PROJECT ACRONYM
CUPIDO

PROJECT TITLE
Cardio Ultraefficient nanoParticles for Inhalation of Drug prOducts

Deliverable 1.2

Functional assessment (compatibility, physic- chemical and biological stability) of CaP restoration and drug release

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1. Executive summary

The prosecution of the program was centered on the preparation of microparticles containing nanoparticles (dpCaPs) loaded with the selected drugs active for the treatment of cardiac disease, i.e. a therapeutic peptide and a clinically-approved small molecular weight drug. Loading, compatibility, characterization and biological stability after microparticle dissolution and nanoparticle restoration have been the direct objectives.

The microparticle preparation containing CaP nanoparticles loaded with peptide has been manufactured with positive results thanks to the strong interaction between CaP and macromolecule. dpCaPs loaded with the peptide have been characterized (content, size, restoration and respirability) and distributed to the partners in charge of the biological assessments.

The loading with the selected clinically-approved drug has been more demanding and required some adjustments of the manufacturing protocol. The efforts for increasing the loading efficiency were borne by CNR-ISTEC and PLU with the support of SAN. The loading of the small molecular weight drug allowed the development of new techniques and procedures of manufacturing and control. Namely, a new HPLC method for the analysis of the selected drugs, allowing to follow their distribution during manufacturing, and a novel spray drying protocol were adapted to the presence of the drug. In addition, the industrial oriented transferable process in regard of nanoparticle purification has been implemented. In vitro methods for determining the release of nanoparticles and drug loading from dpCaPs have been designed and validated. The results will open up to a wider portfolio of relevant novel applications for the biocompatible dpCaPs given by inhalation.

Key deliverable achievements:

1. Small molecule loaded in nanoparticles;
2. Peptide loading of nanoparticles;
3. Microparticles embedding loaded nanoparticles;
4. Respirability and release;
5. *In vitro* functional assessment.

Introduction

The core of this deliverable was devoted to the dpCaP preparation consisting of microparticles embedding nanoparticles, which contain the small molecular weight drug and the therapeutic peptide. The protocol for nanoparticle preparation has been improved and transferred to the partners in charge of their manufacturing and transformation to microparticles. Therefore, the dpCaPs loaded with the two different compounds have been prepared and tested. In particular, dpCaP with peptide has been successfully produced. Then, the dpCaP loading small molecular weight drug after several attempts has been ameliorated and obtained. Drug- and peptide-loaded dpCaPs were functionally assessed *in vitro*.

2. Cooperation between participants

Many partners directly cooperated in this part of the project development.

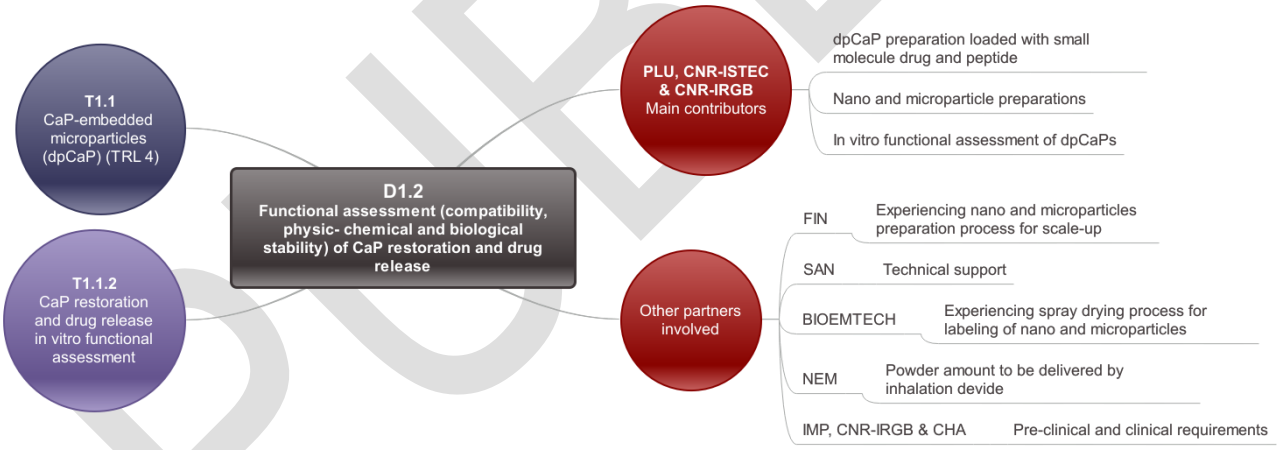
First, the dpCaP preparation loaded with the small molecule drug and peptide required the merging of knowledge and expertise of CNR-ISTEC and PLU. The two partners modified the nano and the microparticle preparation with special attention towards stability and scale up of processes to be transmitted to FIN. FIN participated to several sessions for the preparation of nanoparticles and microparticles. SAN supported the activities and was in tight interaction with CNR-ISTEC, PLU, and FIN.

BIOEMTECH researchers were involved during one week in PLU and CNR-ISTEC for experiencing i) the spray drying process to be applied for the labeling of nanoparticles and microparticles, and ii) nanoparticle and microparticle preparations, respectively.

NEM was involved in the discussion concerning the dpCaP amount to deliver by inhalation, with the goal to anticipate the amount of powder to aerosolize and inhale. This was done in view of the construction of the dry powder device for the delivering of the active drug. NEM consulted CHA for the due clinical requirements.

IMP, CNR-IRGB, and CHA were involved in the evaluation whether the above activities were leading to products satisfying the pre-clinical and clinical needs. CNR-IRGB performed the in vitro functional assessment of dpCaPs.

CNR-IRGB kept the links between all these partners, connecting the efforts for the attainment of the product to administer. All the above partners were periodically interacting via remote teleconferences.



3. Loading of small molecular weight drug

Following the first attempts to load the selected small molecule drug in CaP nanoparticles, the original nanoparticle manufacturing protocol (CaP-002) has been implemented due to the presence of the small molecular weight drug. An adapted protocol for nanoparticle preparation was optimized (CaP-003), paying attention to the physico-chemical properties of the drug, the increase of drug loading efficiency and stability. A number of experimental preparations of nanoparticles have been executed and microparticles made of drug-loaded CaP (dpCaP-drug) were prepared by spray drying to obtain particles characteristics suitable for aerosolization and drug release. The conclusion of this part of the research project constitutes the base for starting the scale up fabrication of drug-loaded dpCaPs.

3.1. Novel protocol for nanoparticle drug loading (CaP-003)

The first batches of drug-loaded CaPs were prepared by CNR-ISTEC with the optimized protocol CaP-003; particle dimensions and morphology were characterized by PLU.

3.1.1. Characterization of drug-loaded nanoparticles

Dimensional analysis by Dynamic Light Scattering (DLS)

Size distribution analysis was performed by DLS measurements and data are shown in Table 1. Collectively, results are in the expected range.

Table 1. Dimensional analysis by DLS. CaP-drug were evaluated before and after filtration with the intent to assess the presence of aggregates. Data are representative for batch ISTEC/CaP-003/190318/drug.

	Z-AVERAGE (nm)	PDI	Z-POTENTIAL (mV)
CaP-drug	86 70 (filtered membrane 0.22 µm)	0.200	-29

Morphology by Scanning Electron Microscopy (SEM)

The Scanning Electron Microscopy (SEM) images of CaPs manufactured according the new CaP-drug protocol (CaP-003) show individual acicular nanoparticles with a length range of 50 nm to 150 nm (Figure 1). Agglomerates of small nanoparticles are present, deriving from the dispersion drying for sample analysis preparation.

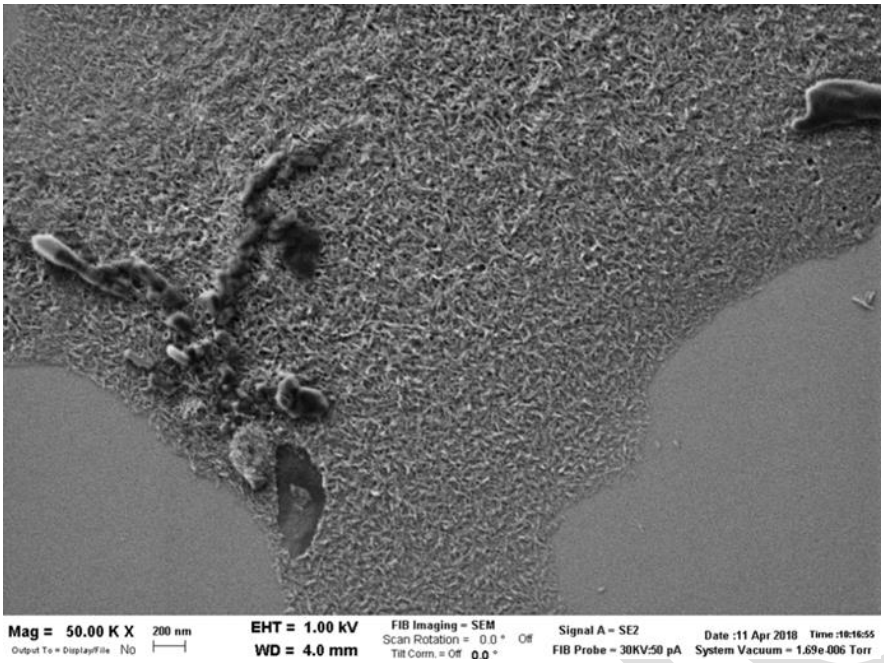


Figure 1. SEM micrograph. Data are representative for batch ISTEC/CaP-003/190318/drug.

4. Scaling up of CaP-003 nanoparticles

4.1. Generation of drug loaded nanoparticles

With the aim to increase the experimental availability of drug loaded nanoparticle dispersions, the CNR-ISTEC protocol CaP-003/drug was transferred to PLU to assess the transferability of the process.

4.1.1. Characterization of drug loaded nanoparticles

PLU prepared additional batches of drug loaded nanoparticle (protocol CaP-003). Size distribution analysis was performed by DLS measurements and, as shown in Table 2, the mean size resulted in the range as generated by CNR-ISTEC.

Table 2. Dimensional analysis by DLS. CaP-drugs were evaluated before and after filtration with the attempt to assess aggregate presence. Data are representative for batch PLU/CaP-003/100418/drug.

	Z-AVERAGE (nm)	PDI	Z-POTENTIAL (mV)
CaP-drug	98 90 (filtered 0.22 µm)	0.300	-30

4.2. Generation of microparticles

Having prepared more significant amounts of nanoparticles, PLU constructed the dpCaP-drug powders following the optimized protocol SD-002. CaP dispersions were spray dried after addition of mannitol resulting in a yield of 70% (data from PLU/CaP-003/100418/drug batch).

4.2.1. Characterization of microparticles

Morphology analysis by SEM

dpCaP-drug microparticles have spherical shape occasionally fused each other (Figure 2). The microparticles surface was rough, signaling the presence on the surface of nanoparticles.

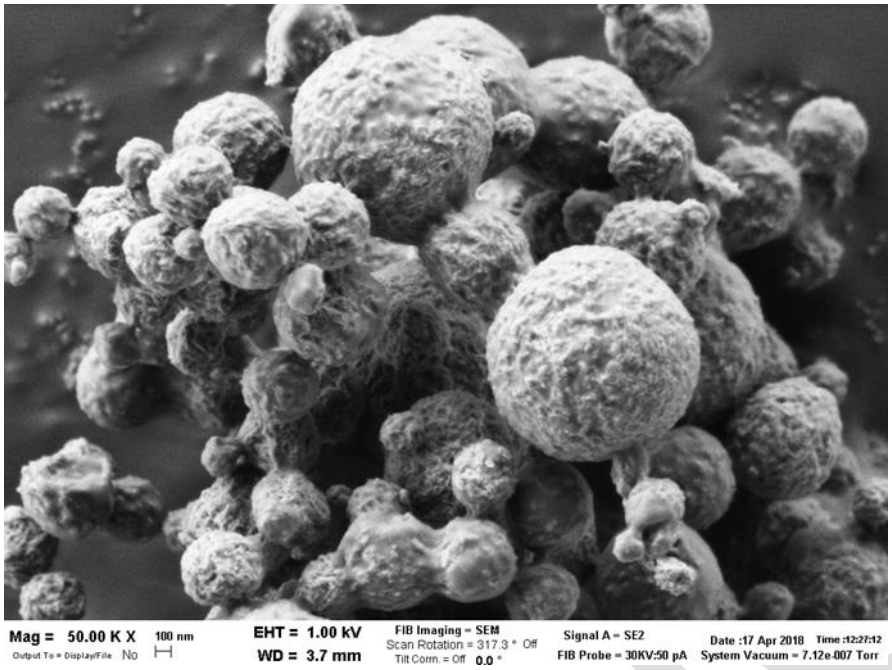


Figure 2. SEM micrograph of PLU/CaP-003/100418/drug batch.

4.2.2. Restoration of nanoparticle from microparticles

To characterize the nanoparticles restored from dpCaP-drugs, a quantity of microparticles were dissolved in water and dimensional analysis performed by DLS. Restored nanoparticles exhibited a mean size that remained unmodified in comparison with the original nanoparticle dispersion.

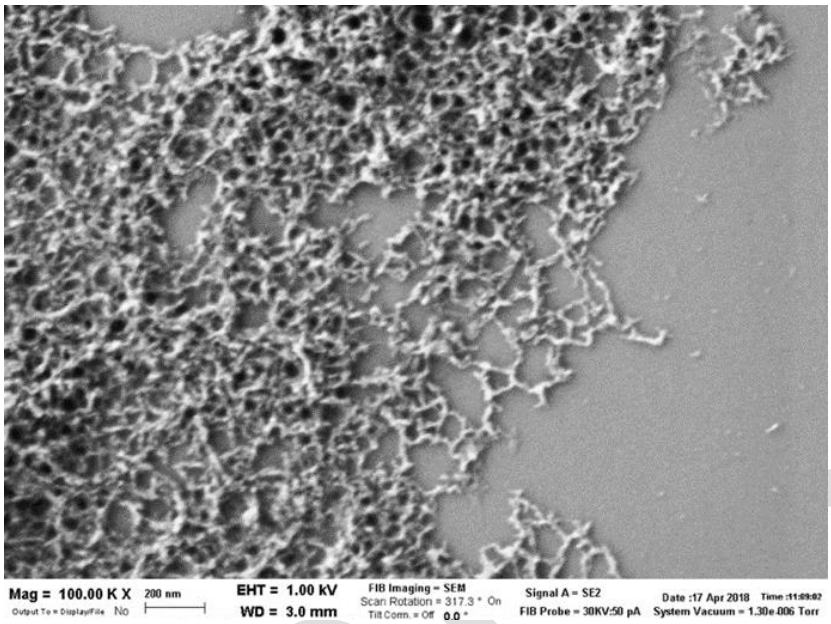


Figure 3. SEM micrograph of restored nanoparticles. Representative image from PLU/CaP-003/100418/drug.

Accordingly, SEM analysis (Figure 3) showed that nanoparticles restored from microparticles dissolved in water present the original CaP morphology, having an acicular shape similar to the first batch of nanoparticles with drug. The presence of mannitol dried during the sample preparation gives rise to a network structure with few visible individual free nanoparticles.

5. Reproducibility of the manufacturing process of microparticles with drug

To investigate the physical reproducibility of the CaP-003/drug protocol and to determine the effective loading of the drug, multiple preparations of dpCaP-drug were performed and characterized by PLU.

5.1. Characterization of drug loaded nanoparticles

Dimensional Analysis by DLS

Nanoparticle dimensions were confirmed to be comparable among several batches, representative data is shown in Table 3.

Table 3. DLS analysis of drug loaded nanoparticles. Representative data from the analysis of PLU/CaP-003/220518/drug. Batches were evaluated as such or following dilution and/or filtration.

	Z-AVERAGE (nm)	PDI
CaP-drug	75.6 ± 14.1 (dilution 1:5 and filtered 0.22 µm)	0.3 ± 0.10

Morphology analysis by SEM

Accordingly to the DLS data shown in Table 3, the size of nanoparticles also evaluated by SEM (Figure 4) was similar to the previous batches (see Figure 1).

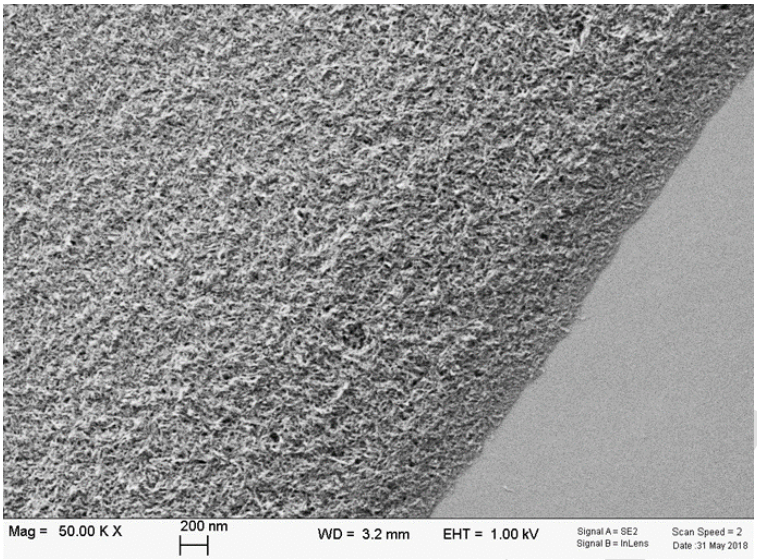


Figure 4. SEM micrograph of restored nanoparticles. Representative image from PLU/CaP-003/220518/drug.

Drug content

An HPLC method for the determination of drug content in the various preparation steps was set up (Table 4, microparticles dispersed in water). The following procedure for drug content by HPLC of CaP-drug has been executed:

1ml of CaP-drug dispersion was treated with 1 ml of HCl 0.1 N to dissolve the CaP and release the drug. At the same time an aliquot of the CaP-drug dispersion was centrifuged and the supernatant analyzed. The precipitate was resuspended with H_2O. The dispersion was centrifugated again and the precipitate, dissolved in 9 ml of HCl 0.1 N, was analyzed.

The same procedure was followed in order to quantify the drug in the nanoparticle dispersion after purification by dialysis.

Table 4. Data represent parameters obtained from batch PLU/CaP-003/220518/drug.

PLU/CaP-003/220518/drug dispersion		
Before dialysis	**Drug content**	
	Drug loaded %(w/w)	**Total (mg)**
Ca/Cit-drug solution	94.5± 0.34	70.87± 0.2
PO4-drug solution	99.2± 2.0	74.4± 1.2
Dispersion after 24h of CaP maturation	36.0± 0.33	52.2± 0.6
Supernatant	12.2± 0.04	17.7± 0.06
Precipitate	23.8± 0.08	34.5 ± 0.08
After dialysis		
Dispersion after 24h of dialysis	-	2.2± 0.26
Supernatant	-	1.9± 0.53
Precipitate	-	0.1± 0.01

The initial determination of the clinically approved drug content in the nanoparticle dispersion and in the microparticles preparation indicated that the active substance in the nanoproduct remains stable during the manufacturing steps. A direct check of the drug stability indicated that the precipitation and spray drying procedure

of a solution theoretically containing 3.2% w/w of drug, resulted to contain 3.16% w/w of drug in the dried powder final product. Further characterization along all manufacturing processing are ongoing.

6. Microparticles embedding CaP nanoparticles loading the therapeutic peptide

Preparations of nanoparticles and microparticles were performed to provide partners with dpCaP loaded with the therapeutic peptide (MP) having characteristics suitable for inhalation and drug release.

6.1. Generation of peptide loaded nanoparticles

CaP-MP prepared by PLU and ICNR-STEC together, showed a nanoparticle concentration post purification estimated in 1 mg/ml with a theoretical concentration of the peptide in the dispersion at 0.045 mg/ml.

6.1.1. Characterization of peptide loaded nanoparticles

Dimensional Analysis by DLS

The intensity distribution profile shows an initial aggregation of the nanoparticles (Z average of 3450 nm by DLS), which however gradually revert to a disaggregated state featuring a small particle size, as demonstrated in our published paper (Miragoli et al. Science Translational Medicine, 2018)

Morphology analysis by SEM

The size and morphology of nanoparticles as verified by SEM (Figure 4) in the nanoparticle dried sample on the SEM stub exhibits a net formation (agglomeration) in this sample with peptide similarly to the sample where mannitol was present.

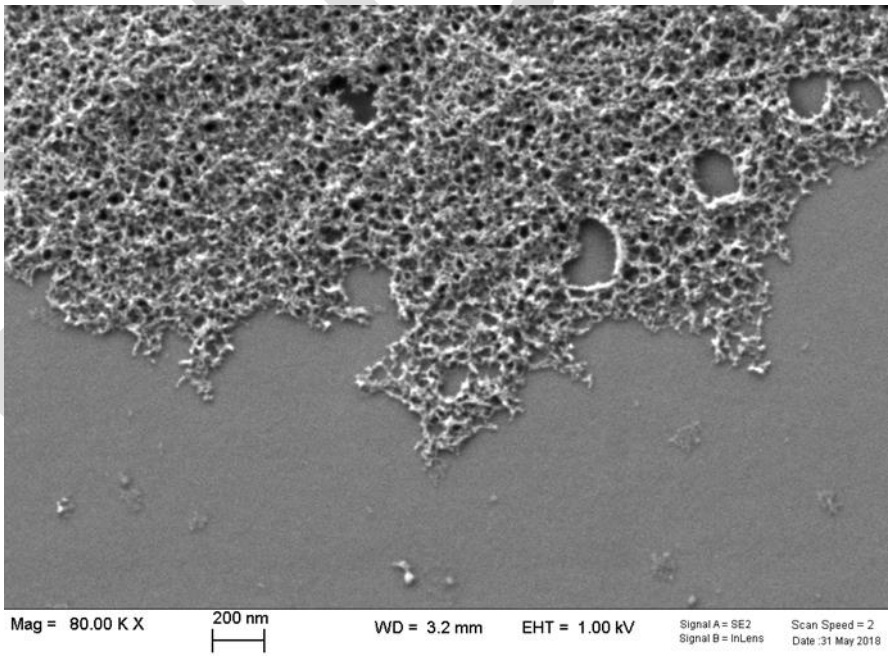


Figure 5. Representative SEM image from PLU/CaP-002/150518/MP.

6.2. Manufacturing of microparticles

CaP-MP dispersions were spray dried with protocol SD-003, where the addition of mannitol contributes to the construction of aerodynamic particles and protection of peptide structure. Generally, the obtained spray-drying

yield show good results with an average value of 80%, as measured for the PLU/SD-003/170518 (PLU/CaP-002/150518/MP) batch.

6.2.1.Characterization of microparticles

dpCaP-MPs exhibit spherical shape, with rough surface indicative of the nanostructures in surface (Figure 6). This structure showed a favorable respirability since the particles did not show a fusion.

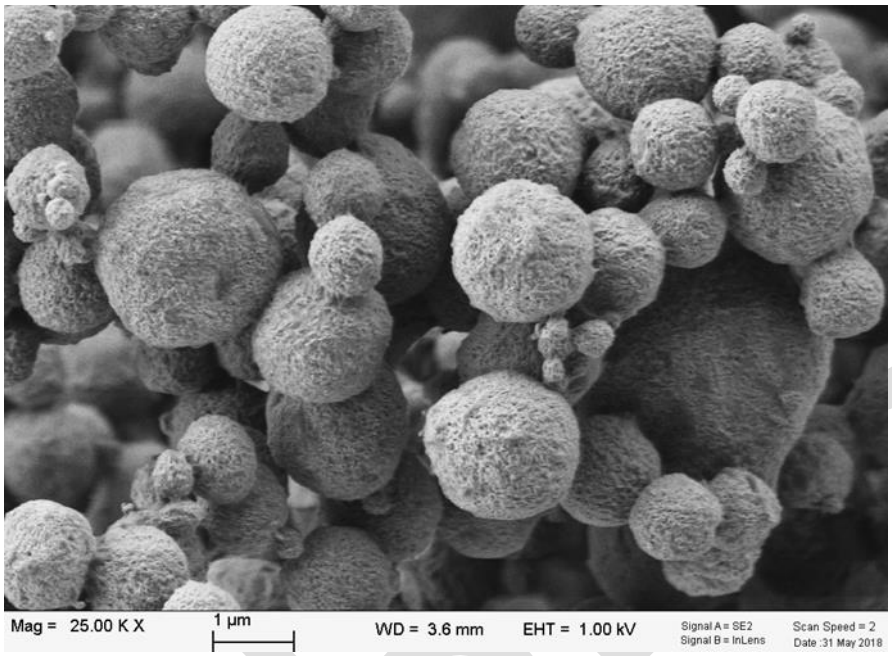


Figure 6. Representative SEM image from PLU/SD-003/170518 (PLU/CaP-002/150518/MP.

The peptide content in the microparticles, measured by HPLC on 10 mg of powder recovered from the spray drier, was 1.02 % w/w [PLU/SD-003/170518 (PLU/CaP-002/150518/MP) batch].

6.2.2.Restoration of nanoparticle

To evaluate nanoparticle restored from dpCaP-MP, aliquots of spray dried powders were dissolved in water and dimensional analysis was performed by DLS.

Dimensional Analysis by DLS

Table 5. Representative data from batch PLU/SD-003/170518 (PLU/CaP-002/150518/MP.

	Z-AVERAGE (nm)	PDI	Z-POTENTIAL (mV)
Restored nanoparticle from dpCap-MP	1000	0.700	-23

The size of restored nanoparticles was smaller than CaPs with peptide before spray drying. However, the size analysis of this peptide sample has to be conducted carefully. The interpretation not only assigned by mean of the Z-average value. The intensity distribution has to be compared, in order to interpret the contribution of the different products in dispersion on the average size (individual particles and agglomerates).

Morphology analysis by SEM

To assess the structure of the CaP-MP, nanoparticles were restored in order to have a concentration as nanoparticle dispersion of 0.25 mg/ml. SEM images after deposition of restored dispersion filtered with 0.22 µm membrane, were taken (Figure 7).

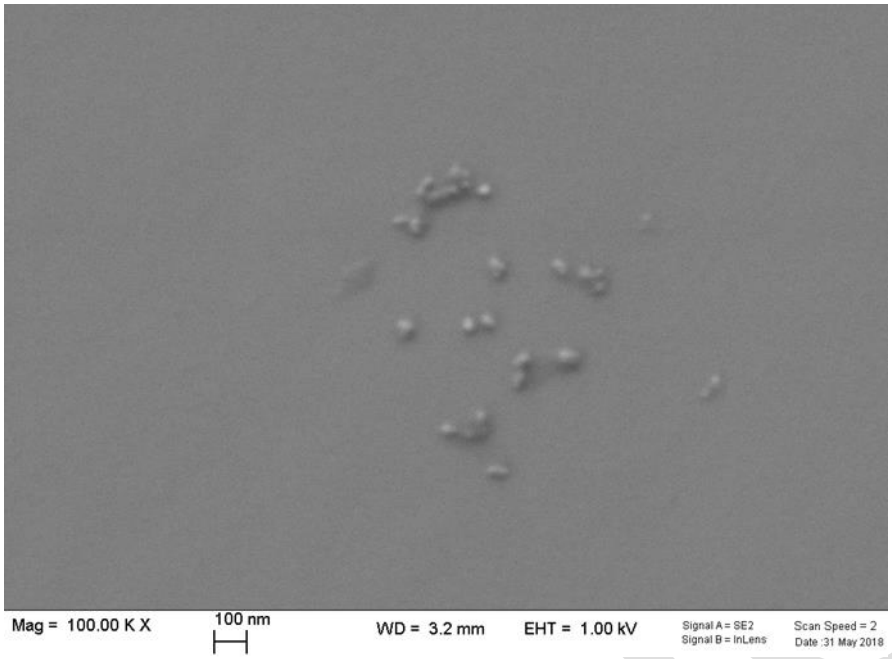


Figure 7. Representative SEM image of nanoparticles restored from PLU/SD-003/170518 (PLU/CaP-002/150518/MP.

CaP-MP show roundish morphology with size in range from 50 to 100 nm.

Finally, the respirability of dpCaP-MPs was determined in the following conditions:

- Fast Screening Impactor
- Capsule HPMC size 3
- 20 mg of powder loaded
- Device: RS01
- Flow rate 60 L/min (pressure drop 4kPa);

The following Table 6 reproduces the emitted dose fraction and the respirable fine fraction of the microparticles loaded with peptide in comparison with the microparticles loaded with small molecular size drug.

The microparticles with drug and the microparticles with peptide exhibited a respirability (fine particle fraction) higher than 60%, in line with the typical powders for inhalation aerodynamic behavior.

Table 6. Representative data of emitted fraction and fine particle fraction of two powder for inhalation with drug and peptide.

Sample	EF %	FPF %
PLU/SD-002/160418(PLU/CaP-003/100418/drug)	70	72
PLU/SD-003/170518 (PLU/CaP-002/150518/MP)	86	65

7. dpCaP functional assessment

Peptide- and drug-loaded dpCaPs were eventually administered to cardiac cells for several in vitro functional assessments such as electrophysiology compatibility, cell vitality, and drug release. Data showed a favorable interaction with the polarized cardiac membrane of cardiac cells and an efficient internalization and release of therapeutic compounds. Effects on the electrophysiological action potential characteristics of cardiac cells were evaluated showing good compatibility. Further deep characterization are currently on going.

8. Conclusions

The multiple preparations performed and the results obtained allow us to confirm that the nanoparticles are chemically and physically compatible with the adjuvant used for their generation and microparticle construction. Mannitol confirmed to be protective for the crucial size properties of the nanoparticles. The most sensitive test for assessing the compatibility is the measurement of particle size distribution and charge after nanoparticles synthesis, in comparison with the nanoparticles obtained from the dissolution of microparticles in water. Size and surface charge are relevant parameters affecting the destiny of this nanomedicine administration.

The size distribution and the respirability obtained with the peptide- and drug-loaded dpCaPs were promising for the final product quality.

Altogether, the obtained results allow us to proceed with the biological characterization and scale up activities. In addition, the future activity will see the manufacturing of additional preparations and relative characterizations devoted to the establishment of the product specifications.