PROJECT ACRONYM

CUPIDO

PROJECT TITLE

Cardio Ultraefficient nanoParticles for Inhalation of Drug products

Deliverable 3.4

In vivo assessment of the studied nanoparticles using different imaging modalities (M30)

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Table of Revisions

REVISION NO.	DATE	WORK PERFORMED	CONTRIBUTOR(S)
1	21/05/2019	*In vivo* assessment of studied NPs	BET
2	14/06/2019	*In vivo* assessment of studied NPs	WP level revision
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6	25/07/2019	Formatting	IN SRL

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Abbreviations

1. BET: Bio-Emission Technology Solutions
2. NSCR: National Centre of Scientific Research
3. ISTEC: Institute of Science and Technology for Ceramic Materials
4. IRGB: Institute of Genetic and Biomedical Research
5. CNR: National Research Council
6. CaP: sample ID ISTEC/CaP-001/050517
7. ^{Fe}CaP: sample ID ISTEC/CaPFe-001/030517/080517
8. NP: Nanoparticle
9. MDP: methyl diphosphonate
10. [^{99m}Tc]Tc-MDP-CaP: CaPs labelled with [^{99m}Tc]Tc-MDP
11. [^{99m}Tc]Tc-MDP-FeCaP: FeCaPs labelled with [^{99m}Tc]Tc-MDP
12. SPECT: Single Photon Emission Tomography
13. Ci: Currie
14. Bq: Becquerel
15. RT: Room Temperature
16. TLC: Thin layer chromatography
17. ROI: Radiochemical incorporation
18. p.p.: post-preparation
19. p.i.: post injection
20. ID: injected dose
21. MPS: Mononuclear phagocyte system
22. MP: mimetic peptide

1. Executive summary

The current deliverable 3.4 (D3.4) outlines the methods followed for understanding the *in vivo* (via imaging and *ex vivo* study) behavior of the different CaP and ^{Fe}CaP candidates and is a continuation of the results extracted from the radiolabeling process and stability tests. Due to the public form of this deliverable, the results of all experiments are partially discussed, data and figures acquired through the imaging studies are not shown as manuscripts are currently in preparation.

Data derived from the handling of CaP and ^{Fe}CaP samples coded PLU/SD-002 -011217, ISTEC-CaP002_230118 (NPs in suspension or Freeze Dried (FD)), BIOEMTECH/CaP-003, ISTEC-^{Fe}CaP001-151117, ISTEC/^{Fe}CaP-001/030517/080517; CITRATE 0.1wt%/090517; Washed/090517 and ISTEC/^{Fe}CaP-001/p.d.030517 CITRATE 0.01wt%/101218; Washed/101218. The intravenous (i.v.) administration route, which constitutes the most common path to deliver pharmaceuticals into the body, was already explored for both types of NPs at D3.3 and serves as a reference result for the experiments carried out at the current deliverable. The intratracheal (i.t.) route, which constitutes the path envisioned by CUPIDO to fast and straightforward deliver pharmaceuticals into the heart through the lungs, was tested for CaP and ^{Fe}CaP samples and are still ongoing for further characterization. The retro-orbital (r.o.) administration route, considered a close pathway to reach the heart through the circulatory system, was additionally explored for the delivery of ^{Fe}CaPs.

D3.4. also presents an upgrade of the imaging platform by BIOMETECH, after the addition of two new tomographic systems – a micro-SPECT and a micro-CT system – allowing a clear visualization and monitoring of internal organ parts and the fully discriminated heart regions. A substantial advantage was gained by this upgrade, as all different regions of the heart can now also be studied for assessing the successful NPs targeting.

This document includes a presentation of the tests conducted and results extracted from the *in vivo* assessment of the different batches mentioned. Experimental activities were performed through 2D and 3D Single Photon Emission Tomography (SPECT) imaging, along with X-Ray and tomographic CT. *Ex vivo* biodistribution experiments were also performed at selected time points and in case of very low uptake of the NPs in specific organs.

The previously validated standarized procedures were now followed for the new optimized batches under study. The *in vivo* imaging allowed to track NP biodistribution, to evaluate possible problems from different NP batches, and to assess magnetic targeting of the ^{Fe}CaP in chosen organs.

Key deliverable achievements:

1. Radiolabelling of CaP002 (sample PLU/SD-002 -011217, ISTEC-CaP002_230118 (NPs in suspension or FD)).
2. *In vivo* assessment through scintigraphy and tomographic imaging of CaP002. (sample PLU/SD-002 -011217, ISTEC-CaP002_230118 (NPs in suspension or FD)).
3. Radiolabelling of CaP003 (sample BIOEMETECH-CaP003).
4. *In vivo* assessment through scintigraphy and tomographic imaging of CaP003. (sample BIOEMETECH-CaP003)
5. *In vivo* assessment through scintigraphy and tomographic imaging of sample ISTEC/ ^{Fe}CaP 001/030517/080517; CITRATE 0.1wt%/090517; Washed/090517 through different administration routes and followed for a time window of 24 hours.

6. *In vivo* assessment through scintigraphy imaging and magnetic targeting of sample ISTEC/ ^{Fe}CaP-001/p.d.030517 CITRATE 0.01wt%/101218; Washed/101218 through intravenous administration route and followed for a time window of 2 hours.

2. Cooperation between participants

BET collaborated closely with CNR-ISTEC, CNR-IRGB, PLU, and CNR-IEIIT-MI for the achievement of this Deliverable, as it can be seen on the following diagram. In more details, the collaboration with CNR-ISTEC was extended on the whole experimental duration with constant sharing of results and analysis of the NPs, aiming to form the most compatible experimental protocols to be followed for the NPs synthesis and purification towards better imaging results. In more details, centrifugation and sonication experiments were carried out in order to establish the best purification protocol for radiolabeling experiments at BET following the input by CNR-ISTEC. For the *in vivo* and *ex vivo* biodistribution assessment, BET results were compared and validated to relevant optical *in vivo* and *ex vivo* results from CNR-IRGB and in collaboration the two partners defined the experiments that should be followed. Moreover, insights on magnets and on how they should be used were provided by CNR-IEIIT-MI.

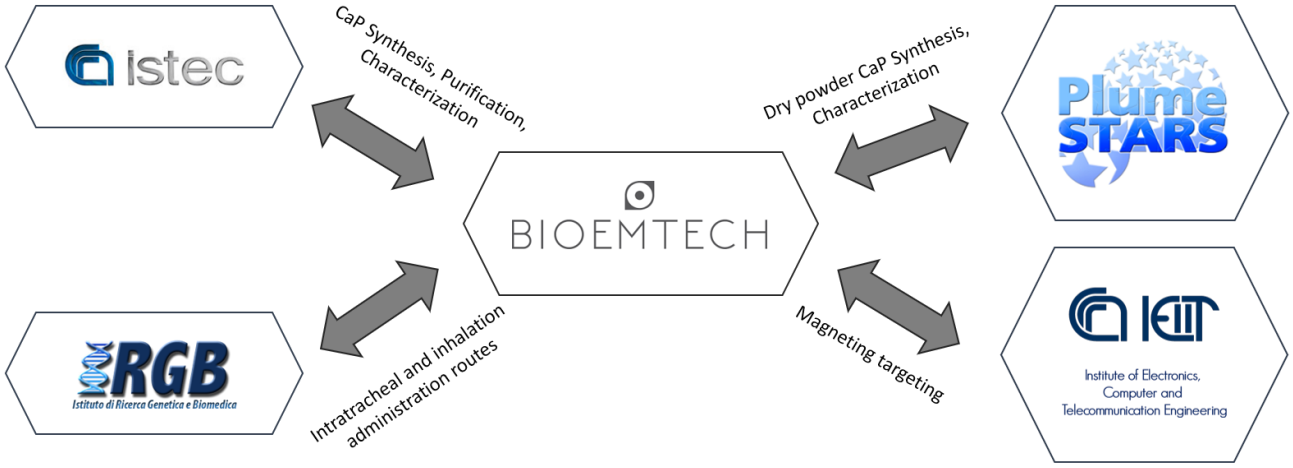

Figure 1: Schematic representation of the cooperation between participants of CUPIDO consortium to acquire this deliverable.

3. *In vivo* assessment of studied nanoparticles (Core report)

3.1. Methods

3.1.1. Radiolabeling Process and Stability tests - Protocols followed

The short-lived and single photon γ-emitting metastable isotope of technetium, Tc ([^{99m}Tc]Tc(III)) was used in order to radiolabel CaPs. The protocol used was the same one as described in deliverable 3.3 for both CaP and ^{Fe}CaP nanoparticles. Only small modifications were applied to the protocol, such as different volume ratio between [^{99m}Tc]Tc-MDP and CaP suspension, purification procedures… aiming a higher labelling of the radioisotope to the NP surface. The labelling was applied to Cap002, CaP003 and ^{Fe}CaP001.

Quality control of the [^{99m}Tc]Tc-MDP-NPs was done with paper chromatography Whatman 3MM (GE Healthcare (UK)) using saline as mobile phase. ITLC analysis was performed on a Scan-RAM radio TLC detector (LabLogic Systems Ltd., (UK)). Chemicals and reagents used were of analytical grade.[1, 2]

3.1.2. Scintigraphy/X-Ray & SPECT/CT imaging

The imaging platform

The imaging studies were performed on a dedicated benchtop mouse-sized gamma camera[3] (γ-eye™ by BIOEMTECH, former BET Solutions), in combination with the X-ray part of a custom-made tri-modal system.[4] The system's field of view is 5 x 10 cm^2, its spatial resolution is 2 mm, and its energy resolution ~25%. The x-ray system consists of an x-ray tube and a CMOS detector, separated by a distance of 30 cm. The minimum pixel size is equal to 0.1 mm and the active area is approximately 12 ×12 cm^2. The combination of the two imaging methods provides a detailed and clear biodistribution and placement of the studied substance throughout time. The radioisotope used for imaging through SPECT (Technicium-99m) allows for a biodistribution monitoring of up to 24 hours.

Along with these imaging systems, already presented in D3.3, the imaging platform was enriched with two new 3D systems: a SPECT and a CT imaging system by Molecubes (Belgium). The upgrade of the imaging platform now allows for a better and clear visualization of the internal part of the organs and for a complete discrimination of the different parts of the heart to be studied.

The micro-SPECT system allows for functional imaging of a variety of radioisotopes, providing a spatial resolution of 0.6 mm for mouse imaging. The micro-CT system allows for anatomical imaging of spatial resolution down to 0.05 mm and for co-registration purposes, when used in combination with SPECT imaging.

Imaging protocol

In vivo studies were performed at the BIOEMTECH laboratories at NCSR Demokritos (Aghia-Paraskevi, Attica Prefecture, Greece), using female normal Swiss-Webster Albino mice (15-25 g) purchased from the Breeding Facilities of the NCSR Demokritos (Permit Number: EL 25 BIO 019, EL 25 BIO 020). The protocol and all the animal procedures were approved by the General Directorate of Veterinary Services (Athens, Attica Prefecture, Greece) and by the Bioethical Committee of the Institution (Permit number: EL 25 BIO 022, EL 25 BIO 042) on the basis of the European Directive 2010/63/EU on the protection of animals used for experimental purposes.

Imaging studies started with planar dynamic imaging on the γ-eye™. Mice were positioned on the animal bed at a <0.5 cm distance from the camera head to allow whole body imaging with maximum spatial resolution and successive 2 min frames were collected for up to 1 h post injection (p.i.). After the first hour, static planar images on the γ-eye™ and static tomographic images were also acquired on the 3D micro-SPECT and micro-CT systems at 1, 2, 4, and 24 h p.i.. This imaging protocol provided both for fast dynamic imaging during the first hour, when kinetics are expected to be faster, and for tomographic, static, higher resolution images at predetermined time points to monitor the heart targeting for a one-day window (up to 24 hours p.i.). Any

potential loss of free technetium-99m from NPs might result with an accumulation in bones, which could occur also from the presence in bone marrow of macrophage entrapping the labelled-NPs.

For i.v. imaging studies, 150 µL of 3.6 MBq of samples were administered to normal mice through the tail-vein injection or 150 µL of 3.6 MBq were administered to normal mice through the retro-orbital injection. Anesthetization was achieved by isoflurane inhalation.

For i.t. studies, due to the closing down of the unique provider of the mouse microSprayer® (Penn-century) in 2015, BET did not have the possibility till now to conduct optimal administration of NP formulations via nebulization like the one used in the fluorescence imaging studies by CNR-IRGB (D3.3 and Miragoli et al., Science Translational Medicine 2018). For these reasons and as an alternative, dosing by liquid instillation have been attempted (data analysis in progress). Fortunately, a new provider was recently identified rendering possible the direct comparison of the instillation process and the previously used intra-tracheal nebulization. Data will be presented and discussed in the future reports

3.1.3. Biodistribution

For Cap002 and FeCaPs distribution studies, labelling was monitored only by *in vivo* imaging.

For CaP003-treated animals and after in vivo imaging, ex vivo biodistribution studies were also performed on the same group of mice, in order to quantify with greater accuracy, the low uptakes to the heart. For any tissue, when in vivo imaging uptake values are close or below 1% (percentage of the administered substance reaching a certain organ), imaging operates close to its detection limits and for these reasons, ex vivo bio-distributions can provide greater accuracy in these ranges of uptake.

Animals were sacrificed at predetermined time intervals from 10 min to 24 h p.i. and main organs or tissues were removed, weighed and counted, together with blood samples, muscle and urine, in a γ-counter system (Cobra II from Packard, Canberra). In comparison to a standard of the injected solution, results were expressed as a percentage of the injected dose (%ID) per organ. For total blood radioactivity calculation, blood is assumed to be 7% of the total body weight.

3.1.4. Magnetic Resonance Imaging

C57BL/6 healthy male mice were injected intravenously with ^{Fe}CaPs at 0.0025 mM of Fe in a 5 wt% glucose solution, to obtain an equivalent dose of 1 mg kg^{-1} of iron per mouse. MRI images were collected at the following time points: pre-injection, and then post-injection at 10 min and 24 h. The signal intensities were measured from a region of interest of 0.10 cm^2 for the liver, 0.07 cm^2 for the spleen and 0.17 cm^2 for the renal cortex (data not shown here please refer to our last publication Adamiano et al., Acta Biomaterialia 2018).

3.2. Results and discussion

3.2.1. Radiolabeling Protocols

The labelling protocol of either of CaP or of ^{Fe}CaP with [^{99m}Tc]Tc-MDP is based on the chemical absorption of the bi-phosphonate arm of the radioligand (MDP) onto the calcium sites found at NPs' surface. The two-strip ITLC-SG method for the analysis that was carried out after the purification of the radiolabelled NPs of [^{99m}Tc]Tc-MDP showed that no amount of either pertechnetate ([^{99m}Tc]TcO_4-) or of colloidal ^{99m}Tc ([^{99m}Tc]TcO_2) was present as given in **Fig. 1 (A.i)** and **(A.ii)**, respectively.

CaP

CaP-002 were labelled following a protocol slightly modified from the one described in D3.2, resulting in the radiochemical incorporation (ROI) of the crude [^{99m}Tc]Tc-MDP-CaP to be 70 %, providing a single radioactive species, based on the amount of activity detected by paper chromatography (Whatman 3MM).

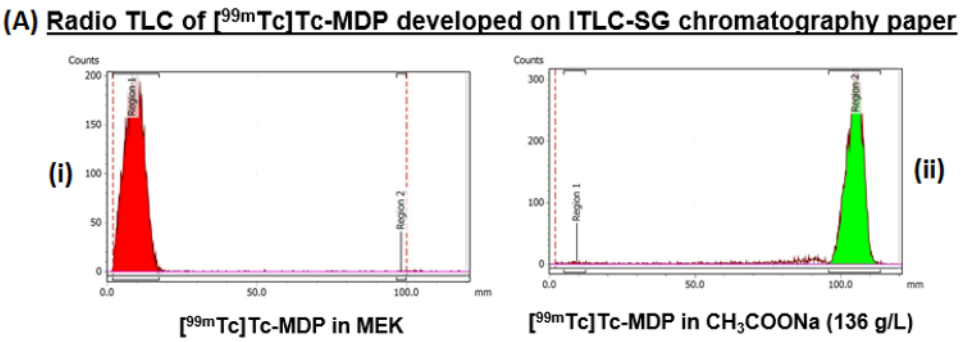

Figure 2: (A) Radio TLC of [^{99m}Tc]Tc-MDP developed on ITLC-SG chromatography paper with **(i)** methyl-ethyl-ketone (MEK) and **(ii)** ethyl-acetate 136 g/L (CH_3COONa) as mobile phases. **(B)** Radio TLC of the crude **(i)** and purified **(ii)** radiolabelled CaP following centrifugation (twice), developed both on Whatman 3MM with saline (0.9 % v/v) as the mobile phase. Sample: PLU/SD-002 -011217.

Not expectedly, CaP003 were proven to be quite unstable over a long time, causing the synthesis of NPs to be carried out within a time frame of two weeks prior to their *in vivo* use. To do so, the CaP003 were synthesized in BIOEMTECH laboratories according to CNR-ISTEC protocol. In close collaboration with CNR-ISTEC, a new purification procedure and labelling protocol were established for CaP003 in order to increase the incorporation of the radioisotope to the NPs and to ensure that the size and physicochemical properties of the CaP003 NPs weren't altered before their use. This new labelling procedure resulted in a high radiochemically incorporated CaP003 NPs (>98%) which could be used without any further purification.

^{Fe}CaPs

^{Fe}CaPs were radiolabeled with [^{99m}Tc]Tc(III) by mixing [^{99m}Tc]Tc-MDP with ^{Fe}CaP solution as described in D3.3. Depending on the sample of ^{Fe}CaP NPs, some small changes have been carried out to ensure over 95% of labelling incorporation of the radioisotope to the NPs surface.

3.2.2. Nuclear Imaging study results

CaPs

[^{99m}Tc]Tc-MDP CaP-002

Scintigraphic/x-ray fused images have been collected at 1, 2, 4 and 24h p.i after CaPs instillation in anaesthetized healthy Swiss mice (data not shown). Ongoing analysis aims to validate the alternative use of the inhaler device versus nebulizer-device. For all experimentations, post-decay and no longer radioactive samples have later been sent to CNR/ISTEC for DLS analysis.

Importantly, a series of repeated analysis have evidenced that centrifugation and freeze dry (a process initially adopted for shipping dry NPs) had a negative impact on NPs, causing their aggregation to a size much larger than the required for *in vivo* experiments. Thus, in collaboration with CNR/ISTEC, the synthesize of NPs started to be performed in Athens, *In vivo* experiments was decided to the executed within a period of two weeks from nanoparticle preparation.

All in all, an optimized protocol was established in collaboration with CNR/ISTEC for purification and radiolabeling of CaP003 NPs. This resulted in [^{99m}Tc]Tc-MDPCaP NPs with a size smaller than 100 nm, suitable for further *in vivo* experiments.

[^{99m}Tc]Tc-MDP CaP-003

An i.v. experiment was performed to compared CaP-003 to previous NP protocols (D3.3.). Data confirmed that the batches follow a very similar distribution *in vivo*. No accumulations in bones were found.

^{Fe}CaPs

Results of the SPECT/CT imaging studies of ^{Fe}CaPs in anaesthetized healthy Swiss mice, at 1 hr, 4 hrs, and 24 hrs p.i. were acquired. The resulting images denoted that, depending on the administration route (either i.v. or i.p.), the biodistribution is affected. Ongoing studies will contribute to better interpret the imaging results and assess the effect when in the presence of an external magnet.

3.2.3. Magnetic Resonance Imaging study results

Preliminary *in vivo* experiments of magnetic resonance evaluation with ^{Fe}CaPs were performed assessing their contrast enhancement at different organs and compared to the one of Endorem® (FDA approved SPION) aiming to demonstrate their potential of ^{Fe}CaPs as multimodal SPECT/MRI imaging agents. Indeed, the obtained images depict an enhanced negative contrast of ^{Fe}CaPs compared to Endorem®, which enables the reduction of the iron amount required for effective contrast enhancement (for more details please refer to our last publication Adamiano et al., Acta Biomaterialia 2018).

3.2.4.Myocardial cell distribution of inhaled CaPs – fluorescence studies

To determine the intra-myocardial uptake and cell distribution after inhalation (via nebulizer) of CaPs, IRGB-CNR is currently applying imaging flow cytometry quantification on cell populations (i.e. cardiomyocytes as well as smooth muscle cells, endothelial cells, fibroblasts, immune cells) isolated from the heart of animals treated with fluorophore-loaded CaPs.

3.2.5.Pulmonary cell distribution

This paragraph is a short update from D3.2 (*in vitro* assessment of nanoparticle behavior at/across the pulmonary barrier) and it is meant to provide further evidence in support of the *in vivo* test by underlying the process of nanoparticle translocation at the pulmonary levels.

TT1 cells uptake and internalization studies after ^{Fe}CaPs 24h exposure.

TT1 cells possible uptake of ^{Fe}CaPs nanoparticles was studied by light microscopy imaging. Prussian Blue, a stain commonly used for detecting ferric iron in tissues, was used for the detection and localization of increasing concentrations of ^{Fe}CaPs at different time points of exposure. Results revealed an increasing number (and blue colour intensity) of iron aggregates associated with TT1 cells, suggesting possible cell uptake for ^{Fe}CaPs and that this internalization was both time- and concentration-dependent. These preliminary results will be confirmed by 3D confocal microscopy analysis.

TT1 cells uptake and internalization studies after CaP-RIS/AF647 NPs 24h exposure

A complete 3D-confocal microscopy analysis was performed by IMP to reveal the possible uptake and degree of internalisation of the new fluorescent CaP nanoparticles (CaP-RIS/AF647) synthetized and provided by CNR-IRGB (end of June 2019). Two concentrations, 10 and 25 µg/ml, were analysed and 3D Z-stack images were taken along the entire height of the sample. The 3D-imaging analysis of TT1 cell monolayers exposed to CaP-RIS/AF647NPs revealed a good fluorescence signal associated to the nanoparticles and proved an actual intracytoplasmatic accumulation after 24h exposure. After 24h exposure to 10 µg /ml, confocal analysis showed distinctively the presence of fluorescent nanoparticles clusters within the cytoplasm of TT1 cells monolayer. However, not all the cells analysed showed NP internalisation. Further studies aim to quantify the amount of CaP-RIS/AF647 uptake and translocation by alveolar epithelial cells.

4. Conclusions

The labeling methods that were standardized and validated at the previous deliverable were used during this period to characterize and test *in vivo* the heart targeting of several NP candidates through different administration routes. The upgraded imaging platform was also exploited to produce 3D heart images, monitoring the accumulation of each substance in the different heart parts.

Different batches of CaP nanoparticles were successfully studied, regarding their radiolabeling, stability and *in vivo* behavior for a time window of 24 hours. The results showed that pilot samples present expected good radiolabeling properties and provide workable imaging results if tissue uptake >1% of the dose. Distribution can be fine-tuned then by *ex vivo* organ quantification. The current ongoing studies, completed soon with sets of data obtained with newly available nebulizer, are aiming to qualify NP fractions which crosses the gas-to-blood barrier and distribute among organs.

The *in vivo* behavior of ^{Fe}CaP NPs administered through different routes and their potentials for magnetic guidance are under exploration. ^{Fe}CaPs were qualified for their potential as multimodal imaging agents (SPECT/MR).

5. References

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