived sequences, linear and branched SAPs, and hybrid SAP-PNA. Because both SAPs and SAP-PNAs share the advantage of not requiring an energy source for self-assembling, SAP-based substitute for spectrin could be the key point for a biomimetic strategy (erythrocytes continuously consume ATP to reassemble their spectrin cytoskeleton). Here we describe the preliminary advances regarding the synthesis and characterization of linear SAPs and SAP-PNAs with particular attention to secondary structure studies. SAP-PNAs were synthesized and purified with high purity and yield. The mechanical properties were characterized using a rotational rheometer. The characterization of secondary structure involved Thioflavin (ThT) binding assays, Fourier-transform infrared spectroscopy in attenuated total reflection mode (ATR-FTIR) analysis, Raman studies and Atomic force microscopy (AFM) analysis.. The primary results showed the evidence of bases pairing contents between different SAP-PNAs chains, demonstrating that this approach does not prevent the standard self-assembling of cross-β structures of the self-assembling peptidic backbones.

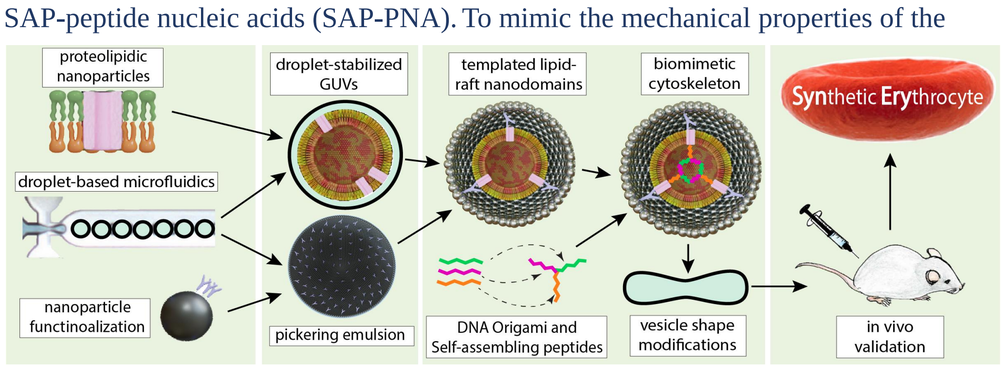

Figure 1. A visual summary of the project

OBJECTIVES

This project is focused, as a biomimetic strategy, on the development of an adequate SAP-based substitute for spectrin, since in contrast to DNA, SAPs can be produced in large-scales in GMP manufacturing facilities, which would be key for the future cost-effectiveness of our proposed synthetic erythrocytes. In detail, SAPs are involved in the biomimetic **reconstruction of a mechanically functional *spectrin cortex*.**

RESULTS AND DISCUSSION

The Principles of Self-Assembly. Self-assembling is a ubiquitous nano-structural phenomenon that occurs in conditions of thermodynamic equilibrium (Figure 2). SAPs self-organize (thanks to hydrogen bonds formation, hydrophobic interactions, π-π stacking, and electrostatic interactions) in well-ordered structures that include fibrils, micelles, vesicles, nanotubes. SAPs are synthetic biomaterials at the forefront of nervous tissue engineering because they are:

- ✓ bioabsorbable
- ✓ highly biocompatible
- ✓ biomimetic

Furthermore, SAPs provide nanostructured microenvironments morphologically resembling the natural extracellular matrices (ECM). By changing the amount and type of peptide decoration, it is also possible to control the density of functional motifs and multi-functionalization, as well as their mechanical properties.

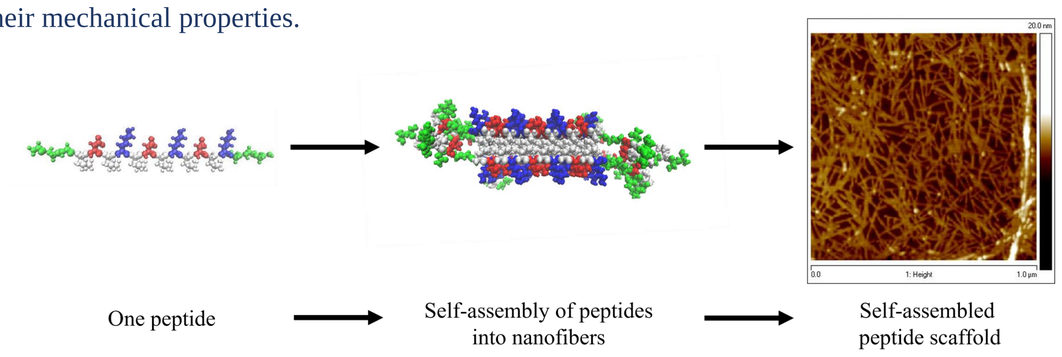

Figure 2. An example of self-assembly of peptides to form nanofibers. The AFM image shows LDLK12 nanofibers.

SAPs Design. In order to mimic mechanical properties of synthetic erythrocyte cytoskeleton we are synthesizing and characterizing a library of biotinylated-Bone Marrow Homing Peptide 1 (BMHP1)-derived sequences (i.e. BM3), non-functionalized (i.e. LDLK12) and functionalized LDLK-based SAPs (i.e. FAQ-LDLK12), branched SAPs, and hybrid SAP-PNAs (i.e. LDLK12-PNA1 and LDLK12-PNA2) (Figure 3 and Figure 4).

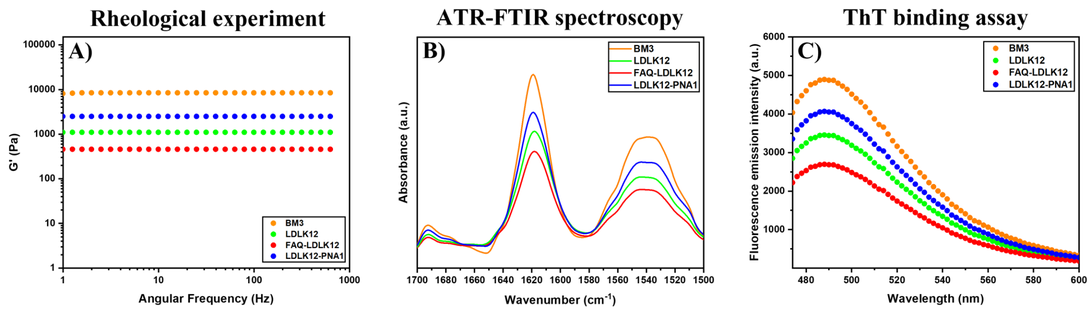

Figure 3. Characterization of SAPs. A) Investigation of mechanical properties by rheological experiments, i.e. frequency sweep test; B) ATR-FTIR absorption spectrum in Amide I and II regions; C) ThT binding assay for the evaluation of β-structuration.

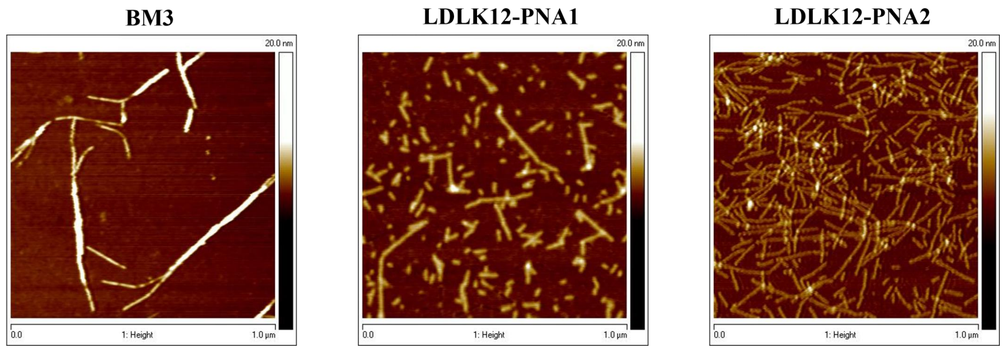

Figure 4. Morphological characterization using AFM.

Self-Assembly Using PNA. PNAs share the specific base-pair recognition characteristic of DNA, representing an important family of building blocks which converges the advantages of both DNA- and peptide nanotechnologies. We are synthesizing a library of hybrid SAP-PNAs to explore both the self-assembly into nanofibers and the hybridization efficiency to reach tunable backbone chain flexibility and, as such, designed mechanical strength. The exploration of SAP-PNAs technology feasible as a **biomimetic *cortex*** starts with the detection of β-sheets and base pairing content (Figure 5).

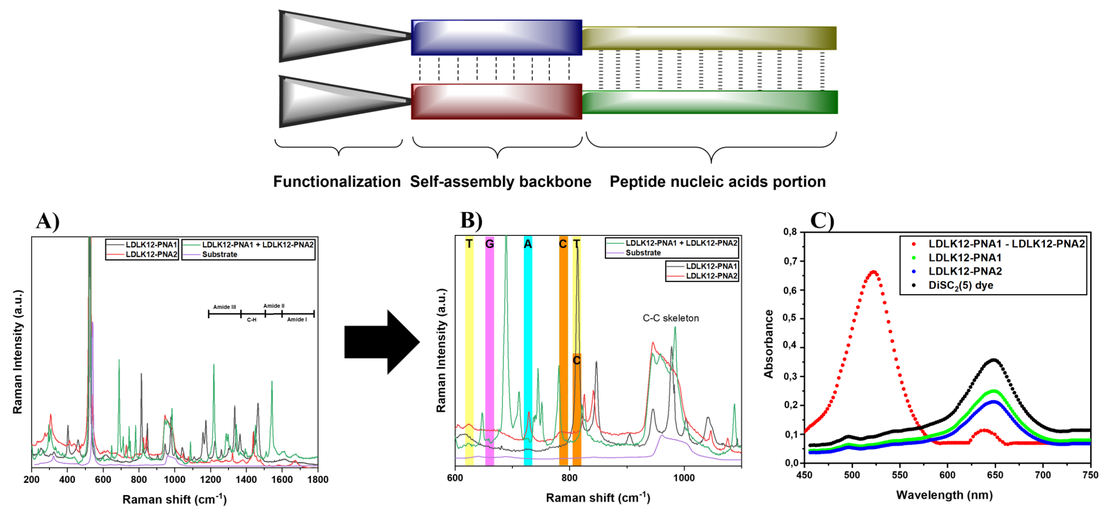

Figure 5. Investigation of the aggregation into complementary LDLK12-PNA1 – LDLK12-PNA2 duplex. A) and B) show Raman spectroscopy spectra where typical bands of single bases are highlighted. Peaks shifts indicate A-T and C-G bases pairing into the hybridized SAP-PNAs. C) Colorimetric test for bases pairing recognition by the use of $DiSC_2(5)$ cyanine dye.

MATERIALS AND METHODS

Peptide Synthesis. All peptides were synthesized *via* microwave assisted Fmoc SPPS chemistry on a Rink Amide resin using a CEM Liberty Blue system (CEM Corp., Matthews, NC, Canada). The peptide derivative was cleaved from the resin, precipitated and lyophilized. The crude peptide was then purified *via* RP-HPLC.

Rheological Characterization. Rheological properties were assessed using a AR-2000ex rheometer (TA Instruments, New Castle, DE, USA) with a truncated cone−plate geometry (diameter, 20 mm; angle, 1°; truncation gap, 34 µm).

ThT Binding Assay. The benzothiol dye ThT was used to detect β-sheets in SAPs. ThT fluorescence measurements were recorded using an Infinite M200 Pro plate reader (Tecan, Mennedorf, Switzerland) with excitation at 440 nm and emission 482 nm.

$DiSC_2(5)$ Test. The cyanine dye $DiSC_2(5)$ was used to detect complementary chains of SAP-PNAs.The absorbance was recorded with excitation at 651 nm.

Raman Spectroscopy. Micro-Raman measurements were carried out at RT by a confocal labRAM (Horiba Jobin-Yvon) spectrometer, operating in backscattering configuration. A He-Ne laser line at 633 nm was used as exciting source with spectral resolution of about 1 cm^{-1}.

ATR-FTIR Spectroscopy Analysis. ATR-FTIR spectra were collected using a Perkin-Elmer Spectrum Two IR spectrometer (PerkinElmer Ltd., Beaconsfield, United Kingdom) equipped with a Perkin-Elmer single-reflection diamond ATR.

AFM. AFM images were captured in tapping mode using a Multimode Nanoscope V (Digital Instruments, Veeco, Plainview, NY, USA), with single-beam silicon cantilever probes (Veeco RFESP MPP-21100-10: resonance frequency 76-90 kHz, nominal tip radius of curvature 8 nm). Image flattening was performed prior to images analysis.

CONCLUSIONS

This project is characterized of scientific, societal and economic impact. While the long-term vision is to provide a global solution in the field of transfusion medicine, the applicability go far beyond. Indeed, strategies that mimic aspects of the erythrocyte could demonstrate as effective drug delivery systems, it will be applicable for a broad set of artificial cells, and it will be broadly applicable for cardiovascular imaging. In these preliminary results we have synthesized and characterized a library of SAPs and hybrid SAP-PNAs. As this approach does not prevent the self-assembling into β-structuration allowing the pairing of the nucleobases, our evidence suggest an exciting possibility to sculpt a biomimetic cytoskeleton, as well as SAP-PNAs could be used as biosensors, probes and microarrays.

ACKNOWLEDGMENT

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