PROJECT ACRONYM

CUPIDO

PROJECT TITLE

Cardio Ultraefficient nanoParticles for Inhalation of Drug prOducts

Deliverable 2.1

Aptamer-mediated guidance of CaP

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Table of Revisions

REVISION NO.	DATE	WORK PERFORMED	CONTRIBUTOR(S)
1	10/01/2018	Document preparation	Michele Iafisco
2	12/01/2018	Document preparation	Daniele Catalucci
3	30/01/2018	Document revision	IPR Team

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1. Executive summary

The current deliverable 2.1 outlines a methodology to improve the specificity of therapeutic drug delivery to the heart using a chemical-based targeting system. To achieve this aim, CaPs from D1.1 were surface functionalized with internalizing aptamers (single stranded synthetic oligonucleotides) to enhance the efficiency of myocardial targeting. Aptamers, which are short single stranded oligonucleotides of modified DNA or RNA are emerging as a very interesting class of molecules that can fold into complex tertiary structures and bind with high affinity to a specific target (Zhou et al., Nature reviews Drug discovery 2017; Catuogno et al., Pharmaceuticals 2016). In particular, isolated from combinatorial libraries, aptamers can be designed for the recognition of any surface receptor thereby serving as cell-selective targeting. If the targeted receptor is undergoing receptor-mediated cell internalization, the conjugation of a secondary reagent to specific aptamers serves also as a carrier for the facilitation of intracellular delivery.

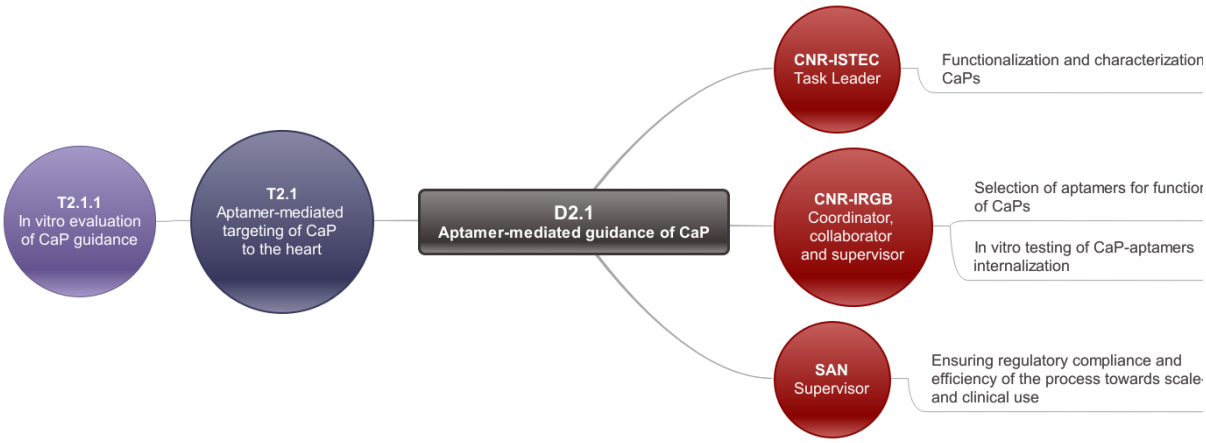
Current results in this document are extracted from the whole activities performed within this D2.1 and provide the evidence for an effective surface functionalization of CaPs with Gint-4, an aptamer previously generated that specifically bind to the platelet-derived growth factor receptor-β, PDGFRβ], which is a receptor expressed in cardiomyocytes (Camorani et al., Mol. Therapy 2014; Chintalgattu et al., JCI 2010). Experimental evaluation of CaP-Gint-4 cell internalization has been obtained and parts of the data are presented. Additional targeting aptamers with a more cardiac specific recognition are currently under evaluation.

Key deliverable achievements:

1. Realization of surface functionalized CaPs with aptamers
2. Characterization of surface functionalized CaPs with aptamers
3. Evaluation of cell internalization of CaP-aptamers

2. Cooperation between participants

CNR-IRGB selected specific aptamers for the initial and subsequent functionalization of CaPs, whereas CNR-ISTEC functionalized and characterized CaPs with the provided aptamers. CNR-IRGB tested in vitro the effective cardiac cell internalization of CaP-aptamer. Remote and physical meetings occurred periodically between the two groups and data were shared with the consortium.



3. Aptamer mediated targeting of CaPs

3.1. Surface functionalized CaPs with aptamers

In a first step designed for a proper measurement of the amount of aptamer, a calibration curve of Gint 4 in water by UV-Vis (Nanodrop, Thermo Scientific, USA) was performed. Starting from a solution with the maximum aptamer concentration (20 nmol/ml equal to 350 ug/ml), analyses from several dilutions have been made as reported in Table 1.

Table 1. Solutions of Gint4 used for the UV-Vis calibration curve.

Sample	Concentration (nmol/ml)	Concentration (μg/ml)	Abs (λ=260nm)
1	20.0	351.4	10.2
2	10.0	175.7	5.3
3	5.0	87.8	2.5
4	2.0	35.1	1.0
5	1.0	17.6	0.4
6	0.5	8.8	0.2

According to the absorbance peak of Gint4 at 260 nm of different solutions, a calibration curve was provided revealing a good linearity (R^2=0.9997) in the analyzed range of concentrations.

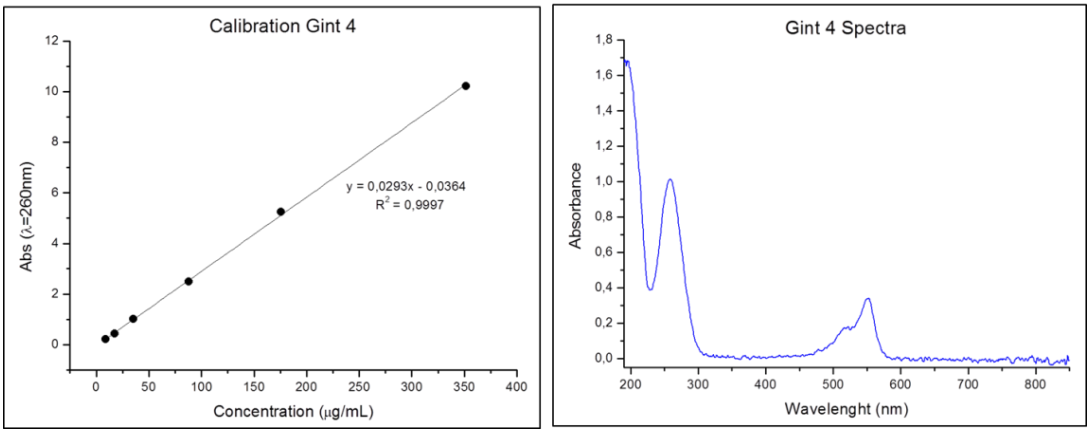


Figure 1. Gint4 calibration curve (left panel) and UV-Vis spectra of Gint4 (right panel).

Nest, the adsorption kinetics of Gint4 on dried CaPs (synthesized according to the protocol CaP-001 reported in D1.1) was investigated. A kinetic overview was possible by the evaluation of the amount of Gint4 adsorbed on CaPs as a function of contact time between nanoparticles and aptamers. 1 ml of Gint4 (60 μg/ml) was put in contact with 0.5 mg of CaPs under shaking at 37°C. At scheduled times, ranging from 5 min to 144 hours, samples were centrifuged and the quantity of Gint4 in the supernatant quantified by Uv-Vis. The amount of Gint4 adsorbed on CaPs was calculated as difference between the initial amount and that in the supernatant. An evaluation of the adsorbed Gint4 (reported as weight percentage respect to the initial amount) versus time is plotted on Figure 2.

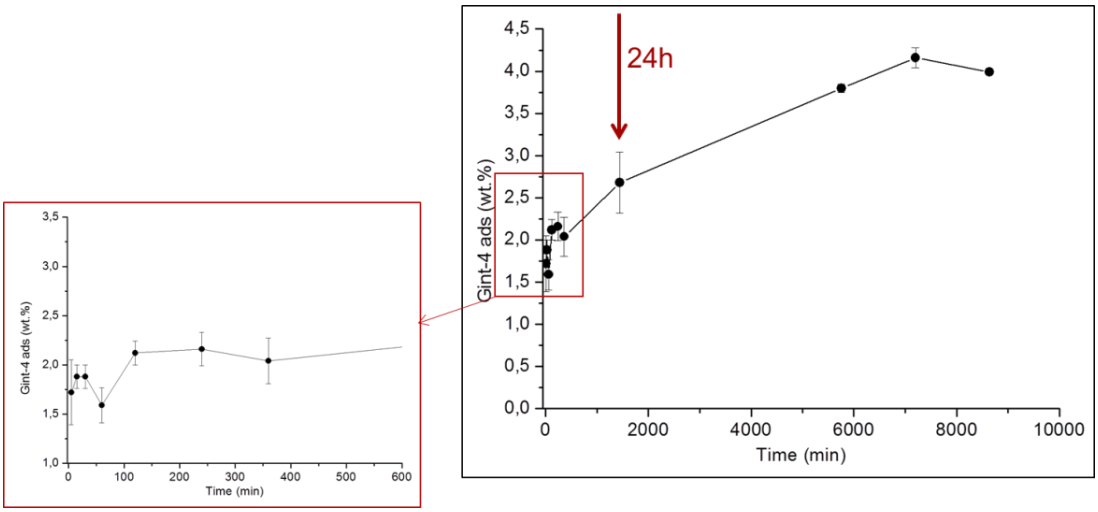


Figure 2. Adsorption kinetics of Gint4 on CaPs.

These data indicated a good affinity of Gint4 for CaPs. In fact, the adsorbed amount of Gint4 increased as a function of contact time with a first rapid rise followed by a progressive stabilization reaching the 4 wt% after 5 days.

In the next step, at the contact time of 24 hours, the adsorption isotherm of Gint4 on CaPs has been analyzed by experiments with three increasing Gint4 concentrations (10, 30, and 60 μg/ml); the amount of CaPs was kept constant at 0.5 mg/ml. The amount of adsorbed Gint4 on CaPs has been evaluated as reported for the adsorption kinetic. Figure 3 shows the obtained isotherms of adsorbed Gint4 (expressed as μg of Gint4 on mg of CaPs) on CaPs as a function of the concentration of Gint4 at equilibrium (expressed as μg/ml).

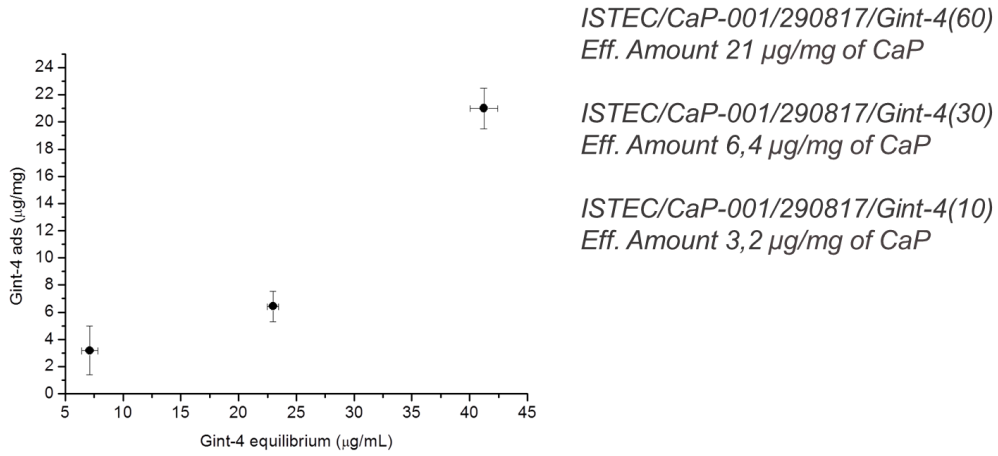


Figure 3. Adsorption isotherm of Gint 4 on CaPs.

The amount of adsorbed Gint4 from ISTEC/CaP-001/290817/Gin-4(60), ISTEC/CaP-001/290817/Gin-4(30), and ISTEC/CaP-001/290817/Gin-4(10) were quantified as 3.2, 6.4, and 21.0 μg/mg, respectively. Finally, these samples were delivered to CNR-IRGB for an in vitro evaluation of CaP-aptamer internalization in cells.

3.2. Characterization of surface functionalized CaPs with aptamers

The surface potential of ISTEC/CaP-001/290817/Gin-4(30) was evaluated through electrophoretic mobility were carried out with a Zetasizer Nano analyzer (Malvern, UK) using disposable folded capillary cells (DTS1061; Malvern, UK) at 25°C, suspending nanoparticles in water. Data reported in Table 1 revealed that the presence of aptamer didn't affect the surface charge of the nanoparticles that remains negative. In this case as example the surface potential of the CaPs loaded with and intermediate concentration of Gint4 (6.4 microgram per mg of CaPs) was measured and compared to that of pristine CaPs.

Table 2. ζ-potential of pristine CaPs and surface functionalized CaPs with aptamers.

Sample	ζ-potential (mV)
ISTEC/CaP001	-23.0 ± 3.4
ISTEC/CaP-001/290817/Gin-4(30)	-23.9 ± 1.4

3.3. In vitro functional evaluation

The capacity of ISTEC/CaP-001/290817/Gin-4(60), ISTEC/CaP-001/290817/Gin-4(30), and ISTEC/CaP-001/290817/Gin-4(10) to target cardiac cells has evaluated in vitro by treatment of HL1 cardiac cell with increasing doses of the three samples. Aptamer cell internalization due to the proper affinity to CaP was evaluated by quantitative real time PCR from total RNA extracted from cells 24 hours post ISTEC/CaP-001/290817/Gin-4(60), ISTEC/CaP-001/290817/Gin-4(30), and ISTEC/CaP-001/290817/Gin-4(10) administration. As shown in figure 4, more comparable levels of internalized aptamer vs total input (corresponding to the total aptamer amount present in the CaP-aptamer formulation used per cell well) were found for the condition ISTEC/CaP-001/290817/Gin-4(30), which is currently used for additional tests as the most promising formulation.

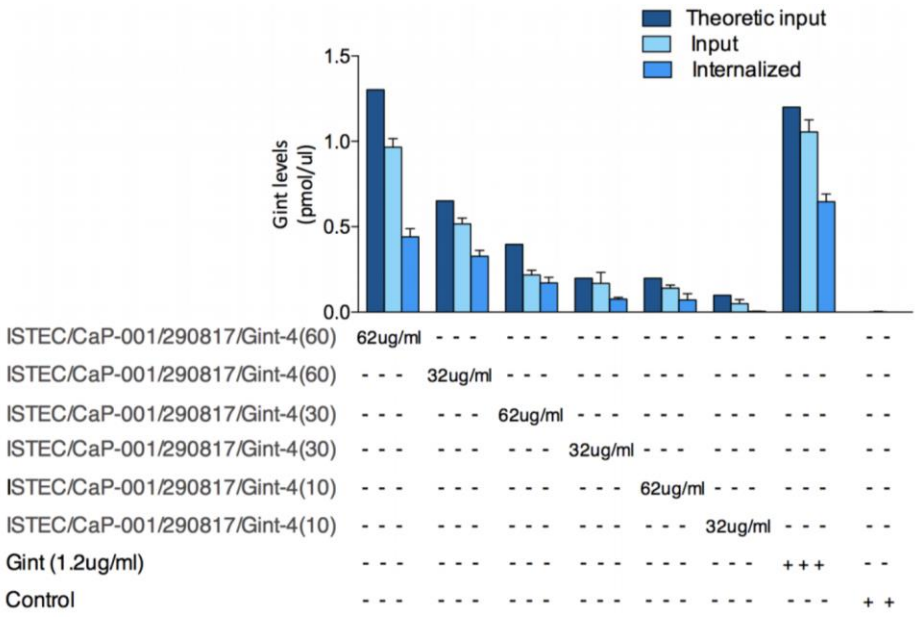


Figure 4. Aptamer-mediated CaP internalization.

4. Conclusions

The activities performed within this deliverable show the identification of a proper protocol of synthesis to effectively functionalize CaPs with cell-targeting aptamers. Extracted results from the currently performed tests in vitro support the evidence that Aptamer functionalized on CaPs remain surface negatively charged and retain their cell-internalizing feature.