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NANOMEDICINE

Release and bioactivity of PACA nanoparticles containing D-Lys^6-GnRH for brushtail possum fertility control

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ABSTRACT

Poly(ethylcyanoacrylate) (PECA) nanoparticles containing the chemical sterilitant D-Lys^6-GnRH were prepared by an *in situ* interfacial polymerization technique. Their potential as a peroral delivery system for biocontrol of the brushtail possum, a major pest species in New Zealand, was evaluated. Peptide release from resulting particles was studied *in vitro* in artificial gastric juice (AGJ), simulated intestinal fluids (SIF) and brushtail possum plasma. The nanoparticles released a small fraction of bioactive over 6 h in AGJ and SIF (<5%), while staying intact and retaining fractions of intact D-Lys^6-GnRH. In contrast, 60% of D-Lys^6-GnRH was released after 1 h in possum plasma. The nanoparticles were also administered *in vivo* into the caecum of brushtail possums. A significant biological response, measured as an increase in plasma luteinizing hormone (LH), was evident 10 min after administration. This demonstrates not only that PECA nanoparticles were able to facilitate the uptake of D-Lys^6-GnRH from the caecum into systemic circulation but also that sufficient bioactive peptide reached the pituitary to exert a significant LH response following GnRH receptor mediated endocytosis. Hence, it can be concluded that PECA nanoparticles comprise a promising formulation strategy for the peroral delivery of the chemical sterilitant D-Lys^6-GnRH to the brushtail possum in New Zealand.

1. Introduction

The common brushtail possum (*Trichosurus vulpecula*) is a marsupial herbivore native to Australia that has become a pest species in New Zealand. The brushtail possum causes significant damage to native flora and fauna and is a reservoir for *Mycobacterium bovis*, the bacterium causing bovine tuberculosis [1]. Current methods of control (poisoning, shooting and trapping) have the disadvantage that they lack target specificity and humaneness, and they have failed to reduce the population of brushtail possums long term. The most publicly accepted strategy of possum population control is reducing their fertility [2]. Fertility control agents are likely to be protein and peptide bioactives [3], such as the neurohormone gonadotropin releasing hormone (GnRH), a key compound in the regulation of reproduction. D-Lys^6-GnRH is a potent GnRH agonist and may be employed as a regulatory hormone causing a downregulation of GnRH receptors and desensitization of pituitary gonadotroph cells. Alternatively, GnRH may be used as a toxin conjugate, such as the GnRH-PAP (pokeweed antiviral protein) conjugate, which is able to target gonadotropin producing cells specifically and cause cell death [1,4].

Any form of biocontrol requiring the individual handling of the animal is not feasible for wildlife. Self disseminating delivery systems (e.g. genetically modified viruses) raise safety concerns considering the proximity of New Zealand to Australia's protected marsupials [5]. In contrast, nondisseminating systems are safer but require aided delivery via bait and include carriers such as bacterial ghosts [6,7], virus like particles (VLPs) [1] and pharmaceutical polymeric nanocarriers [3]. Bacterial ghosts and VLPs largely retain their immunogenicity and so appeal as a delivery system for immunocontraceptives. Chemical sterilitants, such as GnRH derivatives, however, are direct acting and should avoid the immune system. Pharmaceutical nanocarriers, such as poly(alkylcyanoacrylate) (PACA) nanoparticles can be prepared not only to avoid the immune system but also to protect the bioactive and facilitate the uptake in the gut, while maintaining the bioactivity of the drug [8–11]. Furthermore, PACA nanoparticle formulations are easy to scale up, reproducible and affordable [12].

In situ loading of protein and peptide bioactives into PACA nanoparticles prepared by interfacial polymerization results in nanocapsules that confine the bioactive to the aqueous core and the polymer wall [13,14]. High entrapment efficiencies are reported for proteins and peptides [13,15,16]; however, they are accompanied by low release rates of drug in the absence of esterases [17,18]. Previously, we have demonstrated the covalent interference of some

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protein and peptide drugs, including D-Lys^6-GnRH, during the polymerization process, which may explain the low release of bioactive observed in some studies [19,20]. The sustained release of bioactive via the bioerosion of the polymer material has previously been suggested for peptide copolymerized PACA nanoparticles in the presence of esterases and rat plasma [21]. Whether or not peptide released from copolymerized PACA nanoparticles is still bioactive has been investigated only in a few studies. Gibaud et al. [17] demonstrated that the bioactivity of recombinant human granulocyte colony stimulating factor (rhG-CSF) *in situ* polymerized with PACA nanoparticles was lost. However, some authors have suggested that, despite the covalent association of proteins and peptides with PACA polymer, the *in vivo* bioactivity was maintained [18,22]. Tasset et al. [18] demonstrated the hypocalcemic effect of i.v. administered calcitonin-loaded PACA nanoparticles was equivalent to the an i.v. dose of free drug. Peptide copolymerized systems may maintain their bioactivity if the bioactive site remains unobstructed and the folding of the bioactive is unaffected by the modification. For D-Lys^6-GnRH, we have demonstrated that the modification occurs at the histidine residue [20], which is involved in receptor binding [23].

The aim of this study was to investigate the potential of PACA nanoparticles to deliver the fertility control agent D-Lys^6-GnRH to a wild target species, the brushtail possum, and induce a biological response. Poly(ethylcyanoacrylate) (PECA) nanoparticles containing D-Lys^6-GnRH were characterized *in vitro* for the amount of peptide released under physiological conditions. The *in vivo* bioactivity, measured as the plasma concentration of luteinizing hormone, was also determined following intracaecal administration in the brushtail possum.

2. Material and methods

2.1. Materials

D-Lys^6-GnRH (*p*-Glu-His-Trp-Ser-Tyr-Lys^6-Leu-Arg-Pro-GlyNH_2) was purchased from PolyPeptide Laboratories (Torrance, CA, USA). Ethyloleate GPR was sourced from BDH Laboratory Supplies (Poole, England) and surfactants sorbitan monolaurate (Crill 1™) and ethoxy 20 sorbitan mono-oleate (Crillet 4™) were kindly provided by BTB Chemicals (Auckland, New Zealand). The monomer ethylcyanoacrylate (ECA) (Sicomet 40) was kindly donated by Henkel Loctite (Hannover, Germany). Sodium chloride 0.9%, pre-packaged, sterile single dose containers (20 ml) was from David Bull Laboratories (Victoria, Australia). Methanol (HPLC grade), chloroform and hydrochloric acid (HCl) (fuming 37%, GR for analysis) were from Merck KGaA (Darmstadt, Germany). Ethanol was supplied by AnchorEthanol (Auckland, New Zealand). Sodium hydroxide pellets (NaOH) were purchased from Univar, Asia Pacific Specialty Chemicals Limited (Auckland, New Zealand). Potassium *di*hydrogenorthophosphate (KH_2PO_4) was purchased from BDH Laboratory Supplies (Poole, England). Sodium chloride (NaCl) (Sigma Ultra ≥ 99.5%), pepsin (porcine) and pancreatin powder (porcine, meeting the USP testing conditions) were purchased from Sigma Aldrich (St. Louis, MO, USA). For HPLC analysis, trifluoroacetic acid (TFA) was sourced from Sigma Aldrich (St. Louis, MO, USA) and acetonitrile (CH_3CN) (HPLC grade) was purchased from Merck KGaA (Darmstadt, Germany). Distilled, ultra pure water was obtained from a Milli-Q Millipore Purification System (Billerica, Massachusetts, USA) and was used at all times during HPLC analysis and sample preparation. Ovine LH (CY1085) was kindly donated by INRA (Nouzilly, France). Radioactive iodide (^{125}I) was purchased from NEN® radiochemicals (Perkin Elmer, MA, USA). Chloramine T, EDTA, sodium *di*hydrogen orthophosphate, *di*sodium hydrogen orthophosphate and sodium chloride were purchased from BDH Laboratory Supplies (Poole, England). Sodium metabisulphite was from Fluka/Riedel de Haën (Seelze, Germany). Bovine serum albumin (BSA) was purchased from ICPbio Ltd. (Auckland, New Zealand) and polyethylene glycol (PEG) 8000 was obtained from Scharlau Chemicals (Barcelona, Spain). The monoclonal primary antibody (Clone 518B7) was raised in mice against bovine LH (bLH) and purchased from Monoclonal Antibodies, Inc. (Mountain View, CA, USA). The second antibody raised in rabbit against mice (code Z109) was purchased from Dako Immunoglobulins (Glostrup, Denmark). Possum plasma with basal levels of plasma LH was used as filler plasma for the preparation of standard samples. Normal mouse serum was purchased from Sigma Aldrich (St. Louis, MO, USA). Assay buffer pH 7.4 was prepared as a solution of 0.5% (w/v) BSA and 2 mM EDTA in 0.01 M PBS pH 7.4. PEG buffer was prepared as a solution of 5% (w/v) PEG 8000 in assay buffer.

2.2. Methods

2.2.1. Preparation of PECA nanoparticles

The microemulsion used as a polymerization template for the preparation of PECA nanoparticles comprised 54% of a surfactant blend, 36% of oil and 10% of water and was prepared based on the method of Watnasirichaikul et al. [15]. For the preparation of 10 g formulation, 8 mg of D-Lys^6-GnRH was dissolved in the aqueous phase (1 ml). 10 g of microemulsion was polymerized with 200 µl ECA monomer dissolved in 600 µl of chloroform. The mixture was left overnight to complete the interfacial polymerization process and to evaporate the chloroform. Resulting nanocapsules were used for experiments the following day.

2.2.2. In vitro release studies

2.2.2.1. Test media. The passage through the gastrointestinal tract (GIT) following peroral administration was mimicked using simulated gastrointestinal fluids (BP specifications). Artificial gastric juice (AGJ) pH 1.2 and simulated intestinal fluid (SIF) pH 6.8 were prepared according to the BP 2007 (Appendix XII D). In addition to the BP specifications, the SIF was separated by centrifugation at 20 800 g to remove residual particulate pancreas matter from the test medium (Eppendorf Centrifuge 5417 C, t = 20 min, T = 25 °C). The enzymatic activity of the clear SIF was retained (confirmed by incubation of peptide containing microemulsion, resulting in instant degradation, data not shown). The final pH for AGJ and SIF were controlled and adjusted if necessary using 3% HCl or 0.1 M NaOH solutions. Incubation periods in AGJ and SIF were based on McDowell et al. [24], who investigated the residence times of fluids and particulate matter (<125 µm) in different sections of the brushtail possum gut.

In vitro release from isolated nanoparticles, mimicking systemic conditions after peroral absorption, was studied in plasma from brushtail possums collected via cardiac puncture prior to euthanasia (Pentobarb 50, ProVet NZ Pty Limited, Auckland, New Zealand). During blood collection, animals were anaesthetized (Vet isoflurane 100%, Merial New Zealand Ltd., Auckland, New Zealand). The plasma was separated from blood cells by centrifugation at 4 °C and 3000 rpm (Eppendorf Centrifuge Model 5810R) for 20 min. The plasma was pooled and stored at −80 °C until use.

2.2.2.2. Sample preparation for in vitro release studies. An aliquot of 1.0 g of nanoparticles in microemulsion was used for studying the release in 7.5 ml of test medium. Release in AGJ and SIF was studied for isolated PECA nanoparticles and nanoparticles in microemulsion. For nanoparticle isolation, 1.0 g formulation was mixed with 5 ml of ultra pure water pH 2.5 and 5 ml of methanol 80% (v/v, water pH 2.5) and spun in a centrifuge (Beckmann Optima L-80 Ultracentrifuge) at 20 000 g for 20 min. The nanoparticles were washed twice in ethanol (abs.), resuspended in the respective test medium by sonication and transferred to a double walled, temperature controlled beaker at 37 °C under continuous stirring (400 rpm) for up to 6 h. Samples taken

during incubation in AGJ or SIF containing microemulsion were mixed in a 1:1 volume ratio with the same amount of methanol 80% (v/v, water pH 2.5) or water for isolated nanoparticles. Samples taken during incubation in possum plasma were mixed in a 1:2 volume ratio with methanol 80% (v/v, water pH 2.5). All samples were centrifuged at 20 800 g (Eppendorf Centrifuge 5417 C, T = 25 °C) for 20 min. The D-Lys^6-GnRH content was quantified in the clear aqueous phase by gradient RP HPLC [25]. The nanoparticle pellet was used for further characterization by transmission electron microscopy (TEM) and matrix assisted laser desorption/ionization time of flight (MALDI TOF) mass spectrometry.

2.2.3. HPLC analysis

The gradient RP HPLC analysis (Table 1) was carried out using an Agilent™ series 1200 HPLC system (CA, USA) in combination with a Phenomenex (CA, USA) Jupiter C18 column (250 × 4.6 mm i.d., 5 μm particle size, 300 Å) protected by a C18 Phenomenex Analytical Guard cartridge column (KJ0-4282, 4.0 L mm × 3.0 ID mm). The method was linear over the concentration range 2.5–60 μg/ml [25]. The lower limit of detection (LLOD) and lower limit of quantification (LLOQ) were 0.13 μg/ml and 0.38 μg/ml, respectively [25].

2.2.4. Transmission electron microscopy (TEM)

PECA nanoparticles containing D-Lys^6-GnRH were examined before and after incubation in AGJ and SIF using freeze fracture TEM. The pellet remaining after separation from the release medium was washed two to three times with ethanol (abs.). Nanoparticles were resuspended in approximately 100 μl of ultra pure water. 1.5 μl of the concentrated nanoparticle suspension was sandwiched between two recessed copper double replica holders (Bal-Tec AG, Balzers, Liechtenstein). The samples were then frozen rapidly by plunging into liquid propane at −185 °C and were fractured at −110 °C using a Baltec BAF060 freeze fracture machine (Bal-Tec AG, Balzers, Liechtenstein). The samples were etched for 2.5 min at −100 °C prior to cooling to −110 °C. Surface replication was performed using a 2 nm layer of platinum and carbon deposited at an angle of 45° to the specimen surface, followed by a 20 nm layer of carbon deposited at an angle of 90°. The specimen was thawed and the replicas floated in water, which was then gradually mixed with acetonitrile to remove residual sample from the replica. Replicas were viewed using a Philips CM100 transmission electron microscope (Philips/FEI Corporation, Eindhoven, Holland) operating at an accelerating voltage of 100 kV and images captured using a MegaView 3 digital camera (Olympus Soft Imaging Solutions GmbH, Münster, Germany).

2.2.5. MALDI TOF mass spectrometry

PECA nanoparticles containing D-Lys^6-GnRH were examined after incubation in AGJ and SIF using MALDI TOF mass spectrometry (MS). The pellet remaining after separation from the release medium was washed twice with ethanol (abs.). Nanoparticles were dissolved in 20–40 μl acetonitrile. 1.0 μl of sample solution was mixed with 9.0 μl of matrix (10 mg/ml alpha cyano-4-hydroxycinnamic acid dissolved in 60% (v/v) aqueous acetonitrile containing 0.1% (v/v) TFA). An aliquot of 0.8 μl sample/matrix mix was spotted onto the MALDI plate (Opti-TOF 384 well plate, Applied Biosystems, MA, USA) and air dried. Samples were analyzed on a 4800 MALDI tandem TOF Analyzer (Applied Biosystems, MA, USA). The default calibration for each operation mode was updated on six calibration spots. All MS spectra were acquired in positive ion reflector mode with 1000 laser pulses per sample spot.

As previously described [20], the presence of free peptide $[M+H]^+$ and peptide/polymer conjugates $[M+(P)_n+H]^+$ was confirmed by collision induced dissociation tandem mass spectrometry (CID-MS/MS). Precursor ions of interest were selected and spectra acquired with 2000–4000 laser pulses per selected precursor using the 2 kV mode. Air was used as the collision gas at a pressure of $1 \cdot 10^{-6}$ torr. CID-MS/MS spectra were interpreted manually. Only b-, y- and a-type ions and relevant immonium ions were considered according to the Biemann nomenclature [26].

2.2.6. Sample preparation for the in vivo bioactivity study

2.5 g of PECA nanoparticle formulation containing a dose of 2 mg of D-Lys^6-GnRH was administered without the removal of the microemulsion template. Control formulation 1 contained 2 mg of D-Lys^6-GnRH in 5 ml of sodium chloride solution 0.9%. Control formulation 2 consisted of 2.5 g of PECA nanoparticles in microemulsion prepared without bioactive.

2.2.7. Animals

2.2.7.1. Source. Adult, male brushtail possums ($n = 15$) were wild caught in the Otago region of New Zealand. Animals were housed in environmentally enriched pens and given a mixed diet of fruit and cereal and water *ad libitum.* The body weight of the animals ranged between 2.65 and 4.0 kg. Three formulations were administered intracaecally; 2.5 g of the PECA nanoparticle formulation containing a dose of 2 mg of D-Lys^6-GnRH ($n = 5$); 2 mg D-Lys^6-GnRH in 0.9% NaCl ($n = 5$) and empty PECA nanoparticles ($n = 5$). This study had prior approval from the University of Otago Animal Ethics Committee, and conforms to the University of Otago Code of Ethical Conduct for the Manipulation of Animals, 1987.

2.2.7.2. Intracaecal administration. Animals were anaesthetized by an i.m. injection of Zoletil 100 (30 mg/kg) (Virbac Animal Health New Zealand, Auckland, New Zealand) and salivation was controlled by an i.m. injection of atropine (0.3 mg) (Phoenix Pharm Distributors Ltd, Auckland, New Zealand). The abdomen was clipped and a midline incision was made to expose the caecum. A catheter (Angiocath® 14 G × 135 mm, Deseret Pharmaceutical Co., Sandy, Utah, USA) was inserted into the caecum approximately 5 cm from the ileo caecal junction. The formulations were expelled as three aliquots (one third of the dose each) approximately 10, 8 and 6 cm distal to the point of catheter insertion to ensure that the epithelium was not damaged and absorption artificially enhanced. Each dose was expelled adjacent to the epithelium and closest to the side of attachment of the intestinal mesentery and vasculature. Following administration, the site of catheter insertion was sutured and the caecum returned to the abdominal cavity.

2.2.7.3. Blood collection and processing. Blood samples (1.5 ml) were taken via an indwelling jugular canula and collected into heparinized (50 IU/ml) Eppendorf test tubes. Blood samples were spun in a centrifuge (Eppendorf Centrifuge 5415 C) at 25 °C for 5 min at 1700 g. The plasma was aspirated and stored at −80 °C until analysis.

Table 1
Summary of instrumental settings for gradient RP HPLC analysis suitable for detecting D-Lys^6-GnRH in a microemulsion template [25].

Mobile phase	Solvent A: 0.1% TFA in ultra pure water Solvent B: 0.1% TFA in CH_3CN
Gradient	(1) 10–35% B for 12 min (peptide elution) (2) 35–90% B over 3 min (column flush) (3) 90–10% B over 5 min (4) 10% B for 10 min (column re equilibration)
Injection volume	50 μl
Flow rate	1.0 ml/min for steps (1), (2), and (4) 1.2 ml/min for step (3)
Temperature autosampler	4 °C
Temperature column	40 °C
Detector wavelength	220 nm

2.2.8. Radioimmunoassay for analysis of in vivo blood samples (LH levels)

The radioimmunoassay for determining luteinizing hormone (LH) in brushtail possum plasma was based on the method of Moore et al. [27]. For preparation of the radioligand, ovine LH was iodinated by the chloramine T method [28]. The nonspecific binding was 1.47% of the total counts and 13.3% of the radiolabel bound at a zero dose of bLH (0% displacement of radioactive ^{125}I ovine LH).

2.2.9. Statistical analysis

LH plasma concentrations were log transformed in order to normalize the variances before analysis. The differences in biological response (LH concentration) for the different formulations were tested by ANOVA, followed by Tukey's pairwise comparison using Minitab (Release 12.1, Minitab inc., PA, USA). P values<0.05 were considered to be significant.

3. Results and discussion

3.1. Physicochemical characterization of PECA nanoparticles containing D-Lys^6-GnRH

PECA nanoparticles *in situ* loaded with D-Lys^6-GnRH were on average 190 ± 0.5 nm in size with a polydispersity index of 0.077 ± 0.005 and a zeta potential of $+8.78 \pm 0.183$ mV. This is in consensus with our previous work [20]. The positive zeta potential gives a clear indication of the contribution of the positively charged peptide to the overall net charge of the otherwise negatively charged PECA nanoparticles either via the adsorption or copolymerization of peptide. The entrapment efficiency for D-Lys^6-GnRH was high (on average 98.6 ± 0.03%), which is also in agreement with our previous findings [20].

3.2. In vitro release in artificial gastric juice (AGJ)

Release studies in artificial gastric juice pH 1.2 were conducted for both nanoparticles contained in microemulsion and isolated nanoparticles. The peptide release from nanoparticles contained in microemulsion was similar to the release found in water pH 7.4 [20]. For nanoparticles contained in microemulsion, the amount of D-Lys^6-GnRH detected in the medium at t = 0 min is consistent with the amount unentrapped (<3%). No burst release was observed. Following an initial lag phase, the cumulative peptide release after 6 h was less than 5% (Fig. 1).

This release pattern is also in agreement with Aboubakar et al. [29], who found 10% insulin released from poly(butylcyanoacrylate) (PBCA) nanoparticles after 5 h of incubation in AGJ. The *in vitro* release characteristics of a peptide copolymerized PACA nanoparticle system were recently studied in phosphate buffer (PB) by Liang et al. [30]. PACA nanoparticles, copolymerized with a derivative of the peptide representing the antigenic epitope of lymphocytic choriomeningitis virus glycoprotein ($LCMV_{33-41}$), released only 10% of the encapsulated peptide over a period of 6 h in PB pH 8.0. In contrast, no peptide was released from isolated nanoparticles over 6 h (Fig. 1). Thus, peptide release detected was a result of peptide released from the microemulsion rather than from nanoparticles. The comparison of TEM images at $t = 0$ and $t = 6$ h of AGJ incubation indicates that pepsin did not affect the integrity of the PECA nanoparticle for the duration of the experiment (Fig. 1). This is in agreement with the literature [9,31] and desirable, since around 95% of the bioactive is retained and protected within the PECA nanoparticle. The harsh acidic conditions and presence of enzymes (pepsin) in the stomach or in this case, in the AGJ, degrade the peptide, as was observed for the incubation of peptide in solution (Fig. 2).

The presence of intact, free peptide at m/z 1253.7 and peptide/ECA conjugates after 6 h of incubation in AGJ was confirmed by MALDI TOF mass spectrometry (Fig. 3a). For peptide/ECA conjugates, the parent mass is shifted by a multiple of 125 mass units $(P)_n$, which is the equivalent of an ECA monomer subunit. CID fragmentation of copolymer precursors confirmed the identity of histidine modified peptide covalently associated with polymer subunits (data not shown).

Signals corresponding to peptide degradation products were absent (Fig. 3a). Similar to the release of D-Lys^6-GnRH studied in water pH 7.4 [20], the relatively high degree of peptide association with polymer, helps explain the observed low level of peptide released from the nanoparticles in AGJ. The occurrence of free peptide in the mass spectra following a 6 h incubation period (Fig. 3a) can be explained by strong electrostatic interactions between released, positively charged peptide (isoelectric point (pI) 9.99) and the negatively charged PECA nanoparticle material. In the current study, peptide released in AGJ from PECA nanoparticles contained in microemulsion is expected to be stabilized by the microemulsion, since the microemulsion control, containing the equivalent amount of peptide, improved the stability of D-Lys^6-GnRH significantly compared to peptide in solution (Fig. 2). This is consistent with the protective effects of microemulsions reported in the literature [32,33].

3.3. In vitro release in simulated intestinal fluid (SIF)

Release studies in simulated intestinal fluid (SIF) pH 6.8 were also conducted for both nanoparticles in microemulsion and isolated nanoparticles. No release of D-Lys^6-GnRH was detectable for either preparation (Fig. 4). The peptide in microemulsion and solution was

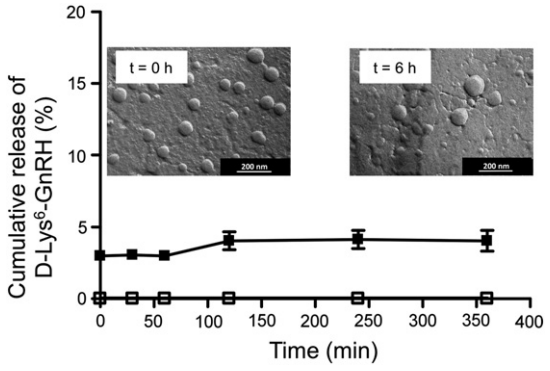

Fig. 1. Cumulative release of D-Lys^6-GnRH from PECA nanoparticles contained in ME (■) and from isolated PECA nanoparticles (□) in artificial gastric juice pH 1.2 monitored over 6 h. Data points represent mean ± SD ($n = 3$). The inserts show TEM micrographs of intact PECA nanoparticles from samples taken at $t = 0$ min and $t = 360$ min.

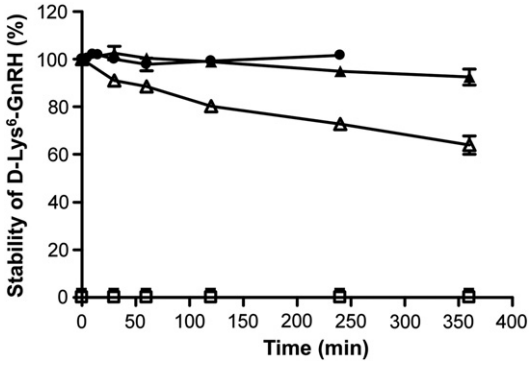

Fig. 2. Stability of D-Lys^6-GnRH monitored in various media. Peptide content was monitored for 6 h for peptide in microemulsion (▲) and peptide in water (△) when incubated in artificial gastric juice pH 1.2 and peptide in microemulsion (■) and peptide in water (□) when incubated in simulated intestinal fluid pH 6.8. Peptide content was monitored for 4 h for peptide in water (●) when incubated in brushtail possum plasma. Data points represent mean ± SD ($n = 3$).

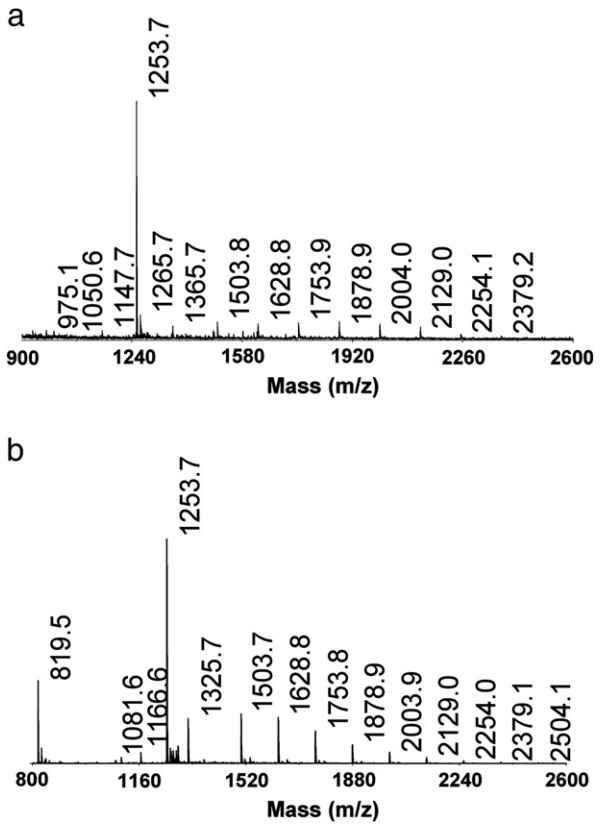

Fig. 3. MS spectra of PECA nanoparticles after incubation in (a) artificial gastric juice pH 1.2 for 6 h and (b) simulated intestinal fluid pH 6.8 for 6 h. In both spectra, free peptide $[M+H]^+$ is visible at m/z 1253.7 and peptide/ECA copolymers $[M+(125)_n+H]^+$ at m/z 1503.8, 1628.8, 1753.9, 1878.9, 2003.0, etc.

degraded instantly upon contact with SIF test medium (Fig. 2). This means that all free peptide is degraded instantly in SIF, and that the true quantity of peptide released from PECA nanoparticles in SIF cannot be accurately quantified.

These findings are in contrast to the study of Aboubakar et al. [29], who investigated the release of fluorescently labelled insulin from PBCA nanoparticles in SIF (USP XXIII) over 5 h. These authors found that the PBCA nanoparticles degraded, releasing a larger fraction (75%) of the bioactive via bioerosion [29]. It is not clear why the enzymes present in the pancreatin in their study did not affect the stability of free insulin in the release medium. It is possible that using fluorescent detection of the label as a method of quantification, rather than the direct quantification of insulin, the release of the label was monitored rather than the release of insulin.

The TEM images taken at $t=0$ h and $t=6$ h of SIF incubation in this study demonstrate that the nanoparticles stayed largely intact upon incubation in SIF for 6 h (inserts in Fig. 4). This is again advantageous, since the intact nanoparticle may continue to protect the unreleased peptide bioactive from enzymatic degradation in the intestine and facilitate the translocation into the systemic circulation. The presence of intact, free peptide and peptide/ECA conjugates after 6 h of incubation in SIF was confirmed by MALDI TOF mass spectrometry (Fig. 3b). This is in agreement with Damge et al. [9], who found PBCA nanocapsules preserved up to 75% of insulin within the nanoparticle after incubation with pepsin, trypsin and chymotrypsin containing media. However, the protective effect of a microemulsion on peptide stability, as demonstrated for the incubation in AGJ, could not be confirmed in this experiment for SIF (Fig. 3).

3.4. In vitro release in possum plasma

Peptide release in possum plasma was studied for isolated nanoparticles only. In contrast to the release studied in other media, PECA nanoparticles released approximately 60% of D-Lys^6-GnRH after 1 h in possum plasma following an initial burst release of about 20% (Fig. 5). After 1 h, no further peptide was released. This might be explained by the action of hydrolytic enzymes present in the plasma to free peptide immobilized via copolymerization with alkylcyanoacrylate monomer. The half life of the PACA polymers, however, seems highly dependent on the nature of the polymer. Mueller et al. [34] found the half life of PECA in calf serum to be <2 min compared to >12 h for PBCA. Another important characteristic when interpreting other release studies is the mode of drug entrapment. Systems with drug copolymerized with polymer may behave differently to homogenous polymer systems [35]. For example, Tasset et al. [18] loaded PBCA nanoparticles *in situ* with calcitonin and found that no bioactive was released after 5 h of incubation in Hank's balanced saline solution pH 7.4 containing esterases.

Released D-Lys^6-GnRH remained stable in possum plasma over the study period of 4 h and was not degraded by plasma enzymes (Fig. 2). The plasma half life of D-Lys^6-GnRH is prolonged compared to endogenous GnRH, which has a half life of 2–8 min in human blood [36,37] and 18 ± 2.5 min the brushtail possum [38]. The exchange of an L-amino acid by a D-amino acid within the bioactive molecule is a common strategy to produce GnRH super agonists with higher receptor affinities and prolonged half lives [23,39]. These results are generally in agreement with the literature, where enhanced release of bioactives from PACA nanoparticles via bioerosion in the presence of esterases is propagated [17,18,21]. In the present study, release reached a plateau after 1 h and no further peptide was released over the next 3 h. Couvreur et al. [40] found 50% of covalently associated

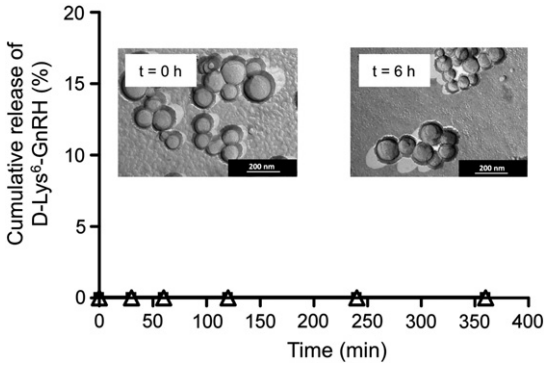

Fig. 4. Cumulative release of D-Lys^6-GnRH from PECA nanoparticles contained in ME (■) and from isolated PECA nanoparticles (Δ) in simulated intestinal fluid pH 6.8 monitored over 6 h. Data points represent mean ± SD ($n=3$). The insert shows TEM micrographs of intact PECA nanoparticles from samples taken at $t=0$ min and $t=360$ min.

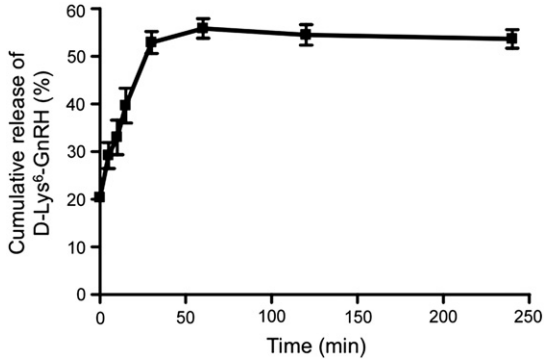

Fig. 5. Cumulative release of D-Lys^6-GnRH from isolated PECA nanoparticles in brushtail possum plasma monitored over 4 h. Data points represent mean ± SD ($n=3$).

drug was unreleased after 2 days of incubation in calf serum, demonstrating slow degradation kinetics for copolymerized PECA nanoparticles. Little is known about the exact composition of possum plasma and the classification of their plasma proteins. It is possible that different fractions of enzymatic proteins are responsible for the mobilization of copolymerized peptide from the nanoparticle and the particle degradation.

The release of 60% of the bioactive after 1 h in plasma, however, is an important result, since the main purpose of a peroral delivery system is to retain and protect the bioactive during the gastrointestinal passage and potentially release the bioactive rapidly following uptake into the blood circulation. In this release study, less than 5% of D-Lys^6-GnRH was released in simulated gastrointestinal fluids, whereas 60% was released in possum plasma. Free d-Ly^6-GnRH thus becomes available in sufficient quantities to show a biological effect.

3.5. In vivo bioactivity determined by radioimmunoassay (RIA)

The LH response following the intracaecal administration of PECA nanoparticles containing D-Lys^6-GnRH was significantly ($P<0.05$) higher than the response to any control formulation (Fig. 6). The AUC of the mean plasma LH concentration was 367.9 compared to 162.2 for empty PECA nanoparticles and 85.95 for D-Lys^6-GnRH in solution (Fig. 6). Basal values of <1 ng/ml, as determined for empty nanoparticles or peptide in solution, are in agreement with values in the literature [27,41]. Basal LH plasma levels are exceeded by the peptide containing nanoparticle formulation after as little as 10 min following intraceacal application (Fig. 6). This demonstrates that incorporation of D-Lys^6-GnRH into PECA nanoparticles facilitated the uptake of the intact bioactive into the systemic circulation. No biological response was observed when 2 mg of D-Lys^6-GnRH was administered in solution (Fig. 6).

The highly hydrophilic character of the D-Lys^6-GnRH molecule may have impaired uptake by gut epithelial cells and peptide in solution would be degraded by enzymes present in the caecum. This theory is supported by the findings of the *in vitro* incubation study that d-Ly^6-GnRH is a substrate of degrading enzymes present in intestinal fluids (Fig. 4). Similar to the current findings, Hillary et al. [22] could not detect any GnRH in plasma after oral dosing of a GnRH-vinylacetate solution to rats. Enzymatic degradation of protein and peptide bioactives in the caecum is reported to occur, however, to a lesser extent compared to the small intestine [16,42,43]. Wen et al. [44] investigated the stability of native GnRH in four regions of the brushtail possum gut. In the hindgut, 50% of GnRH was degraded within 3 h [45]. Thus, the stability of the D-Lys^6-GnRH analogue in solution was also compromised in this current study.

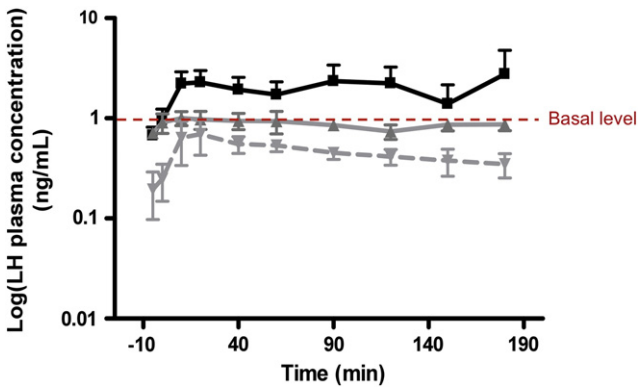

Fig. 6. Plasma concentration of luteinizing hormone (LH) in the brushtail possum following intracaecal administration of D-Lys^6-GnRH contained in PECA nanoparticles (dose was 2 mg D-Lys^6-GnRH) (solid black line) compared to intraceacal administration of D-Lys^6-GnRH in 0.9% sodium chloride solution (2 mg/5 ml) (dashed grey line) and empty PECA nanoparticles (solid grey line). Data points represent mean ± SD ($n=5$). The limit for basal LH level is indicated by the dashed black line.

The significantly increased LH levels achieved by the administration of bioactive copolymerized with PECA nanoparticles in this study, demonstrated that following the translocation of the bioactive into the systemic circulation, D-Lys^6-GnRH was transported within the blood stream to the pituitary. In the pituitary gonadotroph cells, the bioactive was internalized via GnRH receptor mediated endocytosis and produced a significant biological response. This suggests that D-Lys^6-GnRH, either in its free form following the *in vivo* cleavage of the polymer bound peptide or as the polymer conjugated form, still exhibited sufficient potential to react with the GnRH receptor despite the initial conjugation of the His^2 residue to polymer. The efficacy of the receptor binding and subsequent internalization of bioactive into gonadotroph cells was sufficient to produce a significant LH response in the brushtail possum after as little as 10 min.

Increased levels of LH as a response to a GnRH stimulus in the brushtail possum is in agreement with the literature [27,41]. Moore et al. [27] challenged both male and female possums with intravenous doses of mammalian and chicken II GnRH. The animals responded with a significant LH response to both forms of GnRH 15 min following administration. The response in males seemed to be extended compared to that in females and lasted for longer than 60 min [27]. In the current study, the LH levels were maintained for up to 3 h in male possums and so D-Lys^6-GnRH administered in a formulation with PECA nanoparticles is bioactive for an extended period of time. A direct comparison of the current study to previous studies, however, is difficult, since D-Lys^6-GnRH was administered intracaecally compared to i.v. administration in other studies. The dose reaching the systemic circulation is unknown in this study. However, the determination of the bioavailability of D-Lys^6-GnRH was not the subject of this study. Future work should investigate the pharmacokinetics of perorally administered D-Lys^6-GnRH in PECA nanoparticle to the brushtail possum and the dose dependent effects of the LH response.

Another question is whether the potency of the administered formulation is sufficient to induce sterility in the animal. D-Lys^6-GnRH, as a chemical sterilitant, may induce sterility either due to chronic exposure to the hormone analogue and subsequent downregulation of GnRH receptors or as a single dose of a disruptive toxin conjugate. From this study it may be concluded that the internalization efficacy of the bioactive via the GnRH receptor was significant. The conjugation of a toxin to the Lys^6 residue of the bioactive molecule has previously been shown to not affect the receptor binding properties [46]. Thus, from this study it is expected that toxins can be made available within the gonadotroph cells in the pituitary following the D-Lys^6-GnRH mediated cell uptake and disrupt gonadal function.

How chronic exposure of GnRH analogues affects LH levels as a primary response and steroid levels as secondary response in marsupials has been investigated in a few studies [41,47]. In brushtail possums, an implant of the GnRH agonist deslorelin initially increased LH response for both genders [41], followed by a drop to basal levels by 24 h and remained basal for 9 weeks. An i.v. challenge with exogenous GnRH after 9 weeks did not induce an LH response. Steroid levels fell below castration levels both in male and in female animals after 4 weeks [41]. In the current study, only the immediate effects of D-Lys^6-GnRH on LH response were determined, not the long term effects on testosterone levels. However, it is likely that the LH response observed in this study would translate into decreases of testosterone levels below castration levels in male brushtail possums and similar drops of progesterone levels below castration in female brushtail possums when administered chronically (D.C. Eckery, pers. comm.). If D-Lys^6-GnRH is to induce sterility due to the chronic exposure of the animal to the neurohormone, long term studies would be necessary, monitoring steroid plasma levels as an indication of sterility and birth rates in female brushtail possums.

4. Conclusions

PECA nanoparticles comprise a feasible formulation strategy for the peroral delivery of the chemical sterilitant D-Lys^6-GnRH to a target species, the brushtail possum. PECA nanoparticles *in situ* loaded with D-Lys^6-GnRH showed little release (<5%) of bioactive in AGJ and SIF, while the particles stayed intact over the duration of 6 h (TEM) and retained large fractions of intact peptide (MALDI TOF MS). In contrast, about 60% of D-Lys^6-GnRH was released within 1 h when incubated in the presence of particle degrading enzymes in brushtail possum plasma. The important finding in the present study was that PECA nanoparticles, containing the bioactive D-Lys^6-GnRH, were able to facilitate the uptake of the bioactive from the caecum of the brushtail possum followed by delivery of the bioactive to the pituitary gonadotroph cells resulting in a significant biological response *in vivo*. The ability of D-Lys^6-GnRH to bind to the GnRH receptor was not impaired by the chemical modification and conjugation of His^2 to PECA polymer. Thus, PACA nanoparticles, especially when copolymerized with bioactives, are useful as a delivery system with modified release for veterinary applications.

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