



## Inhalation of peptide-loaded nanoparticles improves heart failure

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### Abstract

Peptides are highly selective and efficacious for the treatment of cardiovascular and other diseases. However, it is currently not possible to administer peptides for cardiac-targeting therapy via a non-invasive procedure, thus representing scientific and technological challenges. Here we demonstrate that inhalation of small (<50 nm in diameter) biocompatible and biodegradable calcium phosphate nanoparticles (CaPs) allows for rapid translocation of CaPs from the pulmonary tree to the blood stream and to the myocardium, where their cargo is quickly released. Treatment of a rodent model of diabetic cardiomyopathy by inhalation of CaPs loaded with a therapeutic mimetic peptide (MP) that we previously demonstrated to improve myocardial contraction resulted in restoration of cardiac function. Translation to a porcine large animal model provided evidence that inhalation of a peptide-loaded CaP-formulation is an effective method for targeted administration to the heart.

Altogether, these results demonstrate that inhalation of biocompatible tailored peptide nanocarriers represents a pioneering approach for the pharmacological treatment of heart failure.

**One Sentence Summary:** Inhalation delivers drug-loaded nanoparticles to the heart, improving cardiac function in murine and porcine models.

## INTRODUCTION

The application of nanotechnology to medicine (nanomedicine) is at the forefront of innovation for modern health care and represents a promising approach for efficient delivery of therapeutics (1-3). In line with this, a collection of first-generation nano-products for clinical use have recently been approved by the U.S. Food and Drug Administration, and other liposomal and polymer drug conjugate nanosystems are under clinical and preclinical development (4-7). However, despite remarkable advances in the use of nanoparticles in the cancer field, very few preclinical tests have been reported for nanomedicine applied to the treatment of cardiovascular diseases (CVDs) (8-10) that, claiming 17.5 million lives a year and accounting for an estimated 31% of all deaths globally, stand as the leading cause of death worldwide (11). The identification of innovative therapeutic nano-formulations, use of alternative administrations routes, or a combination of both may help to overcome the limitations associated with current pharmacological treatments for CVDs and lead to more specific and efficient therapies.

Drug administration for the treatment of myocardial disease currently includes either oral or needle-based routes (12). However, enteral resorption is unreliable during acute or chronic gastrointestinal congestion, whereas injection, either intravenous, subcutaneous, or intramuscular, is associated with patient discomfort and malcompliance, rendering these administrations suboptimal for chronic treatment. Moreover, home or ambulatory care is frequently required for injection procedures. To the best of our knowledge, no study has addressed the possibility of using nanoparticles that can treat cardiac conditions when inhaled. In fact, although inhalation delivery is a common procedure for the treatment of respiratory problems, no pharmacological therapies are, as far as we know, currently adopting an inhalation approach for the delivery of therapeutics via nanoparticles to the heart. Furthermore, despite of an increasing interest in peptide therapeutics in pharmaceutical research and development, no effective, non-invasive, peptide-based treatment of cardiac dysfunction has been successfully developed so far, leaving the parental route (injection) the only currently available option (13, 14).

Here we show that inhalation therapy is effective for delivering nanoparticle-based therapeutic peptide to the diseased heart. The approach is based on our previously described biocompatible and biodegradable, negatively charged calcium phosphate nanoparticles (CaPs) that, via a biomineralization-inspired strategy, are produced with a diameter ranging from 20 to 50 nm and are able to deliver bioactive molecules to cardiac cells (15). Without promoting toxicity or interfering with any functional properties of cardiomyocytes, CaPs were shown in vitro to successfully cross the cardiomyocyte cellular membrane and release bioactive molecules (microRNAs) inside the cell (15). We thus sought to assess whether the cardiac targeting of CaPs could be applied for the delivery of therapeutic peptides via the respiratory tree through inhalation (Fig. 1a). The rationale for the use of this unconventional administration route for targeting of the heart is based on the concepts that *i*) during respiration, the oxygenated blood moves from the pulmonary circulation first to the heart via the pulmonary vein, and *ii*) combustion-derived nanoparticles and ultra-fine particulates inhaled through polluted air were recently shown by us (16) and others (17) to be present in the heart and causally associated with cardiac arrhythmia and dysfunction, suggesting that inhaled nanoparticles are deposited in the heart. Furthermore, CaPs can protect peptides from immediate enzymatic degradation and due to their negative surface charge provide cellular permeability via the membrane internalization (15, 18). Thus, we predicted that inhaled small-sized CaPs would be able to cross the alveolar-capillary barrier in the lung and rapidly translocate to the myocardium for effective intracellular release of therapeutic peptides.

## **RESULTS**

### **Characterization of inhalable CaP nanoparticles**

Prior to in vivo administration, CaPs were subjected to a set of in vitro evaluation procedures. First, long-term stability in aqueous suspension was assessed by measurement of CaP surface charge and Z-average as a function of time as well as CaP interaction with proteins. No changes were found in the surface charge, which remained stable ( $-32 \pm 3$  mV) for the whole time of investigation, while the

initial Z-average value of about 125 nm, was found to decrease to about 80 nm after 2 days of storage at 4°C (fig. S1a). Thereafter, Z-average value remained stable with only minor variations ( $\pm 10$  nm) for up to 8 days, which was the time limit for our investigation. To evaluate the potential interaction of proteins with CaPs, nanoparticles were analyzed in aqueous suspension enriched with 10% (v/v) FBS (fig. S1b). Z-average decreased from about 80 nm (in pure water) to about 32 nm, indicating that serum proteins interacting with the nanoparticles increased the dispersion of CaPs. Thereafter, a gradual increase in Z-average was registered, reaching a value of about 280 nm after 7 days. At this later time point, an increment of the Z-potential from -30 to -10 mV corroborated the expected surface interaction of CaPs with proteins. Altogether, these data show that no substantial size alterations occur within the first hours suggesting that an *in vivo* administration featuring a fast targeting of the heart might not face any alteration of CaP properties.

In a second step, near-infrared fluorophore-loaded CaPs (CaP-Cy7) were generated for *in vivo* biodistribution analyses and the effective encapsulation of the fluorophore in the CaPs as well as the stability of CaP-Cy7 were demonstrated (fig. S1c). To determine the effective incorporation of Cy7 into CaPs, comparable amounts of Cy7 were either encapsulated into (CaP-Cy7) or adsorbed post-synthesis to CaPs, and the amount of CaP-associated Cy7 was measured, revealing an amount of  $1.40 \pm 0.13$  % (w/w) encapsulated CaP-Cy7 vs.  $0.85 \pm 0.08$  % (w/w) CaP adsorbed post-synthesis. The analysis of the surface charge of CaP-Cy7 at pH 7.4 revealed the presence of a single population with surface charge of about -20 mV, while CaPs with the fluorophore adsorbed on their surface showed a double population with surface charge of about -34 mV and -6 mV attributed to naked CaPs and fluorophore-coated CaPs (fig. S1c). These data support the conclusion that when included during the synthesis of nanoparticles, Cy7 is effectively encapsulated within the CaPs and not attached to the surface. In addition, CaP-Cy7 did not show any release of fluorophore (limit of detection of 1  $\mu$ g/ml) against an infinite sink in dialysis as well as in aqueous suspension enriched with 10% (v/v) FBS.

### **Inhalation delivers CaPs to the heart**

Following CaP generation and characterization, we assessed the possibility of heart targeting by nanoparticle administration. CaP-Cy7 were delivered to healthy mice and fluorescence measurements were performed to compare distinct administration routes, which, in addition to conventional ways [oral (gavage), intraperitoneal (ip), and intravenous (iv)], included nebulization of CaP-Cy7 via the respiratory route. Whereas enteral administration (gavage) did not result in statistically significant ( $P>0.05$ ) targeting to the heart at 40 min post administration, parenteral administration (ip, iv, and inhalation) resulted in rapid delivery of CaP-Cy7 to the myocardium, with inhalation being the most efficient delivery method (Fig. 1b, c and fig. S2a-d). Notably, the time course of CaP-Cy7 myocardial accumulation was paralleled by a gradual reduction in signal from the lungs as a function of time, suggesting a continuous passage of nanoparticles across the pulmonary barrier (Fig. 1c). Similar results were obtained via an optical in vivo imaging approach, which allowed us to monitor the behavior of inhaled CaP-Cy7 and their gradual accumulation in the mediastinum, the thoracic cavity containing the heart, esophagus, and trachea (Fig. 1d and fig. S2b). A delayed clearance of inhaled CaP-Cy7 was observed compared to Cy7 alone (Fig. 1d and fig. S2e), which was supported by 3D Fluorescence Molecular Tomography (FMT) imaging of the cardio-pulmonary area from mice 60 min after inhalation, revealing marked localization of CaP-Cy7 in the myocardium compared to Cy7 alone (Fig. 1e and movie S1,2). Effective delivery of CaP-Cy7 to the myocardium was further confirmed by fluorescence analyses on explanted hearts (Fig. 1f), whereas no specific cardiac targeting was found of Cy7 alone, which rapidly accumulated in the bladder after rapid and broad diffusion in the lungs and in the rest of the body (Fig. 1d-f). In addition, no CaP-Cy7s were found to cross the blood-brain barrier (Fig. 1d and in fig. S2c). Targeting of CaPs to the myocardium was also confirmed by transmission electron microscopy (TEM) (fig. S2f,g). Notably, in contrast to the toxic outcome associated with inhalation of ultrafine particulates from polluted air and other inorganic nanoparticles such as TiO<sub>2</sub>, SiO<sub>2</sub>, and Co<sub>3</sub>O<sub>4</sub> nanoparticles (19-22), pulmonary CaP administration via single or multiple once-a-day administration protocols did not affect cardiac function as measured by echocardiography (fig. S3 and table S1). In rats, no alterations in cardiac excitability and

refractoriness were detected as shown by the absence of arrhythmogenic events evaluated by in vivo epicardial multiple lead recordings (table S2). Furthermore, no immunological effects were observed, as indicated by no detectable changes in RNA expression of CD11, IL-1, TNF, IL-6, IL-4, and IL-5. In agreement with our recent work (15), this evidence supports the concept that biomimetic CaPs, closely resembling the inorganic phase of bone and teeth (23), are recognized by the body as close to endogenous material, thus being biocompatible and well tolerated in vivo.

### **Efficacy of CaPs to load therapeutic peptides**

Based on our demonstration that CaP inhalation allows nanoparticles to cross the pulmonary barrier and reach the intracellular compartment within the heart, our next objective was to explore the use of peptide-loaded CaPs for cardiac treatment in vivo. We recently demonstrated the therapeutic effect of a cell-penetrating mimetic peptide (R7W-MP) that by targeting the Ca<sub>v</sub>β2 cytosolic subunit of the L-type calcium channel (LTCC) improves cardiac contractility in pathological heart conditions associated with alterations of LTCC levels and function (diabetic cardiomyopathy) via restoration of LTCC density at the plasma membrane (24). Here, we envisioned to test the therapeutic use of CaPs for peptide delivery for the treatment of a cardiac condition via inhalation of CaPs loaded with R7W-free mimetic peptide (CaP-MP). In fact, due to the absence of the cell-internalizing R7W sequence on the MP, its efficiency in rescuing cardiac contractility would require the CaPs to mediate its crossing of the pulmonary barrier and subsequent myocardial cell internalization.

In our previous study (15), CaPs were functionalized with different quantities of microRNA, revealing a slight increase in CaP size as a function of microRNA amount, while comparable values to those of microRNA-free CaPs were obtained for the surface charge and morphology. Here, we performed a similar evaluation and characterized the effect of MP and hemagglutinin (HA) scramble peptides, on size and surface of loaded CaPs. Notably, since our approach is based on a universal nature inspired synthetic strategy, we found that the incorporation mechanism for peptides into CaPs was similar to the one previously observed for microRNAs. This mechanism consists of a first step

of interaction in which, within a basic reaction environment (pH 8.5), the  $\text{Ca}^{2+}$  and/or  $\text{PO}_4^{3-}$  ions interact via a strong electrostatic interaction with the chemical groups (i.e. carboxylate, amine, and sulfonic groups, etc.) of the analyzed biomolecules. While in the case of Cy7, mainly sulfonic and amine groups are likely to be involved in the interaction, the negative charge of MP and HA peptides, which at the pH of the reaction have a calculated isoelectric point of 7.06 (25), generally interacts with  $\text{Ca}^{2+}$  ions. Afterwards, the reaction of  $\text{PO}_4^{3-}$  with  $\text{Ca}^{2+}$  ions or *vice versa* triggers the nucleation of particles and their growth, resulting in the mineralization of biomolecules (i.e. creation of a matrix of mineral phase surrounding the biomolecule-rich nucleus). During this process, citrate stabilizes the nanoparticles and modulates their growth through its binding on the surface at the early stage of crystallization as previously reported (15).

In line with our previous results obtained with microRNA (15), immediately post dialysis higher Z-average, but similar surface charge, was obtained for functionalized CaPs compared to peptide-free CaPs. CaP-MP and CaP-HA showed comparable values of surface charges ( $-31 \pm 2$  mV and  $-32 \pm 1$  mV), respectively, indicating that their surface is homogeneously covered with citrate. Z-averages for the functionalized CaP-MP and CaP-HA were  $210 \pm 6$  nm and  $220 \pm 6$  nm, respectively, where the effective interaction of peptides with CaPs might be responsible for the slight difference in size in respect to peptide-free CaPs (i.e. 125 nm). Also in this case, the Z-average of CaP-HA and CaP-MP immediately after dialysis decreased as a function of time, passing from about 200 nm to 90 nm after 2 days (fig. S1a). This value remained stable with small variation ( $\pm 10$  nm) for up to 8 days, which was the time limit for our investigation. Consistently, also in this case the surface charge of the CaP-peptides ( $-31 \pm 3$  mV) did not change as a function of time. Transmission electron microscopy (TEM) images of CaP-MPs and CaP-HAs (fig. S4a) collected immediately after dialysis showed round shaped particles of approximately 20 - 50 nm in diameter. The selected area electron diffraction (SAED) patterns collected from the same CaPs (fig. S4a) demonstrated their amorphous nature due to the presence of diffuse rings rather than spots. In addition, energy dispersive X-ray spectrometry (EDS) spectra (fig. S4a) revealed that both CaP-MP and CaP-HA are mainly composed



of calcium and phosphate. All these features are comparable with our previously prepared CaPs (15) and we therefore assumed that the functionalized CaPs synthesized in this work have similar behavior towards cardiomyocytes and cardiac tissue.

To evaluate peptide loading, the total MP in CaP-MP preparations was quantified. To distinguish the amount of CaP-loaded MP from the possible CaP-free MP present in the preparations, peptide quantification was performed both from pellet (CaP-MP) and supernatant (CaP-free MP) fractions obtained by centrifugation. Finally, acidic treatment was performed to completely dissolve CaP-MPs from both fractions, thereby facilitating the total release and effective quantification of total MP. The same approach was used for both the pre- and post-dialysis preparations and data was compared to the theoretical maximum loading. As shown in Table S3, MP loading from the pellet fraction (corresponding to the CaP-loaded MP) was comparable to the theoretical value, leading to the conclusion that CaP provides high loading capacity, keeping the amount of unloaded peptide (CaP-free MP) at a minimum. To evaluate MP stability within nanoparticles, peptide quantification was performed on CaP-MP aliquots stored at different amounts of time at room temperature using the approach described above. As shown in fig. S4b, no reduction in MP loading was found till 10 hours of storage at room temperature, while a slight decrease was observed at 24 hours of storage, supporting the conclusion that CaP-MPs stably maintain their payload in an encapsulated state. Thereafter, following a time-dependent response to CaP dissolution under acidic conditions, we evaluated release kinetics of encapsulated peptide from nanoparticles. As shown in fig. S4c, a gradual release of MP from CaP-MPs was obtained, showing an inverse relationship between the reduction in the pellet fraction (CaP-loaded MP) and an increase in the supernatant fraction (CaP-free MP). These data are in line with results from Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) analyses (fig. S4d) that, by measuring the cumulating  $\text{Ca}^{2+}$  ion release under physiological (pH 7.4) and slightly acidic (pH 5.5) condition, provide degradation rate of CaPs and peptide-loaded CaPs as a function of time. Degradation and consequently peptide release is triggered by a drop in

pH. Altogether, these data provide the evidence that CaPs possess high loading capacity, keeping the amount of unloaded peptide (CaP-free MP) at a minimum.

### **Efficacy of inhaled CaPs loaded with a therapeutic mimetic peptide to improve myocardial contraction**

To test the therapeutic use of CaP-MPs for the treatment of a cardiac condition, we treated a mouse model of streptozotocin (STZ)-induced diabetic cardiomyopathy via inhalation of CaPs loaded with R7W-free therapeutic peptide. Consistent with the ability of CaPs to mediate cardiomyocyte internalization (15), stimulated emission depletion (STED) 3D z-stack microscopy of isolated adult cardiomyocytes from mice subjected to inhalation of MP-Rhodamine-loaded CaPs showed effective targeting of MP to cardiomyocytes, accounting for  $124 \pm 52$  fluorescent signals (corresponding to the internalized MP-Rhodamine-loaded CaPs) per single cell (Fig. 2, movie S3, and fig. S5). In agreement with this, CaP-MP inhalation treatment of diabetic mice (Fig. 3a and fig. S6a,b) led to a complete recovery of cardiac function as shown by echocardiographic assessment of left ventricular function (Fig. 3b and table S4). Improvement of molecular and cellular defects associated with diabetic cardiomyopathy was achieved as a result of the direct targeting of MP to LTCC (24), including restoration of LTCC protein levels (Fig. 3c and fig. S6c) as well as recovery of LTCC currents (Fig. 3d, fig. S6d, and Table S5) and LTCC-related contractile properties (Fig. 3e) as determined in isolated cardiomyocytes from CaP-MP-treated mice. In contrast, as expected, no therapeutic effects were obtained by administration of CaP-HA or unloaded (free) MP (Fig. 3b-e, fig. S6d and table S4). In addition, no induction of oxidative stress was observed (table S6). Altogether, these data provide evidence that inhalation of MP-loaded CaPs is an effective approach for delivery of therapeutic peptides to the heart (Fig. 3f) and demonstrate the benefit of CaP-MP inhalation in recovering LTCC-associated cardiac dysfunctions.

## **Effectiveness of peptide-loaded CaP-formulation for targeted administration to the heart in a porcine model**

Finally, to advance towards clinical research in humans and generate the first proof-of-concept in large animals, feasibility of cardiac targeting via our nanoparticle-based inhalation approach was assessed by nebulization of CaP-HA in anaesthetized healthy landrace pigs (Fig. 4a). Similar to our results in mice, no changes in heart rate, mean arterial blood pressure, cardiac output ( $9.8 \pm 0.8$  vs.  $9.9 \pm 0.8$  l/min), or systemic and pulmonary vascular resistance were observed 5 hours after a single inhalation of CaP-HA (Fig. 4b). Left ventricular (LV) function, as assessed by hemodynamic measurements of pressure-time derived parameters (LV maximum pressure, maximum and minimum LV dP/dt) and pressure-volume analyses (end-diastolic and end-systolic pressure-volume relationships) did not change (Fig. 4c). No arrhythmia occurred, and surface electrocardiography (ECG) derived PQ ( $133 \pm 6$  vs.  $135 \pm 5$  msec) and QT intervals ( $349 \pm 12$  vs.  $345 \pm 19$  msec) as well as QRS duration ( $56 \pm 5$  vs.  $51 \pm 5$  msec) remained constant. Finally, respiratory function (end-tidal carbon dioxide partial pressure and arterial oxygen saturation) remained stable at constant mechanical respiration. In a separate group of open-chest pigs, LV free wall biopsies from the beating heart were taken at baseline and sequentially after treatment. 300 min post inhalation, a marked HA signal was detected in the myocardium, providing evidence for effective delivery of CaP-HA to the heart (Fig. 4d). These data were supported by Western blot (Fig. 4e) and confocal microscopy (Fig. 4f) analyses of cardiac tissue. Altogether, these data confirm the efficiency of our CaP inhalation approach for intra-myocardial delivery of compounds in a large animal.

## **DISCUSSION**

Here, we provide the proof-of-concept for an innovative and unconventional nanotechnological inhalation approach for cardiac delivery of peptides for the treatment of pathological heart conditions. Inhalation *per se* has long been established for the treatment of pulmonary diseases (26, 27), but its

use for targeting of the heart and management of cardiac failing conditions has not previously been explored. We demonstrated that inhalation of our recently developed bioresorbable and negatively charged CaP nanocarriers (15) allows for effective targeting to and treatment of the heart, resulting in higher, faster, and more selective cardiac accumulation of CaPs compared to other delivery routes, such as gavage, ip or iv. The delivery of drugs through CaP-facilitated inhalation is thus likely to reduce the amount of required therapeutic compound, which together with more cardiac-specific delivery may minimize side effects compared to systemic delivery. Reduced first-pass metabolism may explain the more efficient delivery of CaPs to the heart by inhalation.

Although the feasibility for a novel therapeutic application is envisaged, we acknowledge that some limitations are still present. For instance, pathological conditions affecting the lungs such as chronic obstructive pulmonary disease might affect CaP persistence and their translocation toward the pulmonary bloodstream, thereby limiting the efficiency of the targeted administration to the heart. Additionally, the mechanistic events by which CaPs interact with and cross through the pulmonary barrier are still unknown and further efforts to understand these processes are necessary. CaPs might be subjected to physical modifications following in vivo administration. However, it is important to remark that no substantial size alterations occurred within the time required for CaPs to target the heart after in vivo inhalation, which is within the first hours (fig S1b). Last, despite our encouraging results, chronic administration and pharmacokinetic studies will be required to investigate the safety of this new delivery approach.

In conclusion, this study identifies a novel approach currently not available for targeting of the heart and may open up new avenues of investigation and potential uses of nanomaterials for the treatment of CVD with therapeutic peptides. Inhalation therapy is easily administered and free from any physical or emotional burden associated with injection or other minimally-invasive procedures, thus facilitating patient compliance, convenience, and home-based chronic treatment. A broader use

of CaPs for carrying other drug/diagnostic compounds with different release kinetics (therapeutic polypharmacy) or even with different tissue targeting can be envisaged.

## **Materials and Methods**

### **Study design**

The overall hypothesis is to target the diseased heart via inhaled nanoparticles carrying therapeutic peptides. Herein we designed a study centered to 1) synthesize a proper CaP-based MP carriers, 2) test the effectiveness of CaP inhalation in small (mice), medium (rats) and large (pigs) animals, and 3) treat a typical diabetes-induced cardiomyopathy via inhalation of CaP-MPs. In the setting for functional, histological, molecular evaluations, and cardiac activity monitoring, the number of animals per each individual group were minimized to 6-11 mice, 11 rats, and 9 pigs. For the therapeutic application, the sample size of diabetes-induced heart failure model was determined based on previous experience (24) and taking into account the mortality rate. The study, which included 10 mice per each individual group, was blinded and randomized. To ensure comparable degrees of cardiac dysfunction, non-invasive approaches such as echocardiography or ECG was assessed at each experimental time point. To ensure that neither the nanocarrier or therapeutic peptide alone were responsible for the improved cardiac phenotype, we included mice inhaled with CaP-HA and MP, respectively. The large animal experiments were designed to achieve a proof-of-concept of the feasibility of our new nanocarrier-mediated approach using a small number of animals (9 in total). The statistical analysis is therefore primarily descriptive in nature. The first series of animals was aimed at testing inhaled CaPs' ability to enrich the heart at a dose derived from previous experiments in rodents. After collecting evidence of heart-enrichment via Western blotting, a separate set of animals was used to investigate the hemodynamic and electrophysiological effect of the carrier *per se*. Numbers were determined by the investigator according to previous experimental experience. All experimental procedures were approved by the Institutional Animal Care and Use Committee (Permit

N° 920/2015-PR, 746/2017-PR, 59/2012, and G0063/16) and conducted in accordance with the guidelines of the National councils where experiments were performed.

**Nanoparticle preparation.** CaPs were generated according to our previous work (15) through a biomineralization-inspired strategy consisting of mixing (1:1 v/v, 20 mL total) two aqueous solutions of (a) CaCl<sub>2</sub> (100 mM) + Na<sub>3</sub>Cit (400 mM) and (b) Na<sub>2</sub>HPO<sub>4</sub> (120 mM) for 5 min at 37 °C. pH was adjusted to 8.5 by adding 0.1M NaOH (0.1M). The mixed solution was kept in a water bath at 37 °C for 5 min. For conjugation with the fluorophore, Sulfo-Cyanine7 amine (Cy7, Lumiprobe GmbH) (125 µg/ml) was added, while for drug conjugation, hemagglutinin (HA), or mimetic peptide (MP) (500 µg/ml) (HA: YPYDVPDYA; MP: DQRPDREAPRS. Genescript) was added. To remove unreacted reagents, the CaP suspension was dialyzed for 6 hours across a cellulose dialysis membrane with a cut-off of 3,500 Da and immersed in 400 mL Milli-Q water followed by multiple water exchanges. The suspension was recovered and stored at 4 °C until further use. The final concentration of CaP suspensions was 600 µg/ml in all cases. The final concentrations of Cy7 and peptides within the CaP suspension were 75 µg/ml and 150 µg/ml, respectively. Surface decoration of CaPs with Cy7 was also tested by adding an aqueous solution of Cy7 to the CaP suspension (final concentration of 15 µg/ml similar to the amount effectively encapsulated). The suspension was maintained overnight under shaking at 37°C. After that, the un-adsorbed fluorophore was removed by extensively washings by centrifugation.

**Nanoparticles characterization.** The amount of CaPs after dialysis was evaluated by freeze-drying the sample suspensions (5 aliquots of 1 ml) and weighting the solid content. The amount of HA and MP loaded on CaPs was detected using the DC protein Assay (Bio-Rad) according to the manufacturer's instructions. The amount of Cy7 loaded on CaPs was measured using a UV-Vis spectrophotometer (Nanodrop One, Thermo Scientific, USA) ( $\lambda=750$  nm;  $\epsilon_{\text{cy7}} = 240600$  1/M ·cm). The concentration of Cy7 was calculated as the difference between the concentration of the initial

solution and that of the supernatant after sample centrifugation at 5000 rpm for 15 min. Size distribution and surface charge of unloaded and loaded CaPs were evaluated by DLS (Zetasizer Nano Series, Malvern, UK) and  $\zeta$ -potential (Zetasizer Nano analyzer (Malvern, UK) analysis, respectively. CaPs particle size was measured by backscatter detection ( $\lambda = 630$  nm,  $\theta = 173^\circ$ ) and reported as Z-average of hydrodynamic diameter of three measurements of 10 runs for 10 sec at 25°C.  $\zeta$ -potential measurements through electrophoretic mobility were carried out using disposable folded capillary cells (DTS1061; Malvern, UK) at 25°C, suspending CaP in 10 mM HEPES buffer at pH 7.4. The  $\zeta$ -potential was calculated from three separate measurements (100 runs each) in each case. Transmission electron microscopy (TEM) images were collected using a FEI Tecnai F20 ST microscope operating at 80 kV. An aliquot of 20  $\mu$ l of nanoparticle suspension was placed on a carbon/formvar coated-copper grid for 5 min. The grid was then washed with ultrapure water and dried by manual blotting. The stability of CaP, CaP-MP, and CaP-HA in aqueous solution (600  $\mu$ g/ml) starting immediately after dialysis was evaluated by measuring the Z-average and the surface charge of the suspensions as a function of time (up to 8 days) at 4°C by DLS and  $\zeta$ -potential analysis. The stability of the unloaded CaP suspension 2 days after dialysis enriched with 10% (v/v) of fetal bovine serum (FBS, Sigma Aldrich) was also evaluated for up to 7 days at room temperature by DLS and  $\zeta$ -potential analysis. The degradation of CaPs and CaPs-peptide at different pH values was evaluated by mixing 5 mg of freeze-dried nanoparticles with 10 ml of 0.1 M HEPES buffer solution (pH 7.4) or 10 ml of 0.1 M Acetate buffer solution (pH 5.5). At scheduled times for up to 24 h, 1 ml of the supernatant (that was well separated from the solid phase by 15 min of centrifugation at 5000 rpm) were removed for  $\text{Ca}^{2+}$  quantification and replaced with fresh buffer. Calcium content was determined by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) using a 5100 spectrometer (Agilent Technologies, Santa Clara, USA).

To evaluate peptide loading, the DC protein Assay (Bio-Rad) was adopted according to the manufacturer's instructions. Quantification of both the pre- and post-dialysis preparations was performed from both pellet (CaP-MP) and supernatant (CaP-free MP) fractions obtained by

centrifugation (14 min at 20000g) of CaP-MPs. Before the peptide quantification assay, both pellet and supernatant fractions were subjected to an acidic treatment (0.05 M HCl for 4 hours) to completely dissolve CaP-MPs and facilitate the total release and effective quantification of total MP.

The release of Cy7 from CaP-Cy7 was tested against an infinite sink in dialysis by transferring the particles into 3500 Da MWCO cellulose membrane dialysis tubes and dialyzing against water for 24 h with multiple water exchanges. At scheduled times, 10  $\mu$ l of the supernatant (that was well separated from the solid phase by 15 min of centrifugation at 5000 rpm) was analyzed for fluorophore quantification using a UV-Vis spectrophotometer.

The release of Cy7 from CaP-Cy7 was also tested in aqueous solution (600  $\mu$ g/ml) enriched with 10% (v/v) of fetal bovine serum (FBS). At scheduled times, ranging from 1 min to 24 h, 10  $\mu$ l of the supernatant (that was well separated from the solid phase by 15 min of centrifugation at 5000 rpm) were analyzed for fluorophore quantification using a UV-Vis spectrophotometer.

**Experimental Animals.** *Mice.* All procedures on mice were performed according to institutional guidelines in compliance with national (D.L. 04/03/2014, n.26) and international law and policies (directive 2010/63/EU). The protocol was approved by the Italian Ministry of Health. Special attention was paid to animal welfare and to minimize the number of animals used and their suffering. All experiments were performed on 10-week-old C57B6J male mice, CD1 male mice, and CD1-Foxn1 nude mice. All animals were obtained from the Charles River Laboratories. Therapeutic treatment of mice was performed as follows: Type-1 diabetes was induced in adult C57B6/J male mice by intraperitoneal injection of streptozotocin (STZ, 50 mg/kg per day) for 5 consecutive days as previously described (24). 7 days after the last STZ injection, whole blood was obtained from the mouse tail vein and glucose levels were measured using the Accu-check Aviva blood glucose monitoring system (Roche). Mice were fasting for 8 hours before blood collection. At 3 weeks post STZ-treatment, mice were considered diabetic and used for the study only if glucose > 250 mg/dL and %FS < 35% (29 out of 36 STZ-treated mice were considered suitable for the study). STZ diabetic



mice were randomly divided into three separate groups and 50 µl of solution (CaP-MP: 0.5 mg/kg/day; CaP-HA: 0.5 mg/kg/day; or MP: 0.5 mg/kg/day) was administered by inhalation via intratracheal nebulization for ten consecutive days, as indicated.

*Rats.* The study population consisted of 11 male Wistar rats bred in the animal facility at the University of Parma, aged 12-14 weeks and weighing 300-350 g. Briefly, animals were anesthetized by intraperitoneal injection with a mixture of 40 mg/kg ketamine chloride (Imalgene, Merial, Milano, Italy) and 0.15 mg/kg medetomidine hydrochloride (Domitor, Pfizer Italia S.r.l., Latina, Italy). All efforts were made to minimize suffering. This study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institute of Health. The protocol was approved by the Veterinary Animal Care and Use Committee of the University of Parma and conforms to the National Ethical Guidelines of the Italian Ministry of Health (Permit number: 59/2012).

*Landrace pigs.* The experimental protocol was approved by the local bioethics committee of Berlin, Germany (G 0063/16), and conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996). A total of nine healthy animals were anaesthetized as previously described (28). Briefly, after administration of 1 mg/kg propofol, the animals were intubated and anaesthesia was continued with 1-1.5% isoflurane, 20 µg/kg/hour fentanyl and 0.2 µg/kg/hour pancuronium. The animals were ventilated (Cato Dräger medical, Germany) with an FiO<sub>2</sub> of 0.5, an I:E-ratio of 1:1.5, a positive end-expiratory pressure of 5 mmHg, and a tidal volume of 10 ml/kg. If necessary, the respiratory rate was adjusted to maintain an end-tidal carbon dioxide partial pressure between 35 and 40 mmHg. Sheath accesses of the left internal carotid artery and jugular vein were surgically prepared. Heart rate and arterial blood pressure were continuously monitored. A balanced crystalloid infusion (Sterofundin ISO, B. Braun Melsungen AG, Melsungen, Germany) was administered at a fixed rate of 10 ml/kg/hour throughout the protocol. A body temperature of 37.5 - 38.5 °C was maintained by either surface cooling or a warming blanket.

**Nanocarrier administration.** *Mice.* CD1 mice were divided into four different groups of 6 mice and 100 µl of CaP-Cy7 were administered by gavage, intraperitoneal, and intravenous injection (Cap-IP) as well as by intratracheal nebulization. For the intratracheal treatment MicroSprayer Aerosolizer – Model IA-1C and a FMJ-250 High Pressure Syringe (Penn-Century™) was used. Mice were anesthetized with 3% isoflurane to allow nebulization. For each administration method, 11 mice administered with an aqueous Cy7 solution were used as controls. At the end of the experimentation, mice were sacrificed and tissues collected.

*Rats.* Administration was performed via a 16-gauge catheter connected to a rodent ventilator (Rodent ventilator UB 7025, Ugo Basile, Comerio, Italy) for artificial respiration. One dose of 20 µl/100 g BW solution (3 mg/kg CaP or saline) was administered via the catheter. After CaP administration, animals were woken up with a single intraperitoneal injection of 0.15 mg/kg atipamezole hydrochloride (Antisedan, Pfizer, Italy) and left conscious for four hours before electrophysiological in vivo experiments were performed. Before exposing the heart, pre-cordial ECGs (3 unipolar and 3 bipolar leads) were recorded in order to screen for possible anoxic effects on cardiac electrical activity due to the tracheal instillation.

*Landrace pigs.* A left lateral thoracotomy was performed and the left ventricle was exposed in a pericardial cradle. The protocol is described in Fig. 4a. After stabilisation for 30 min, two transmural left ventricular free wall biopsies were taken from the beating heart. Next, a 10 ml solution containing 10 mg/ml CaP-HA (150 µg/ml HA) was nebulized over 30 min with the Aeronex Professional Nebulizer System (Aeronex Pro, Aerogen Ltd., Galway, Ireland), connected with an adult T-piece into the inspiratory limb of the breathing hoses. At 30, 60, 180, and 300 min after the beginning of inhalation, further biopsies were taken. All biopsies were rinsed carefully in saline, immediately frozen in liquid nitrogen, and stored at -80 °C. Finally, the animals were sacrificed by a bolus injection of 100 mmol/l potassium chloride and samples were collected.

**Echocardiography.** A Vevo 2100 high-resolution in vivo imaging system (VisualSonics Fujifilm) with a MS550S probe "high frame" scanhead was used for echocardiographic analysis. Mice were anesthetized with 1.0% isoflurane and imaged in M-mode as previously described (24).

**Invasive hemodynamics in Landrace pigs.** A separate group of pigs ( $n = 9$ ,  $88 \pm 4$  kg, Fig.4a) was acutely instrumented closed-chest under fluoroscopic guidance with a pulmonary artery flotation catheter (Edwards Lifesciences CCO connected to Vigilance II, Edwards Lifesciences, Irvine, CA), a LV conductance catheter (5F, 12 electrodes, 7-mm spacing; MPVS Ultra, Millar Instruments, Houston, TX), and a valvuloplasty catheter (24 mL; Osypka, Rheinfelden, Germany) in the descending aorta as previously described (28). A 3-lead surface ECG was recorded throughout the whole experiment and used for offline analysis. Offline analysis included measurements of PQ intervals during spontaneous heart rates, QRS duration, and QT duration. After stabilisation for 30 min, steady-state hemodynamics were acquired over three respiratory cycles. Pressure-volume relationships were generated by briefly inflating the intraaortic balloon catheter three times. Next, 10 ml solution containing 10 mg/ml CaP-HA (150  $\mu$ g/ml HA) was nebulized over 30 min with the Aeronex Professional Nebulizer System as described above. Measurements were then repeated after 300 min. Pressure-volume data and time intervals were analyzed off-line by CircLab Software (custom made by Prof. P. Steendjik). The conductance catheter measurements were calibrated by hypertonic saline solution (3 boluses of 3 mL at 10%) and cardiac output was continuously derived from the pulmonary artery flotation catheter.

**Statistical Analysis.** Assessment of normality of the data was calculated with the Kolmogorov-Smirnov (K-S) test. Statistical comparisons were performed using one-way or two-way ANOVA and Student's t-test for unpaired samples in Patch Clamp experiments. Prism 6.0 software (GraphPad

Software, CA) was used to assess the normality of the data and for statistical calculation. A P value < 0.05 was considered significant unless otherwise indicated.

### **Supplementary Materials:**

Supplementary Material and Methods; Western blot analyses; RNA isolation and quantitative real-time PCR (q-RT-PCR) analysis; thiobarbituric acid reactive substance detection (TBARS); Transmission Electron Microscopy (TEM) analysis; patch-clamp recordings; cardiomyocyte contractility measurements; in vivo epicardial potential mapping; confocal Microscopy; Cy7 detection, in vivo imaging.

Figures S1. Size stability in proteins free and proteins enriched aqueous medium of CaPs and CaPs functionalized with MP and HA. Surface charge of CaP-Cy7 and CaPs with Cy7 adsorbed on their surface.

Figures S2. *In vivo* and *ex vivo* characterization of CaPs.

Figures S3. Echocardiographic analysis of CaP-treated mice.

Figures S4. Stability, loading, degradation, and structural characterization of CaPs and CaPs functionalized with MP and HA.

Figures S5. Stimulated emission depletion (STED) microscopy of isolated cardiomyocytes from mice treated with unloaded CaPs.

Figures S6. MP-CaP treatment of diabetic mice.

Table S1. Echocardiographic analysis of 10-week-old male WT mice treated with CaP.

Table S2. Excitability and refractoriness parameters obtained by epicardial multiple lead recording in untreated rats or rats treated with CaP.

Table S3. CaP-MP loading.

Table S4. Echocardiographic analysis of 10-week-old male WT and STZ mice treated with MP, CaP-HA, and CaP-MP.

Table S5. Characterization of LTCC current during rectangular double-pulse protocols and membrane capacities measured in adult cardiomyocytes isolated from treated mice.

Table S6. Thiobarbituric acid reactive substance detection in mice treated with MP, CaP-HA, and CaP-MP.

Movie S1. 3D Fluorescence Molecular Tomography (FMT) imaging of the cardio-pulmonary area from Cy7-treated mice.

Movie S2. 3D FMT imaging of the cardio-pulmonary area from CaP-Cy7-treated mice.

Movie S3. Stimulated emission depletion (STED) microscopy of isolated cardiomyocytes from mice treated with MP-Rhodamine-loaded CaPs.

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T.P., L.D.E. S.P., S.R., H.P., and M.M. carried out experiments. In particular, P.Ce., carried out

several of the experiments and analyses; N.S., S.R., and M.M. were responsible for electrophysiology

studies and analyses; M.V. performed all mouse setups and treatments, physiological analyses, and

graphic representations; P.Ca. performed echocardiographic analyses and cardiomyocyte isolation;

M.V. and M.E. performed *in vivo* imaging; M.E. performed STED; F.R and F.R. performed TEM on

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and L.D.E. prepared and characterized nanoparticle formulations; S.P. and R.A. performed T-bars

analysis; M.I designed and supervised the synthesis and functionalization of nanoparticles; M.M. and

D.C. designed the study and supervised most of the experiments; M.M., M.I, G.C., and D.C. wrote

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**interests:** D.C and G.C. are authors on a patent application n. MI2014A000097 and

PCT/EP2015/051376 (WO2015/110589) “Mimetic peptides and their use in medicine” submitted by

the Italian National Research Council, which covers mimetic peptides (MPs) that through a novel

mechanism directly targets the L-type calcium channel (LTCC) and are suitable for use in the

treatment of conditions where LTCC density and function is altered. D.C., M.M., M.II, and A.T. are

authors on a patent application n. MI2014A002207 and PCT/EP2015/080991 (WO2016/102576)

“Products for the delivery of therapeutic/diagnostic compounds to the heart” submitted by the Italian National Research Council (75%) and the Italian Istituto nazionale Assicurazione Infortuni sul Lavoro (25%), which covers a process for the preparation of a product comprising one or more nanoparticles of calcium phosphate and are suitable for use as a vehicle for one or more diagnostic/therapeutic compounds for the treatment of cardiovascular diseases through inhalation administration. **Data and materials availability:** All data reported in the paper are included in the manuscript or available in the Supplementary Materials. Requests for materials should be addressed to D.C (daniele.catalucci@cnr.it) and will be handled according to a material transfer agreement through the Italian National Research Council.

**Fig. 1. Inhalation delivers CaPs to the heart.** (a) Schematic representation of CaP heart targeting via the inhalation route. 1 = Nanoparticle inhalation; 2 = Nanoparticle deposition in lungs and translocation through the air-blood pulmonary barrier; 3 = Heart targeting and drug release into the heart. (b) Quantification of Cy7 fluorescence signals from heart tissue of mice treated with CaP-Cy7 via gavage, intraperitoneal (ip), intravenous (iv), and inhalation administration. All data are shown as mean  $\pm$  SEM. \*P < 0.05 and \*\*\*P < 0.001 compared to gavage (one-way ANOVA test) (n = 5). (c) Time-course quantification of Cy7 fluorescence signals from heart and lung tissue of mice treated with CaP-Cy7 via inhalation administration. All data are shown as mean  $\pm$  SEM. \*P < 0.05 and \*\*P < 0.01 compared to the 10 min time point (two-way ANOVA test) (n = 5). (d) Whole body optical *in vivo* imaging to evaluate the distribution of CaP-Cy7 and Cy7 in CD1 nude mice at different time points after inhalation. Data are representative of at least 6 independent experiments. Values are expressed as radiance efficiency ( $\times 10^9$ ). (e) 3D Fluorescence Molecular Tomography (FMT) imaging of mice as presented in d and analyzed 60 min post CaP-Cy7 and Cy7 administration. T = trachea, H = heart, L = lung. (f) Fluorescence imaging of explanted hearts from perfused mice analyzed 60 min post inhalation of 50 and 100  $\mu$ l of CaP-Cy7. NT = heart from non-treated mouse. Values are expressed as radiance efficiency. Scale values for Heart: max 1.04e7 - min 3.56e6; Lung: max 7.78e7 - min 6.17e6.9 (n = 4).

**Fig. 2. Stimulated emission depletion (STED) microscopy of isolated cardiomyocytes from mice treated with MP-Rhodamine-loaded CaPs.** (a) STED microscopy on an isolated cardiomyocyte from mice treated with MP-Rhodamine-loaded CaPs. Green: LTCC; Red: MP-Rhodamine. Representative of 10 different acquisitions. Original magnification: 100X; Scale bar = 15  $\mu$ m. (b) Enlargement of yellow dotted-line square shown in panel a. Scale bar = 5  $\mu$ m. (c) Orthogonal view of isolated cardiomyocyte shown in panel a and b. Original magnification: 100X; Scale bar = 5  $\mu$ m.

**Fig. 3. Inhaled CaP-MPs restores cardiac function in a mouse model of diabetic cardiomyopathy.** (a) Design of the study. (b) Left ventricular fractional shortening and ejection fraction (indices of cardiac contractile function) as determined by echocardiographic analysis on streptozotocin (STZ)-treated mice treated as indicated. All data are shown as mean  $\pm$  SEM. \*\*\*P < 0.001 compared to NT (non-treated) mice (two-way ANOVA test) (n = 10). (c) Western blot analysis for the LTCC pore unit (Ca<sub>v</sub> $\alpha$ 1.2) on adult cardiomyocytes isolated from treated mice. (d) Average peak of LTCC current density as a function of voltage command measured in adult cardiomyocytes isolated from treated mice. All values are shown as mean  $\pm$  SD for n cells. \*P < 0.02 for NT compared to MP and CaP-HA mice (n = 16 - 21). (e) Contractility of adult cardiomyocytes isolated from treated mice. All data are shown as mean  $\pm$  SEM. \*P < 0.05 and \*\*P < 0.01 compared to NT mice (two-way ANOVA test) (n = 20). (f) Z-stack confocal laser scanning microscopy images showing HA peptide in myocardial tissue from mouse treated by CaP-HA inhalation. Immunofluorescence staining for HA (Red), LTCC (green), and cell nuclei (DAPI, blue). Bar = 20  $\mu$ m (n = 3).

**Fig. 4. CaP-HA inhalation in landrace pigs.** (a) Design of the study. (b) Systemic, pulmonary and left ventricular hemodynamics (n = 6, all data are shown as mean  $\pm$  SEM). Steady-state data before and after CaPs inhalation were compared by 1-way analysis of variance for repeated measurements. Pressure-volume relationships were compared by analysis of covariance. Post-hoc testing was performed by Tukey's test. A p-value < 0.05 was considered significant. (c) Original registrations from one representative animal of left ventricular pressure-volume loops before (pre, top) and after (post, bottom) CaP-HA inhalation. All registrations were recorded at spontaneous heart rate. ESPVR: end-systolic pressure-volume relationship. EDPVR: end-diastolic pressure-volume relationship. (d) Kinetics of inhaled CaP-HA cardiac targeting as examined by dot blot analysis from biopsies obtained at the indicated times after inhalation. (e) Western blot analysis for HA on tissues of treated pigs. (f) Z-stack confocal laser scanning microscopy images showing HA peptide in myocardial tissue from

pigs treated by CaP-HA inhalation. Immunofluorescence staining for HA (Red), LTCC (green), and cell nuclei (DAPI, blue). Bar = 20  $\mu\text{m}$  (n = 6).