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# A novel approach for the intravenous delivery of leuprolide using core-cross-linked polymeric micelles

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ABSTRACT

Therapeutic peptides are highly attractive drugs for the treatment of various diseases. However, their poor pharmacokinetics due to rapid renal elimination limits their clinical applications. In this study, a model hormone peptide, leuprolide, was covalently linked to core-cross-linked polymeric micelles (CCL-PMs) via two different hydrolysable ester linkages, thereby yielding a nanoparticulate system with tuneable drug release kinetics. The ester linkage that provided the slowest peptide release kinetics was selected for in vivo evaluation. Compared to the soluble peptide, the leuprolide-entrapped CCL-PMs showed a prolonged circulation half-life (14.4 h) following a single intravenous injection in healthy rats and the released leuprolide was detected in blood for 3 days. In addition, the area under the plasma concentration–time curve (AUC) value was >100-fold higher for leuprolide-entrapped CCL-PMs than for soluble leuprolide. Importantly, the released peptide remained biologically active as demonstrated by increased and long-lasting plasma testosterone levels.
This study shows that covalent linkage of peptides to CCL-PMs via hydrolytically sensitive ester bonds is a promising approach to achieving sustained systemic levels of peptides after intravenous administration.
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## 1. Introduction

Over the past decades, peptides have emerged as promising therapeutic agents for the treatment of cancer, metabolic disorders, cardiovascular and a variety of other society-burdening diseases [1]. Compared to other biologics (e.g., antibodies), peptides have many advantages such as higher potency, less immunogenicity and easier synthesis and modification [2–4]. However, the development of therapeutic peptides for clinical application still faces substantial challenges. To mention, peptides generally have poor pharmacokinetics. Due to their small molecular size, peptides are rapidly eliminated through the kidneys leading to their short plasma half-life, typically ranging from few hours to minutes [5]. For this reason, frequent dosing is required to achieve therapeutic effects. Moreover, peptides are also susceptible to proteolytic degradation which renders them ineffective [5].

To overcome these limitations of therapeutic peptides, various delivery systems have been developed to enhance the efficacy of peptides through the improvement of their pharmacokinetics and biodistribution profile [6]. For example, the circulation kinetics of peptides can be improved through conjugation to polymers (e.g., polyethylene glycol, polysialic acid), oligosaccharides (e.g., cyclodextrins) or proteins (e.g., human serum albumin) [7–10]. Besides chemical conjugation, peptides can also be noncovalently incorporated into biodegradable long-acting release matrices, such as poly(D,L-lactide-co-glycolide) (PLGA) microparticles, which protects them against degradation and allows their sustained release [6,11]. To maintain prolonged therapeutically relevant plasma levels of peptides (essential for e.g., peptide hormones), peptide formulations are often administered via the subcutaneous (s.c.) or intramuscular (i.m.) route. Such routes of administration allow sustained release of peptides from the locally administered formulations leading to prolonged systemic exposure to the peptide. In the present study, we propose a novel approach for obtaining sustained plasma levels of a peptide by attaching the peptide via a hydrolytically sensitive bond to long-circulating core-cross-linked polymeric micelles (CCL-PMs) after intravenous (i.v.) administration.

Polymeric micelles are self-assembled colloidal particles composed of amphiphilic block copolymers. Their size, typically <100 nm, depends on the molecular weight and the characteristics of the amphiphilic block copolymers [12,13]. Owing to the steric stability provided by the hydrophilic shell and their small size, polymeric micelles can circulate in

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blood for extended periods by evading the mononuclear phagocytic system (MPS) and yet not excreted by kidneys [14–18]. Several polymeric micellar formulations have undergone clinical evaluations as recently reviewed by Cabral et al. [19]. However, a major challenge for polymeric micelles after i.v. administration is their poor in vivo stability as a result of dilution and adsorption of unimers to plasma proteins (e.g., albumin and lipoproteins) [15,20]. To stabilize polymeric micelles for in vivo applications block copolymers can be cross-linked in the micellar core [21,22]. Furthermore, instead of physical encapsulation, drugs can be covalently entrapped in polymeric micelles to prevent premature drug release from the micelles [23–25].

In this study, CCL-PMs were explored to prevent the rapid renal elimination of therapeutic peptides and to slowly release these peptides in the systemic circulation. Previously, micellar systems based on block copolymers of poly(ethylene glycol) (PEG) and poly(N-(2-hydroxypropyl)methacrylamide-oligolactates) (pHPMAmLac$_n$) have been successfully applied to target dexamethasone and the anticancer drug doxorubicin for the treatment of rheumatoid arthritis and tumours in animals, respectively [23,24,26]. Using this technology, in the present study a model peptide (leuprolide) was covalently linked to CCL-PMs via hydrolysable linkers.

Leuprolide is a potent agonistic analogue of gonadotropin releasing hormone (GnRH), which inhibits the secretion of pituitary gonadotropin and suppresses testicular and ovarian steroidogenesis when administered at therapeutic doses [27,28]. Interestingly, short-term use of leuprolide stimulates pituitary gonadotropin release and briefly increases testosterone levels, while long-term administration induces inhibition of the pituitary-gonadal axis due to down-regulation of the GnRH pituitary receptors leading to reduced systemic testosterone levels and so-called 'chemical castration' in men [29]. However, leuprolide in its free form is rapidly cleared from the bloodstream following parenteral administration, with a biological half-life of ~3 h in healthy male volunteers [30]. The use of CCL-PMs aims to prevent the rapid elimination of leuprolide and achieve sustained bioactive leuprolide levels in the systemic circulation.

In the present study, leuprolide was covalently linked to the micellar core via two different hydrolysable linkages based on either a sulfide or a sulfoxide ester. The in vitro release profiles of both micellar dispersions were compared, and the leuprolide-entrapped CCL-PMs with the slower release kinetics was selected for in vivo assessment. The pharmacokinetic profile of the selected leuprolide-entrapped CCL-PMs was evaluated in healthy rats. Furthermore, the bioactivity of released peptide from these leuprolide-entrapped CCL-PMs was determined by measuring plasma testosterone levels.

## 2. Materials and methods

### 2.1. Materials

Leuprolide HCl (pGlu-His-Trp-Ser-Tyr-Leu-Leu-Arg-Pro-$NHC_2H_5$, molecular mass 1209.5 Da) and the internal standard for leuprolide (pGlu-His-Trp-Ser-Tyr-Ala-Leu-Arg-Pro$NHC_2H_5$) were obtained from Bachem AG (Bubendorf, Switzerland). Testosterone-17β (androst-4-ene-17β-ol-3-one) and internal standard testosterone-17β-d3 were obtained from Steraloids (Newport, RI) and CDN-Isotopes (Quebec, Canada) respectively. N,N′-dicyclohexylcarbodiimide (DCC), 4-dimethylaminopyridine (DMAP), 4-methoxyphenol, methacrylic anhydride, potassium persulfate (KPS), tetramethylethylenediamine (TEMED), trifluoroacetic acid (TFA), ammonium acetate and formic acid were purchased from Sigma Aldrich (Zwijndrecht, The Netherlands). N,N-dimethylformamide (DMF) and acetonitrile (ACN) were purchased from Biosolve (Valkenswaard, The Netherlands). Triethylamine (TEA) was purchased from Merck (Darmstadt, Germany). The monomers N-2-hydroxypropyl methacrylamide monolactate (HPMAmLac$_1$) and N-2-hydroxypropyl methacrylamide dilactate (HPMAmLac$_2$) as well as the initiator (mPEG$_{5000}$)$_2$-ABCPA were synthesized as described previously [31]. The other chemicals were used as received.

### 2.2. Synthesis and analysis of leuprolide-derivatives

#### 2.2.1. Synthesis of leuprolide-L1

2-((2-(Methacryloyloxy)ethyl)thio) acetic acid (L1) was synthesized as described previously [24] (Fig. 1A). Next, leuprolide was conjugated to L1 as illustrated in Fig. 1B. In brief, leuprolide (0.12 mmol), L1 (0.30 mmol) and DMAP (0.30 mmol) were dissolved in 3.7 mL DMF. Subsequently, DCC (0.33 mmol) was added and the resulting mixture was stirred for 16 h at room temperature. Next, the reaction mixture was filtered and then evaporated at 45 °C under reduced pressure. The residual oil was purified using preparative HPLC (Agilent 1100/1200 integrated with Waters Sunfire Prep C18 5 μm OBD 30 × 50 mm column; eluent A: 95% $H_2O$/5% ACN/0.1% formic acid; eluent B: 5% $H_2O$/95% ACN/0.1% formic acid) and freeze-dried to obtain leuprolide-L1 (LeuL1) as fluffy white powder.

#### 2.2.2. Synthesis of leuprolide-L2

2-((2-(Methacryloyloxy)ethyl) sulfinyl)acetic acid (L2) was synthesized essentially as previously described with minor modifications [24] (Fig. 2A). In brief, compound 2 (5.12 mmol) was dissolved in ACN (18 mL) and mixed with a solution of sodium periodate (7.68 mmol) in $H_2O$ (18 mL). The reaction mixture was stirred for 16 h at room temperature and then filtered. The filtrate was extracted three times with ethyl acetate. The combined organic layers were washed with brine, dried over $Na_2SO_4$ and evaporated to dryness to obtain an oil which solidified upon standing. The resulting solid was dissolved in diethyl ether (50 mL) and cooled to −40 °C. The obtained precipitate was filtered, washed with cold diethyl ether and dried to obtain compound 3 (61% yield) as a white solid. Next, compound 3 (4.67 mmol) and a trace amount of 4-methoxyphenol (to prevent premature polymerization) were dissolved in cold TFA (2.95 mL) under nitrogen and stirred in an ice bath for 2 h. Thereafter, TFA was removed by evaporation in vacuo and coevaporation with toluene. The resulting yellow oil was stirred with diethyl ether (20 mL) for 15 min and the precipitate was filtered to obtain L2 as a white solid (38% yield). Subsequently leuprolide was conjugated to L2 to obtain leuprolide-L2 (LeuL2) using the same method as described in Section 2.2.1. (Fig. 2B).

#### 2.2.3. Analysis of leuprolide derivatives

The molecular mass of leuprolide derivatives was determined using electrospray ionization mass spectrometry (ESI-MS) on a Shimadzu liquid chromatography–mass spectrometry (LC–MS) QP8000 in positive ion mode. A Gemini® 3 μm C18 column (150 × 3 mm) (Phenomenex) was used with a gradient from 100% eluent A (95% $H_2O$/5% ACN/0.1% TFA) to 100% B (5% $H_2O$/95% ACN/0.1% TFA) in one hour with a flow of 1 mL/min and UV-detection at 253 nm. The mass used to identify leuprolide-L1 and leuprolide-L2 was m/z 1396 $(M + H)^+$ and m/z 1412 $(M + H)^+$, respectively.

The synthesized leuprolide derivatives were measured by nuclear magnetic resonance (NMR) spectroscopy on a Bruker AVANCE III HD 700 MHz spectrometer equipped with a TCI cryoprobe, using DMSO-d6 as solvent. For each leuprolide derivative, the numbering scheme of the linker is given in Supplementary Fig. 1 and a full NMR characterisation was carried out by applying various homo- and heteronuclear two-dimensional experiments to obtain complete sets of $^1H$, $^{15}N$ and $^{13}C$ resonance assignments (Supplementary Table S1–S3).

### 2.3. Synthesis of mPEG$_{5000}$-b-pHPMAmLac$_n$ block copolymer

Block copolymer containing a hydrophilic block of monomethoxy poly(ethylene glycol) (mPEG, $M_n$ = 5000) and a thermosensitive block composed a random copolymer of HPMAmLac$_1$ and HPMAmLac$_2$ (Fig. 3) was synthesized. The feed molar ratio of HPMAmLac$_1$: HPMAmLac$_2$ was

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