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Coating of PLA-nanoparticles with cyclic, arginine-rich cell penetrating peptides enables oral delivery of liraglutide

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Abstract

Until today, the oral delivery of peptide drugs is hampered due to their instability in the gastrointestinal tract and low mucosal penetration. To overcome these hurdles, PLA (polylactide acid)-nanoparticles were coated with a cyclic, polyarginine-rich, cell penetrating peptide (cyclic R9-CPP). These surface-modified nanoparticles showed a size and polydispersity index comparable to standard PLA-nanoparticles. The zeta potential showed a significant increase indicating successful CPP-coupling to the surface of the nanoparticles. Cryo-EM micrographs confirmed the appropriate size and morphology of the modified nanoparticles. A high encapsulation efficiency of liraglutide could be achieved. In vitro tests using Caco-2 cells showed high viability indicating the tolerability of this novel formulation. A strongly enhanced mucosal binding and penetration was demonstrated by a Caco-2 binding and uptake assay. In Wistar rats, the novel nanoparticles showed a substantial, 4.5-fold increase in the oral bioavailability of liraglutide revealing great potential for the oral delivery of peptide drugs.

Key words: Nanoparticles; Polylactide acid; Cell penetrating peptides; Oral delivery; Peptide drugs; Liraglutide

The oral administration of macromolecular drugs (i.e. peptides, proteins, and antibodies) proves to be a current challenge in the field of drug discovery and development.[1] Both, the drugs` instability in the acidic milieu of the stomach as well as the low absorption across the intestinal barrier hamper the oral bioavailability of these therapeutics.[2] For this reason, only parenteral delivery routes (such as subcutaneous or intravenous injection) are possible, which are expensive, time-consuming and result in low patient compliance.[3,4] To overcome these hurdles and to facilitate the oral delivery, several approaches have been studied in recent years, including nano- or micro-emulsions,[5] polymeric micelles,[6] hydrogel micro-particles,[7] tetraether-lipid liposomes[2,8] or surface-modified liposomes[9–11] - but with limited success.

For this reason, the aim was to search for improved formulation technologies. A promising approach is the use of nanoparticles (NP) consisting of biodegradable materials such as polylactide acid (PLA) or poly lactic-co-glycolic acid (PLGA). Despite several attempts to enable oral drug delivery,[12–15] none of the previous PLA or PLGA iterations achieved a breakthrough.[13,16–19] Reasons for this phenomenon are primarily the poor mucosal penetration in the intestine and also the rapid removal by the mucus.[4] A novel strategy to enhance the mucosal uptake of these nanoparticles is the surface-modification with cell penetrating peptides (CPPs).[20] CPPs are short peptides of less than 30 amino acids (mostly positively charged[21]). They are able to penetrate cell membranes and translocate different

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cargoes into cells.[22] The potential cargoes can range from small molecules to proteins.[23] Arginine rich CPPs are currently exhaustively investigated.[24,25] Recently, Herce et al.[26] reported the successful development of functional cell-permeable nanobodies by the use of cyclic arginine-rich CPPs. Other studies showed enhanced absorption rates of insulin after oral co-administration with CPPs.[27–29]

In the present study, both strategies were combined and the surface of PLA nanoparticles was coated with a cyclic R9-CPP derivative to facilitate the intestinal retention and mucosal penetration of peptide therapeutics. As one developmental step of this study, a cyclic CPP was used, because it is less susceptible to hydrolysis by peptidases. In addition, cyclic peptides have been shown to be enzymatically more stable.[30] The peptide liraglutide (LRT), a widely used antidiabetic drug, was studied as a model drug for animal studies.[31] LRT can be also used as drug against obesity,[32,33] though requiring long term administration, where patient compliance is demonstrably reduced.[34] The application of LRT is still hampered due to its limitation to subcutaneous administration.

Based on this urgent need, an oral formulation of LRT using a novel class of CPP-coated PLA nanoparticles was investigated. Maleimide coupling of the CPPs to PLA was chosen due to the high stability of the covalent binding as previously shown by Youngblood et al.[35] PLA nanoparticles were systematically prepared using surfactants covering the entire hydrophilic–lipophilic balance (HLB) chart in order to determine the most suitable preparation technique for CPP-nanoparticles. Nanoparticle characterization by dynamic light scattering demonstrated that Tween® 85 resulted in smallest nanoparticles, which is advantageous for mucosal penetration. By the use of this formulation strategy, a high encapsulation efficiency of the model drug LRT was achieved. The successful coating of the CPP on the nanoparticle surface was verified by the increase in zeta potential of the particles. Additionally, these nanoparticles were successfully freeze-dried for long term storage providing an additional benefit.[36] In a Caco-2 binding assay the strongly enhanced mucosal adhesion of the CPP-modified nanoparticles was clearly demonstrated. Animal trials using Wistar rats were performed and showed a strongly improved uptake of LRT after oral administration as compared to the free peptide.

Methods

Materials

Maleimide-modified polylactide acid (MW = 5000 g/mol) was obtained from Sigma Aldrich (Steinheim, Germany) and Amicon® Ultra-4 centrifugal filters from Merck Millipore (Tullagreen, Ireland) while Filtropur S 0.2 sterile filters were purchased from Sarstedt (Nümbrecht, Germany). Dulbecco's phosphate buffered saline was applied from Gibco® by life technologies™ (Paisley, UK); Tween® 85 from Sigma Aldrich (Steinheim, Germany), Atto dyes from Atto-tec GmbH (Siegen, Germany), size-exclusion (NAP™-5) columns from GE Healthcare (Buckinghamshire, UK) were obtained and radioiodine I-125 was purchased from Hartmann Analytic GmbH® (Braunschweig, Germany), while cholesterol, Triton™ X-100, chloroform, methanol and all other solvents were obtained from Sigma Aldrich (Taufkirchen, Germany). Liraglutide was isolated of Victoza®-pens by preparative HPLC and the cyclic R9-CPP was synthesized by SPPS (solid phase synthesis) in our laboratory. For the cell binding assay, DMEM medium (high glucose), Fetal Bovine Serum and Trypsin–EDTA was purchased from Thermo Fisher (Waltham, Massachusetts, USA) while glass coverslips were obtained from Greiner-Bio one (Frickenhausen, Germany). The dye DAPI was obtained from Sigma Aldrich (Steinheim, Germany), Mowiol 4–88 from Roth (Karlsruhe, Germany) and alamarBlue® from Bio-Rad antibodies (Puchheim, Germany).

Nanoparticles

Preparation of nanoparticles

Nanoparticles were prepared by a modified double emulsion technique according to Zambaux et al.[37] and Li et al.[38] First, the required amount of the peptide drug LRT (0–2 mg) and in total 10 mg of the polymers (6 mg of the polymer PLA and 4 mg of a maleimide-modified PLA) were dissolved in 1 ml acetone followed by sonication for 5 min. Afterwards, the resulting mixture was added dropwise into 2 ml of a Tween® 85 aqueous solution (0.5% v/v) under constant and fast stirring. The required amount of the CPP (0.5 mg) was dissolved in another 2 ml of an aqueous solution of Tween® 85 and finally added to the obtained mixture (coupling principle see Figure 1). The coupling of the CPP could be performed by the reaction of the thiol group of its cysteine to the maleimide-modified PLA. Prior to characterization, the nanoparticles were stirred overnight to ensure the evaporation of the organic solvent. In order to remove free LRT and free CPP, the nanoparticles were purified by Sephadex G-25 gel filtration chromatography (NAP™-5 columns). As elution buffer, 0.5% (v/v) Tween® 85 in water was used.

Characterization of nanoparticles

The particle size, PDI and zeta potential of the nanoparticles were determined at room temperature using the automatic mode of a Zetasizer Nano ZS from Malvern™ (Malvern Instruments Ltd., Worcestershire, United Kingdom). Nanoparticle characteristics (size, PDI) were measured after dilution to a PLA concentration of 0.10 mg/ml with phosphate buffer (10 mM; pH 7.4), while the zeta potential was determined after dilution to a PLA concentration of 0.20 mg/ml by phosphate buffer (50 mM; pH 7.4). For default settings of the automatic mode of the Zetasizer Nano ZS see supplementary data.

Nanoparticle characteristics using various surfactants

Various surfactants for the preparation of PLA-nanoparticles have been intensively examined, above all polyvinyl alcohol (PVA) in several concentrations,[32,34–36] but nevertheless, until now, no comprehensive data covering the whole range of the HLB-value regarding size and PDI of PLA-nanoparticles exist. For this reason, several surfactants covering the whole HLB-range were examined. The surfactants used for this study are listed in the supplementary data table S1. All nanoparticles were prepared by the modified double emulsion method and the size and PDI were determined as described previously.

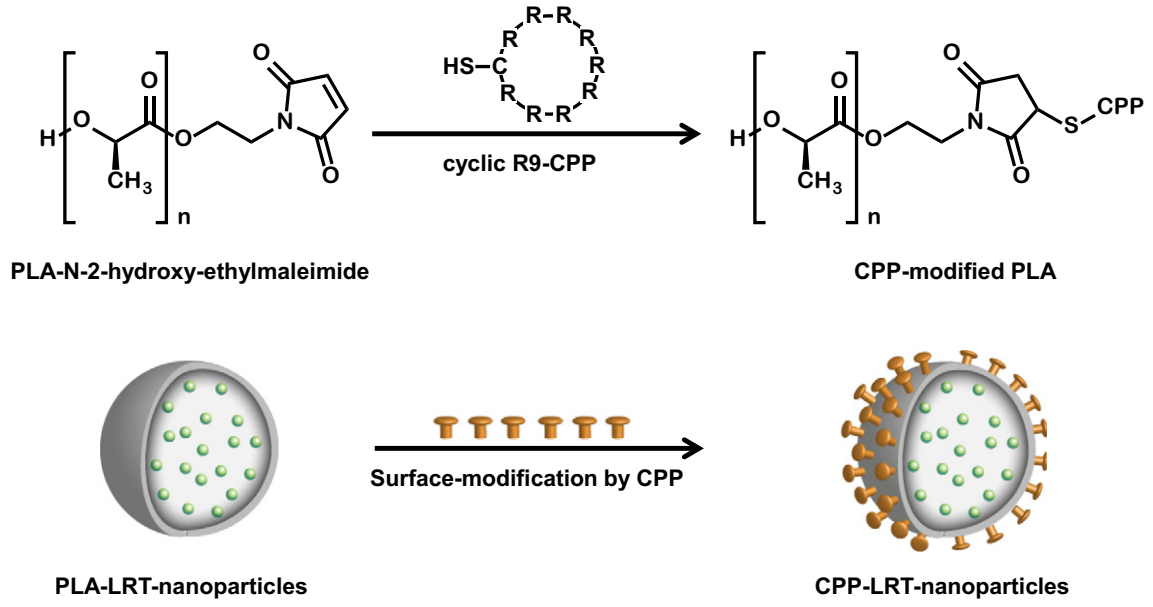

Figure 1. Illustration of the nanoparticle surface modification with the cyclic R9-CPP. For this coupling strategy, the cyclic R9-CPP contained an additional cysteine. Covalent binding was achieved by the reaction of the free thiol group of the cysteine with the maleimide group of the modified PLA.

Encapsulation efficiency of LRT

The determination of the encapsulation efficiency of LRT was performed by reversed phase HPLC (Agilent 1100 Series) using a C18 column (Chromolith® Performance RP-18e, 100–3 mm; linear gradient of 0.1% TFA in water (eluent A) to 0.1% TFA in acetonitrile (eluent B) within 5 minutes) according to Uhl et al.[2] After preparation, the nanoparticles were divided into two samples (1 ml each). The first sample was used to calculate the 100%-value after dissolving the nanoparticles by acetonitrile (1:1 v/v), while the other sample was purified from non-entrapped LRT by NAP™-5 size exclusion gel chromatography according to Uhl et al.[2] After following dissolution by acetonitrile (1:10 v/v), the sample was injected in the HPLC in order to calculate the X-% value of entrapped LRT by the following equation under consideration of different sample volumes:

$$E(\%) = \frac{[AUC]\text{LRT part 2}}{[AUC]\text{LRT part 1}}$$

Whereas [AUC] LRT part 2 is considered as the concentration of LRT in the nanoparticles after purification and [AUC] LRT part 1 is considered as the concentration of LRT in the nanoparticles before purification.

Release of LRT

The release of LRT out of the CPP nanoparticles was determined over a period of 28 d. Therefore, the nanoparticles were prepared as described above and the free LRT was removed by size exclusion chromatography (SEC). The amount of LRT after the purification step (day 0) was determined by HPLC and considered as 100% value. Afterwards the nanoparticles were stored at 2–8 °C and samples were taken at 7, 14 and 28 d. These samples were purified again and the remaining amount of LRT was compared with the amount of day 0.

Cryo-EM micrographs

Cryo-TEM was performed as previously described by Kulkarni et al.[39] Concentrated particles were applied to glow-discharged copper grids and plunge-frozen using a FEI Mark IV Vitrobot (FEI, Hillsboro, OR). For storage, the grids were kept in liquid nitrogen until imaged. The grids were afterwards moved into a Gatan 70° cryo-tilt transfer system and then inserted into the microscope. For imaging of all samples, an FEI LaB6 G2 TEM (FEI, Hillsboro, OR) operating at 200 kV under low-dose conditions was used. A bottom-mount FEI Eagle 4 K CCD camera was used to capture all images. They were imaged at a 55,000 × magnification with a nominal under-focus of 1–3 µm to enhance contrast.

Synthesis of peptides and radiolabeling

Synthesis of cyclic R9-CPP by solid phase synthesis

The cyclic cell penetrating peptide Cys-$(Arg)_9$ (cyc R9-CPP) was synthesized via solid phase peptide synthesis (SPPS) on a chlorotrityl resin (2-CTC) employing the fluorenylmethyloxycarbonyl/tert-butyl (Fmoc/tBu) strategy; 117 mg (0.2 mmol) Fmoc-Cys(Trt)-OH dissolved in dichloromethane (DCM) with 5 eq. diisopropylethylamine (DIPEA) were loaded onto 250 mg of 2-CTC for 90 min at RT in order to yield a loading of 0.8 mmol/g. The resin was pre-swelled in DCM. After the coupling of Fmoc-Cys(Trt)-OH to the resin, the uncoupled part of the amino acid was removed by washing with DCM for three times. Subsequently, free reactive sites on the resin were blocked by the addition of DCM/methanol/DIPEA (17/2/1 v/v) for 30 min. Then, the resin was washed with DCM and DMF followed by nine consecutive steps of coupling Fmoc-Arg(Pbf)-OH in DMF, using an excess of 5 eq. amino acid, 4.75 equivalents 2-(1H-

benzotriazol-1-yl)-1,1,3,3-tetramethyluroniumhexafluoro–phosphate (HBTU) and 4 equivalents DIPEA. Fmoc-groups were removed after each coupling by treatment with 20% piperidine in DMF. In between steps, the resin was washed rigorously with DMF. The resulting peptide was then cleaved from the resin with 10% acetic acid and 20% trifluoroethanol in DCM. The procedure was repeated two times for 3 h. The cleavage solution was subsequently evaporated with an excess of toluene for three times for quantitative removal of acetic acid.

The side chain protected peptide was dissolved in DMF in a peptide concentration of 3 mg/ml. Afterwards, the cyclization was performed with 4 equivalents of (7-azabenzotriazol-1-yloxy) tripyrrolidinophosphonium hexafluoro–phosphate (PyAOP) and DIPEA at RT overnight. After stopping the reaction with water, the solution was concentrated to a fiftieth to hundredth of the starting volume and the side chain protected cyclic peptide precipitated by pouring into cold tButyl-methylether. The precipitated protected cyclic peptide was dried and subsequently deprotected with a mixture of 5% ethandithiol in trifluoroacetic acid (TFA). Finally, the peptide was precipitated in diethyl ether and centrifuged two times for 5 min at 3000 g. The pellet was dried under vacuum and the peptide was purified via preparative HPLC (Reprosil Pur 120 C18-AQ, 5 μm (250 × 25 mm), 0–30% acetonitrile +0.1% TFA in 25 min) and the purity of the peptide was confirmed by high resolution mass spectrometry (see supplementary data Figure S1).

Purification of LRT

LRT was isolated out of Victoza®-pens by preparative HPLC (Reprosil Pur 120 C18-AQ, 5 μm (250 × 25 mm), 50–80% acetonitrile +0.1% TFA in 25 min) and the purity of LRT was confirmed by high resolution mass spectrometry (see supplementary data Figure S2).

Radiolabeling of LRT

For radiolabeling, the chloramine T method according to Crim et al.[40] was used; 25 μl of LRT (1 mM stock solution in 0.25 M phosphate buffer pH 7.5) and approximately 5 megabecquerel (MBq) of I-125 iodide were mixed with 10 μl of a 1 mM solution of chloramine T and the mixture was shaken for 30 s. Subsequently, the reaction was stopped by the addition of 20 μl of a saturated methionine solution. Purification was done by semi preparative HPLC as described by Schieck et al.[41] Afterwards, the purity of the radiolabeling was verified by radio-HPLC (Agilent 1100 series) as described previously.[2] The radio-HPLC analysis of the unpurified and the purified I-125-LRT is shown in the supplementary data, Figure S3.

Lyophilisation to obtain long term storage stability

To avoid the release of LRT out of the CPP nanoparticles and to enable the storage for longer times, the CPP nanoparticles (polymer concentration = 2.5 mg/ml) were freeze dried (main drying was carried out at −20 °C for 2 days followed by a secondary drying step at 0 °C for at least 6 hours) in a Delta 1–20 KD from Martin Christ (Osterode, Germany). Sucrose and trehalose in concentrations of 100–700 mM were used as lyoprotector as described previously.[42] Briefly, the nanoparticles were prepared and sucrose respectively trehalose was added to the aliquots (50 μl each). Afterwards, the aliquots were shock frozen in liquid nitrogen and freeze dried. In order to assess the quality of the freeze-dried products, the nanoparticles were rehydrated with 50 μl 0.5% (v/v) Tween® 85 in water the nanoparticle characteristics (size, PDI) were determined by Zetasizer measurements as described above.

Cell binding assays of nanoparticles

Labeling of CPP-modified nanoparticles and LRT with fluorescent dyes

For this cell binding assay, the model substance LRT was labeled with a fluorescent dye (NHS-Atto495, green color). Furthermore, another dye (NHS-Atto610, red color) was coupled to an amine-modified PLA. Both reactions took place in a mixture of PBS (pH 8.3) and DMF (4:1 v/v). After coupling under constant shaking overnight, the mixture of LRT and NHS-Atto495 was lyophilized and dissolved again in a mixture of water and acetonitrile (50:50 v/v). This mixture was purified by preparative HPLC (Reprosil Pur 120 C18-AQ, 5 μm (250 × 25 mm), 35–70% acetonitrile +0.1% TFA, 25 min). The success of the purification step was verified by HPLC/MS-analysis. In case of the mixture of amine-modified PLA and NHS-Atto610, the purification was performed by solvent extraction using various volumes of water/chloroform (1:1 v/v).

Caco-2 cell binding assay

Caco-2 cells were kept in culture medium comprising DMEM medium (high glucose) supplied with 20% Fetal Bovine Serum at 37 °C. When cells reached a confluency of 50%, they were washed in pre-warmed DPBS for 10 minutes. Dissociation of the cells was performed using Trypsin–EDTA (0.25%) at 37 °C. The cell suspension was counted using a Neubauer counting chamber; 100.000 cells were seeded into each well of a 48-well plate already containing glass coverslips. On the next day, cells were washed in DPBS and kept in 50% culture medium/50% (v/v) DPBS during the experiment. Cells were incubated with a nanoparticle suspension (initial polymer concentration = 2.5 mg/ml) diluted 1:40 for the time periods indicated. Afterwards, Caco-2 cells were washed four times in DPBS for 5 minutes each and subsequently fixed in 4% w/v formaldehyde/DPBS for 10 minutes on ice. Fixative was removed by washing four times in DPBS at room temperature (RT). Cell nuclei were stained using DAPI (5 mg/ml) diluted 1:1000 in DPBS for 10 minutes at RT. Cells were mounted in Mowiol 4–88 and analyzed using a Nikon Eclipse Ti microscope.

Particle retention assay using porcine intestine

In order to evaluate the mucoadhesion of the CPP nanoparticles in comparison to unmodified particles, a particle retention assay according to Preisig et al.[43,44] was performed. Intestinal tissue was prepared according to Preisig et al.[44] Duodenum was removed (first 50 cm) and tissue samples were prepared from the jejunum (approx. 2 m). In contrast to the procedure described in,[44] outer muscle layers were not removed. The dimensions of the tissue, on which the different formulations were applied, were 17 mm in width and 80 mm in length. In brief, the nanoparticle suspension (equal amounts of nanoparticle-lyophilisates with a polymer concentration of 2.5

mg/ml were suspended in 250 µl of water) was applied on the pig intestine and incubated for 10 min at 37 °C without any flow. For the incubation time, the flow channel was kept in horizontal position during the experiment, the assembly was tilted to 45° and the flow was started. The flow (5 ml/min water; total volume = 25 ml; recirculating system) was initiated and samples (200 µl each) were withdrawn at 5, 15, 30, 60 and 120 min. This mucoadhesion assay was performed for each formulation in triplicates. Analysis of the samples (in triplicates) took place by Zetasizer measurements. For these measurements, 50 µl of each sample were diluted with 950 µl of a 10 mM PBS. By means of the derived count rate, the part of the detached nanoparticles could be determined. For the analysis of this assay, the amount of measured particles for each time point was compared with the previous time point (% increase in free particles).

Cytotoxicity assay

Cell cultivation

The cultivation of the Caco-2 cells took place in DMEM (20% Fetal Bovine Serum, 1 mM sodium pyruvate, GlutaMAX®, 4 mM L-alanyl-glutamine and 1% non-essential amino acids as supplement). The cells were cultured at 37 °C in an atmosphere of 95% air and 5% CO_2. When cells reached 80% confluence, subcultures were taken.

Cytotoxicity assay

Before testing, the Caco-2 cells were seeded into 96 well plates and grown for 14 days after the formation of a monolayer. Therefore, the medium was changed every 2 days. Nanoparticles were added in appropriate concentrations and incubated for 3 hours. At this stage, the medium was replaced by growth medium (supplemented with 10% alamarBlue®) and the cells were incubated for 3 h. Fluorescence measurement was performed on an Infinite® Tecan Plate reader (wavelength of 590 nm with an excitation wavelength of 560 nm). Normalization of the cell viability was done with respect to wells containing untreated cells and wells without cells as blank.

Proof of concept study: animal trials

The procedures of this study were approved by the Animal Care and Use Committee at Regierungspräsidium Karlsruhe (Karlsruhe, Germany). For this study, female Wistar rats with a body weight of about 150–200 g were used. The model substance LRT was labeled with I-125 and incorporated into the nanoparticles as described previously; 0–24 h post oral administration, the LRT uptake was measured by counting of the radioactivity in the blood samples. Briefly, three groups (n = 3; female Wistar rats) were formed. Prior to 12 h of the experiment, the rats were kept without food, but with free access to water. Oral application of the nanoparticles and the free peptide took place by gavage. Each rat of the first group obtained a dose corresponding to 0.5 megabecquerel of the labeled free peptide (negative control). All rats of the second group obtained a dose corresponding to 0.5 MBq of the unmodified nanoparticles. The rats of group 3 obtained a dose corresponding to 0.5 MBq of the nanoparticles coated with the cyclic R9-CPP. The rats were sacrificed 24 h post oral administration and the radioactivity of all blood samples was measured using a Cobra Auto γ-Counter (Packard Bioscience, USA) in comparison with standards. The radioactivity of the blood samples was related to the total injected dose (ID) and expressed as a percentage of the total injected dose per gram of tissue (% ID/g) as described previously.[2,41]

Statistical analyses

Statistical analysis was performed using Prism® software (GraphPad Software, San Diego, CA, USA). Analysis is presented as mean ± standard deviation of the mean (S.D.). The different groups of the animal trial were compared by unpaired t-test and considered significant at $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

Results

Nanoparticle characterization

For the preparation of the surface-modified PLA-nanoparticles by double emulsion technique, various surfactants covering the entire HLB-range (1.8–29) were examined (see supplement table S1). Tween® 85 and Cremophor® EL (HLB-values = 11–12) provided favorable particle size and PDI (data see supplement Figure S5). Interestingly, the combination of PLA and PVA, a popular combination for nanoparticle preparation,[37,45–47] did not provide comparable results. The average size of the unmodified nanoparticles (prepared using Tween® 85) was in a range of 120–220 nm depending on the amount of the model drug LRT incorporated (Figure 2, *A*). In general, as the LRT concentrations increased, so did the size of the nanoparticles. The size of the CPP-PLA particles was slightly higher than the unmodified PLA particles and showed as well an increase in size with increasing amounts of encapsulated LRT. The same effect was observed on the PDI of the nanoparticles. Compared with the work of Zambaux et al.[37] and Lamprecht et al.,[45] where PVA was used as a surfactant, unmodified particles (without drug) had a size of 200–250 nm. In contrast, the utilization of Tween® 85 as surfactant provided nanoparticles with a significant smaller size (approximately 120 nm). This demonstrates the significant influence of the surfactant for the nanoparticle characteristics (see supplement Figure S5). The optimal amount of CPP for the surface modification was determined by coupling different amounts of CPP and investigating the change of the zeta-potential. By statistical analyses, 0.5 mg of the CPP was selected to be sufficient (Figure S6).

The zeta-potential of the CPP-modified (LRT encapsulated) nanoparticles showed a strong increase compared to the unmodified nanoparticles due to the positively charged amino acid arginine (pK_a arginine = 2.17) of the cyclic R9-CPP (Figure 2, *B*) confirming the successful coupling of the CPP on the surface of the particles.[48,49]

Encapsulation efficiency of LRT

The CPP-modified nanoparticles showed an encapsulation efficiency of 70–77% depending on the amount of LRT (Figure 3, *A*). Notably, the amount of LRT in the range of 0.5–2 mg per 4 ml batch had no significant influence on the

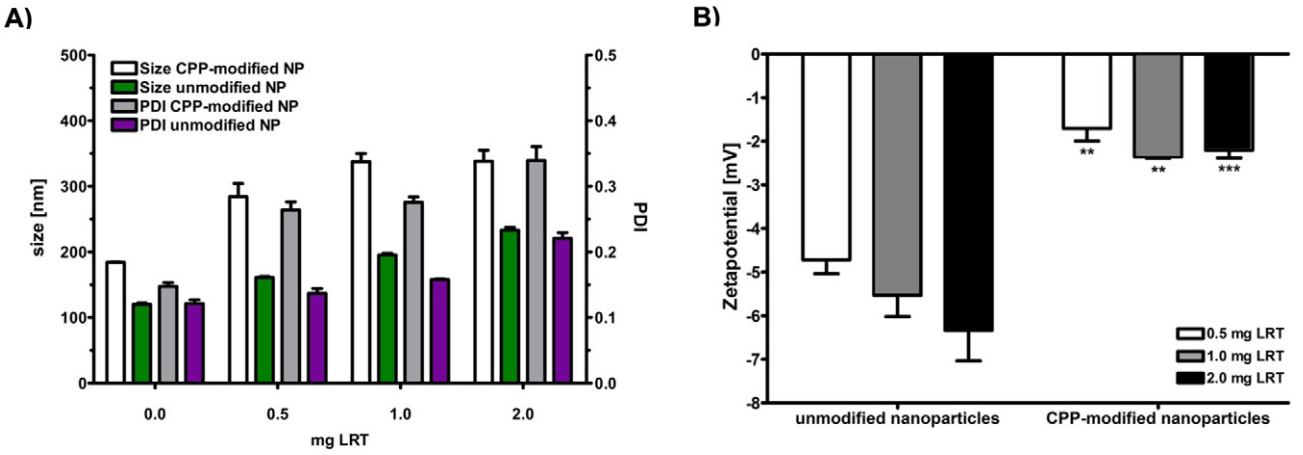

Figure 2. A) Size and PDI of unmodified and CPP-modified NP (n = 5) without LRT and at different LRT concentrations (0.5, 1.0 and 2.0 mg of LRT). It was shown that both, the surface modification of the NP and the incorporation of LRT led to a moderate increase in the size of the NP. In general, CPP-modified NP showed an increased size compared to unmodified NP. B) Zeta potential of different concentrations of LRT (induced by micelle effect) in solution (0.5% Tween® 85 in water) in comparison with the same amounts of LRT incorporated into unmodified NP and CPP-modified NP (n = 3). The significant increase of the zeta potential for the CPP-modified NP can be traced to the highly positive charge of the cyclic R9-CPP and therefore demonstrates the successful surface modification of the NP.

encapsulation efficiency. In comparison to encapsulation efficiencies obtained for other drugs such as doxorubicin (40–70%[50]) and insulin (40%[51] – 70%[18]), the CPP-modified nanoparticles for LRT provided comparable values.

Release of LRT

The release of LRT at various time points is shown in Figure 3, *B*. A time-depending release of LRT out of nanoparticles could be clearly demonstrated. For this reason, the nanoparticles were freeze-dried to obtain a dosage form with increased storage and particle stability.

Cryo-TEM

The Cryo-TEM images of unmodified and CPP-modified nanoparticles verified the size of the particles obtained by dynamic light scattering measurements (Figure 4). Furthermore, image analysis revealed a high homogeneity of the particle size and surface characteristics. Besides, the samples of both modified and unmodified nanoparticles showed additionally the occurrence of filaments, which are probably caused by the used polymer PLA. Nevertheless, these additional filaments do not hamper the use of the nanoparticles as oral drug delivery system. Additionally, after sterile filtration of the nanoparticles and subsequent Cryo-TEM imaging, the number of filaments could be decreased.

Freeze-drying

Previous findings[36,52,53] also showed, that saccharides as lyoprotectors can provide encouraging results for freeze-drying of nanoparticles. The freeze-drying of CPP-modified nanoparticles suspended in sucrose and trehalose as cryo protective agents was compared at different molar concentrations (100–700 mM). At ≥500 mM sucrose, particle sizes and PDI remained similar to pre-lyophilisation (Figure 5, *A*). With regard to trehalose, the highest concentration of the lyoprotector (700 mM) suggested some protection from an increase in particle size (120

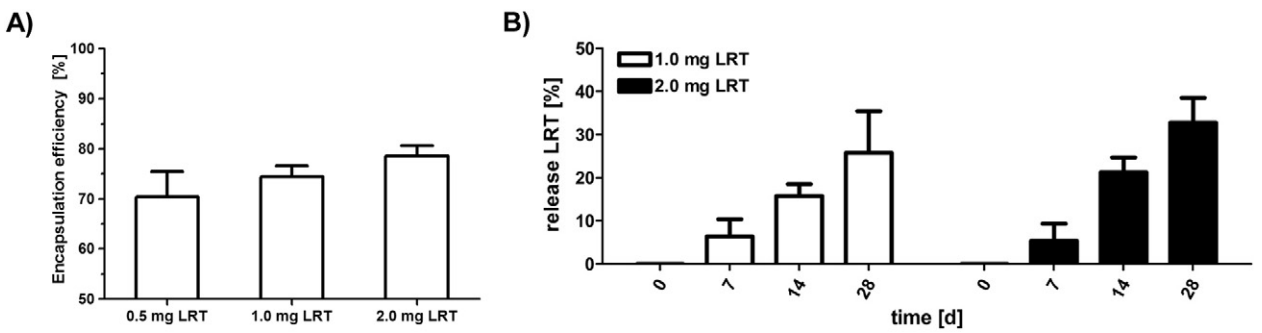

Figure 3. A) Encapsulation efficiency of various amounts of LRT in the CPP-modified NP (n = 5). No significant difference between the different LRT concentrations was observed, demonstrating that the encapsulation efficiency of the CPP-modified NP does not significantly depend on the amount of LRT used in this study. B) Release of LRT at different time points (n = 3). It is clearly shown, that LRT leaks out of the NP by storage at 2–8 °C independent of the LRT concentration. Therefore, in following studies, the nanoparticles were freeze-dried in order to obtain a solid dosage form for long term storage.

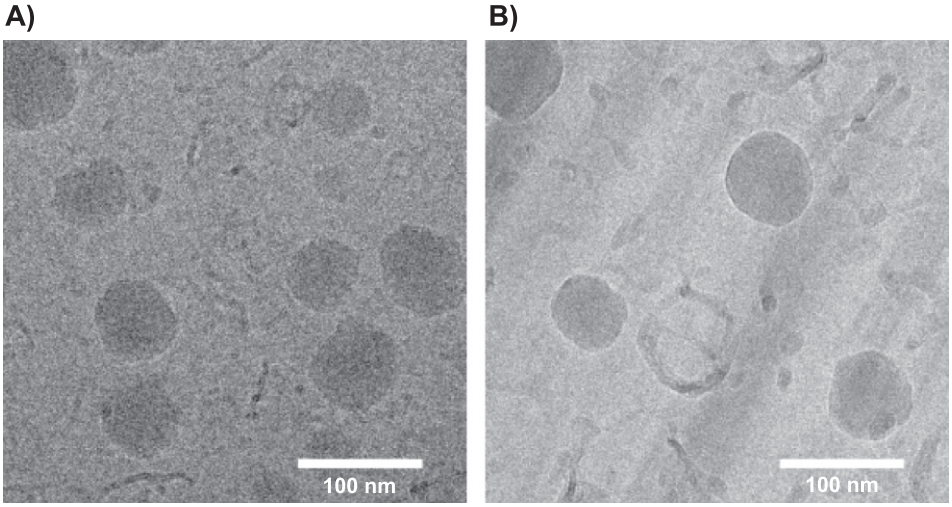

Figure 4. Cryo-TEM micrographs of unmodified (left, A) and CPP-modified NP (right, B). The micrographs show no noticeable difference in surface characteristics and size between modified and unmodified NP. For both formulations the high homogeneity of the particles is obvious.

nm prior to lyophilisation and ~180 nm after), but sucrose afforded much better preservation of the size and the PDI. Nevertheless, a further increase in the concentration of the lyoprotector (700 mM sucrose) did not result in vast improvements in protection of the size and PDI.

Particle retention, cytotoxicity and cell-binding assays

The particle retention assay using pig intestine could clearly confirm the prolonged adhesion to pig intestine of the CPP-modified nanoparticles compared to the unmodified particles (Figure 5, *B*).

Regarding the alamarBlue® assay, both, modified and unmodified PLA-nanoparticles showed no significant toxicity in all tested concentrations (Figure 6, *A*). Therefore, it could be claimed that the surface-modified nanoparticles are non-toxic as previously shown for PLA-nanoparticles in general.[54]

The cell binding studies using fluorescently-labeled LRT (green fluorescence, Atto495) and PLA (red fluorescence, Atto610) showed a strongly enhanced binding to Caco-2 cells for the CPP-modified nanoparticles over a period of 60 min (Figure 6, *B*) compared to the unmodified nanoparticles. The quantification of depicted images revealed a significantly enhanced uptake of fluorescently-labeled LRT-Atto495 for the CPP-modified nanoparticles in comparison to the unmodified ones (Figure S8). Importantly, this enhanced binding of the nanoparticles mediated by the CPP enables a prolonged retention time on the mucus of the intestine probably leading to increased uptake values. Permeation (Transwell®) studies highlight these findings, as after 3 hours of the nanoparticle exposure, the CPP-

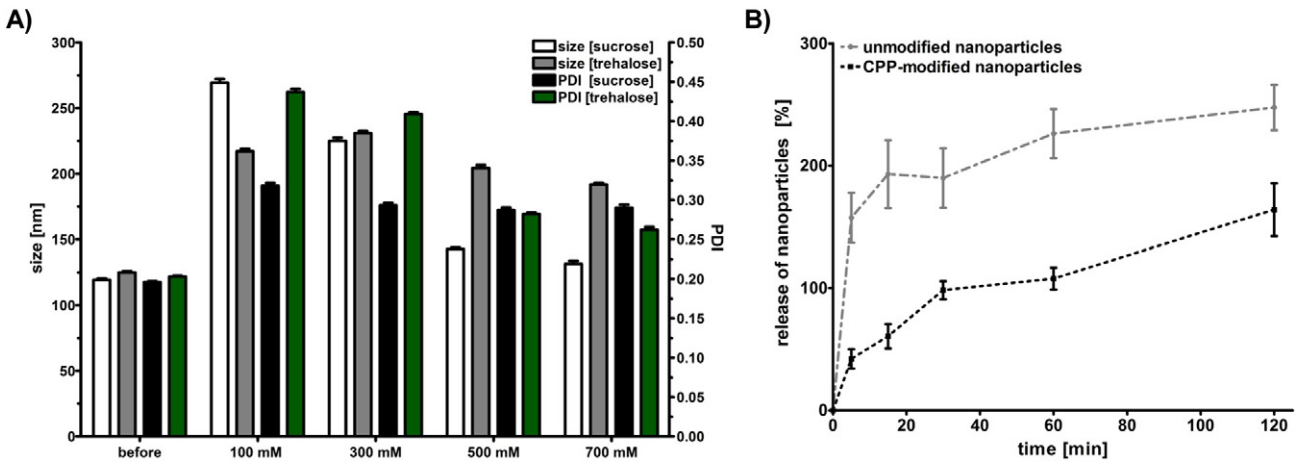

Figure 5. A) Size and PDI of the CPP-modified NP before and after freeze drying with two different lyoprotectors (sucrose and trehalose) at various molar ratios (n = 3). It is clearly shown that in general, both lyoprotectors show promising results at ≥500 mM concentration. Higher concentrations of the lyoprotector did not provide better results for NP characteristics. B) Particle retention assay of CPP-modified and unmodified NP using porcine intestine (n = 3). The amount of measured particles for each time point was compared with the previous time point (% increase in amount of free particles). A prolonged retention time of the CPP-modified nanoparticles in comparison to unmodified NP is shown.

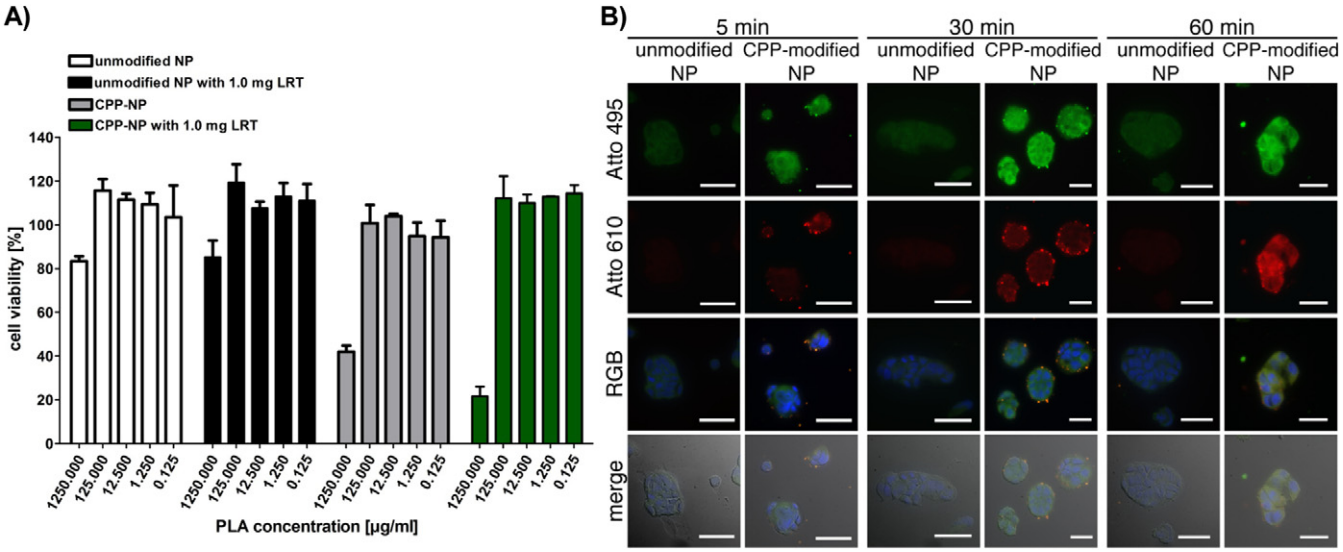

Figure 6. A) Cytotoxicity studies of nanoparticles with and without LRT (n = 3). It is clearly shown that both LRT and the surface modification of the nanoparticles with the cyclic R9-CPP do not influence the cytotoxicity of the NP. The highest concentration of the undiluted particles (1.25 mg/ml) is far beyond nanoparticle concentrations achievable in a clinical setting. B) Caco-2 cell binding assay of nanoparticles containing 0.5 mol-% of an Atto610 dye (red) on their surface. Incorporation of LRT coupled to an Atto495 dye (green) was determined at various time points (5 min, 30 min and 60 min). Nuclei were visualized by DAPI (blue). The accumulation of LRT inside Caco-2 cells is dramatically increased (green signal) upon use of the CPP-modified NP. Scale bar indicates 50 µm.

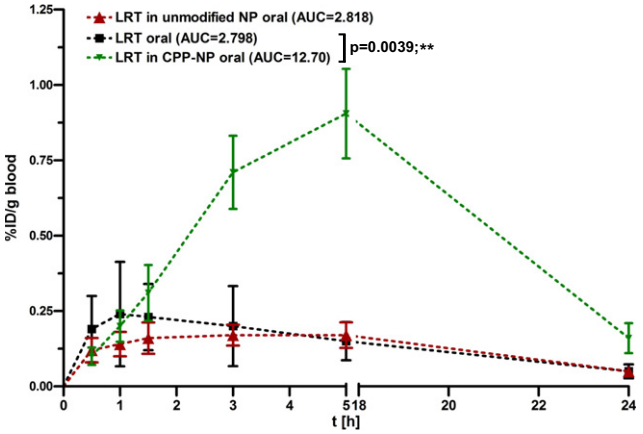

Figure 7. Blood levels of I-125 radiolabeled LRT in Wistar rats 0–24 h post oral administration (n = 3). LRT encapsulated into the CPP-modified NP showed significantly higher blood levels of LRT than free LRT after oral administration (P = 0.0039). Unmodified NP did not enhance the LRT bioavailability.

modified nanoparticles showed 1.5-fold higher permeation of LRT-Atto495, persisting till the endpoint. The permeation of the particles after 3 hours was twice as high, however slightly decreased to a 1.8-fold improvement of the CPP-modified nanoparticles at the end of the experiment (Figure S9 and Figure S10).

Proof of concept: animal study with female Wistar rats

The results of this study demonstrated a significant increase in the blood levels of I-125 labeled LRT for the nanoparticles modified with the cyclic R9-CPP (after oral administration) when compared with free I-125 labeled LRT (Figure 7). The stability of I-125-radiolabeled LRT was determined by a serum stability assay over 24 h. This assay revealed the stability of the radiolabeled compound used in this study (Figure S4). With respect to the animal study, the blood levels of LRT were significantly higher (P = 0.0039), which was also observed for the area under the curves (AUCs). The AUC of LRT using CPP-modified nanoparticles (12.70) was 4.5-fold higher than the AUC of free LRT (2.798). Interestingly, unmodified nanoparticles (AUC = 2.818) resulted in nearly the same oral.

bioavailability as the free peptide drug LRT demonstrating the need of the CPP surface-modification for enhancing oral delivery of peptide drugs. These results strongly highlight the benefit for oral administration of LRT by the use of the nanoparticles surface-modified by the cyclic R9-CPP.

Discussion

Thus far, only few examples for oral peptide delivery with CPPs exist. Nielsen et al.[27] and Morishita/Kamei et al.[28,29] investigated a co-administration strategy of insulin and CPPs and showed a significant benefit for the co-administration strategy. In these studies, linear CPPs were used. In order to improve this, we used a cyclic, arginine-rich cell penetrating peptide with improved enzymatic stability, which was covalently linked to the surface of the nanoparticles. Other studies tried to enhance the peptide stability in the intestine by the incorporation of D-form amino acids,[55] which is a less cost-effective strategy when compared to cyclized peptides. Furthermore, we could demonstrate that the cyclized CPP shows enhanced stability in simulated gastric and intestinal fluid compared with linear L- and also D-penetratin used in previous studies as demonstrated by HPLC/MS measurements (Figure S7). By the CPP-modification strategy used in this study, a strongly enhanced mucosal binding and penetration was achieved as demonstrated by the Caco-2 binding and uptake assay (Figure 6, *B*) leading to

strongly increased oral availability of LRT (Figure 7). For a linear R8 peptide, Kamei et al.[56] performed mechanistic studies for the uptake across membranes of Caco-2 cells and postulated an intestinal epithelial transport via energy-independent pathways. It can be assumed that related pathways also play a role in the penetration of CPP-modified nanoparticles. A particle retention assay using pig intestine demonstrated the prolonged retention time on the mucus (Figure 5, *B*), which might influence nanoparticle uptake and therefore the oral bioavailability of the incorporated peptide drug LRT.

Therefore, it could be claimed that the addition of CPPs for oral peptide delivery enhances the mucosal penetration and could therefore be a promising tool for oral delivery of poorly resorbed drugs.[29,57] Our studies showed no relevant cytotoxicity for the calculated target concentrations of the model R9-CPP tested in our Caco-2 assays (Figure 6, *A*). Only the highest concentration used in this assay (polymer concentration = 1.25 mg/ml), which is far beyond nanoparticle concentrations achievable in a clinical setting, showed a cytotoxic effect. Nevertheless, with respect to the dilution of the particles after oral administration by gastric and intestinal fluid, the nanoparticles showed a high cytocompatibility up to the calculated target concentrations. These encouraging results strongly recommend the further evaluation of cyclic CPPs as so-called resorption enhancers for peptide delivery.

But with respect to oral peptide delivery and to obtain a bioavailability sufficient for clinical use, further improvements such as prolonged mucosal adhesion obtained possibly by PEGylation as shown by Yang et al.[58] and Wang et al.[59] should be implemented. In addition, further studies need to focus on up-scaling and testing of these nanoparticles in larger mammals.

In this study, a promising oral delivery system for peptide drugs such as the model substance LRT was developed by the surface-modification of common PLA nanoparticles with a cyclic, arginine-rich CPP. A significant benefit of this novel formulation is the absence of toxicity in the target concentrations on Caco-2 cell assays. The optimized formulation method enabled the fast and reproducible preparation of nanoparticles. Interestingly, a strong dependence of the surfactants` HLB-value for nanoparticle characteristics (size, PDI) was demonstrated.

The main benefit of this nano-formulation was highlighted by animal trials using female Wistar rats, which showed a significant, 4.5-fold increase of the oral bioavailability of LRT using the surface-modified cyclic-R9 nanoparticles when compared to the free peptide or unmodified nanoparticles.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nano.2019.102132.

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