CORE-CROSS-LINKED POLYMERIC MICELLES:

*A versatile nanomedicine
platform with broad applicability*

ANTICANCER
DOCETAXEL
INTRA-VEINOUS
SUBCUTANEOUS
PEPTIDE
LEUPROLIDE
PACLITAXEL
TUNEABLE
LIGAND
NANOMEDICINE
DOCETAXEL

Title: Core-cross-linked polymeric micelles:
a versatile nanomedicine platform with broad applicability
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ISBN: 978-90-365-3947-0
DOI: 10.3990/1.9789036539470

The research in this thesis was carried out in

UNIVERSITY OF TWENTE. Department of Biomaterials Science and Technology,
MIRA Institute for Biomedical Technology and
Technical Medicine, University of Twente, Enschede,
The Netherlands

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Cover design by Sasja Verhoog (sasjaverhoog@hotmail.com)

Printed by CPI Koninklijke Wöhrmann

The printing of this thesis was financially supported by
Faculty of Science and Technology, University of Twente, Enschede, The Netherlands
Cristal Therapeutics, Maastricht, The Netherlands

**CORE-CROSS-LINKED POLYMERIC MICELLES:
A VERSATILE NANOMEDICINE PLATFORM WITH
BROAD APPLICABILITY**

DISSERTATION

to obtain
the degree of doctor at the University of Twente,
on the authority of the rector magnificus
Prof. dr. H. Brinksma,
on account of the decision of the graduation committee,
to be publicly defended
on Thursday 29 October 2015 at 14:45

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ISBN: 978-90-365-3947-0

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To my family

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Chapter 1

General introduction

1. General introduction

1.1. Nanomedicines

Nanomedicine, the application of nanotechnology to medicine, concerns the use of nano-sized materials to develop products for the diagnosis and treatment of diseases [1-5]. To date, a few nanoparticle-based therapeutics are already on the market, and many more are currently under clinical development [6, 7]. Nanoparticles for therapeutic applications are generally characterised by a small size (< 200 nm). They are employed to enhance the solubility of poorly-soluble drugs, improve drug stability and allow for targeted and controlled drug release. Ultimately, the utilisation of nanomedicines may improve the drug disposition profile in the body by enhancing the drug levels at the target sites and/or reducing drug exposure to healthy tissues, leading to improved therapeutic outcomes. So far, a myriad of nanocarriers have been employed for the development of nanomedicinal products, such as liposomes, polymer-drug conjugates, polymeric nanoparticles and inorganic nanoparticles [8].

Among these platforms, polymeric nanoparticles have gained considerable attention. Polymeric nanoparticles are structurally defined as solid nanoparticles, micelles, polyplexes and dendrimers [9]. Through modulation of the chemical composition and/or physical structure of the polymer, the physicochemical properties of polymeric nanoparticles can be completely tuned, yielding a large array of nanomedicinal products for various therapeutic applications [9, 10].

1.2. Core-cross-linked polymeric micelles

As a representative of this nanoparticle class, polymeric micelles (PMs) composed of methoxy poly(ethylene glycol)-*b*-poly[N-(2-hydroxypropyl) methacrylamide-lactate] (mPEG-*b*-PHPMAmLac_n) block copolymers have been extensively studied in the last decade [2, 11-15]. These block copolymers are thermosensitive. This means that below a unique temperature called critical micelle temperature (CMT), the block polymers are hydrated and soluble in aqueous solutions. Above the CMT, the thermosensitive blocks become insoluble and the block copolymers self-assemble into PMs at concentrations above the so-called critical micelle concentration (CMC). PMs formed by these amphiphilic block copolymers are also biodegradable, which under physiological conditions can degrade into known fragments such as lactic acid [14, 16, 17]. These PMs have a shell-core architecture and are generally characterised by a small hydrodynamic size (i.e. < 80 nm) [11, 14]. Considering the dynamic nature of the PMs, the micellar structure should be stabilised to prevent premature disintegration of the carrier *in vivo*. This can be achieved by cross-linking the block copolymers in the micellar core. Core-cross-linking (CCL) ensures the stability and

prolongs the residence time of the PMs in the systemic circulation [18], which in turn allows the PMs to passively target tumour and/or inflammatory tissues via the enhanced permeability and retention (EPR) effect [19, 20].

1.3. Covalent entrapment of drug using stimuli responsive linkers for controlled drug release

Although core-cross-linked polymeric micelles (CCL-PMs) are very stable in the circulation, drugs physically loaded in the micellar core are not stably retained in the circulating CCL-PMs *in vivo*, as demonstrated by Rijcken *et al.* using a similar CCL-PMs system [18]. Accordingly, drug molecules should be covalently entrapped to ensure their retention in the CCL-PMs prior to reaching the target sites. Covalent attachment of drug molecules to the micellar core can be achieved by using stimuli-responsive linkages, which may also allow for tailored drug release profiles with excellent spatial and temporal control [21, 22]. In particular, hydrolytically sensitive ester linkages enable the release of native drug molecules under physiological conditions and thus have been successfully employed in several recent studies [2, 13]. To date, the combination of CCL-PMs and covalent attachment of small molecule drugs (**Figure 1**) have proven to be an attractive strategy to attain excellent therapeutic efficacy in preclinical animal models following intravenous route of administration [2, 12, 13].

Figure 1. Schematic presentation of covalent drug entrapment in core-cross-linked polymeric micelles

1.4. Tuneable nanomedicine platform and broad applications

As the field of nanomedicines matures, a great number of nanoparticle-based therapeutics are being developed for various clinical settings [6]. The design of nanomedicinal products is largely dictated by the pathological site and the nature of the disease. To attain a desired drug disposition profile and thereby optimal therapeutic outcome, nanomedicines should possess distinct properties. Taking tumour targeting as an example, ideally, the hydrodynamic size of a nanoparticle

should be ≤ 50 nm to maximally exploit the EPR effect and to allow for deep tumour penetration [23, 24]. To realise the full potential of nanomedicines, a highly tuneable nanoparticulate platform with respect to physicochemical and other pharmaceutical properties are in great need. This versatile platform should enable the generation of a library of nanoparticulate systems, out of which specific pharmaceutical properties can be readily attained. CCL-PMs hold great potential to become such a tuneable platform. Similarly as in the case of other polymeric nanoparticles, the physicochemical properties of the CCL-PMs may be modulated by altering the chemical compositions and physical architecture of the block copolymers. The use of stimuli-responsive linkage for covalent drug attachment also serves as an attractive approach to attain a tailorable drug release profile.

2. Aim of this thesis

Nanomedicines based on CCL-PMs have shown excellent therapeutic performance in several preclinical models. The aim of this thesis is to expand this nanomedicine platform based on CCL-PMs for broad applications regarding the following objectives:

- Tailor various key pharmaceutical properties of the CCL-PMs system, to attain a versatile nanomedicine platform for broad therapeutic applications.
- Improve the therapeutic performance of the anticancer drug docetaxel in preclinical animal models, in order to support its clinical development and translation.
- Next to small molecule drugs, covalently entrap a model therapeutic peptide in the CCL-PMs to demonstrate the applicability of the CCL-PMs system for biologicals and to improve the pharmacokinetic profile of the peptide after intravenous administration.
- Investigate the pharmacokinetic profiles and systemic availability of nanomedicines based on CCL-PMs following subcutaneous administration, to expand their therapeutic opportunities following non-intravenous routes of administration.

3. Thesis outline

In **Chapter 2**, tailoring of the key pharmaceutical properties of nanomedicines based on CCL-PMs is addressed. The pharmaceutical properties investigated in this chapter include particle size, drug release kinetics and carrier degradation characteristics.

Chapter 3 deals with the characteristics and *in vivo* therapeutic performance of the anticancer drug docetaxel covalently linked to CCL-PMs through an ester bond. The antitumour efficacy and tolerability of this nanomedicinal product after a single intravenous injection in preclinical animal models are presented. Mechanistic aspects contributing to the *in vivo* therapeutic performance were investigated.

Chapter 4 addresses the possibility of covalently entrapping a therapeutic peptide in the CCL-PMs and releasing the peptide in its bioactive form in a sustained manner after intravenous administration. Leuprolide was used as the model peptide, which was covalently attached to the CCL-PMs via two ester linkages of divergent hydrolytic sensitivity. One of the formulations was selected for *in vivo* pharmacokinetic evaluation at escalating doses and blood levels of testosterone were used as an indicator for the bioactivity of the released peptide.

In **Chapter 5**, the feasibility of attaining high systemic availability of CCL-PMs following subcutaneous administration was investigated. Both the glucocorticoid dexamethasone and the taxane paclitaxel were covalently entrapped in CCL-PMs and assessed in this study. Moreover, using the former drug, the influence of linker type on the pharmacokinetic profile of the subcutaneously administered nanomedicines was examined.

Chapter 6 summarises the results of the thesis and provides perspectives for the future development of nanomedicines based on the CCL-PMs towards broad (clinical) applications.

Abbreviations

CCL	Core-cross-linking
CCL-PMs	Core-cross-linked polymeric micelles
CMC	Critical micelle concentration
CMT	Critical micelle temperature
EPR	Enhanced permeability and retention
mPEG- <i>b</i> -pHPMAmLac _n	Methoxy poly(ethylene glycol)- <i>b</i> -poly[N-(2-hydroxypropyl) methacrylamide-lactate]
PMs	Polymeric micelles

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Chapter 2

Core-cross-linked polymeric micelles: a highly versatile platform to generate nanomedicines with divergent properties

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Abstract

Nanomedicines hold great potential to substantially improve the therapeutic index of drugs. To attain desired drug disposition in the body and sufficient drug levels at the target site, the physicochemical properties of nanomedicines should be tailor-made for each of their therapeutic applications. Accordingly, a highly tuneable nanoparticulate platform is needed to allow for the development of nanomedicinal products with optimal pharmaceutical properties yielding excellent therapeutic outcomes. In this study, we developed a series of core-cross-linked polymeric micelles (CCL-PMs) composed of methoxy poly(ethylene glycol)-*b*-poly[N-(2-hydroxypropyl) methacrylamide-lactate] (mPEG-*b*-pHPMAMLa_n) block copolymers that exhibit varying and tailorable pharmaceutical properties. First, by altering the molecular weight of the block copolymer, the particle size of CCL-PMs can be controlled in the range of 30-90 nm. To enable a tuneable drug release profile, a model drug, docetaxel, was covalently attached to the core of CCL-PMs through various ester linkages. As a result, divergent drug release profiles were attained (10-90% of the coupled drug was released within 8 days, at pH 7.4, 37 °C). Further, by employing crosslinkers of different types or densities, the degradation characteristics of CCL-PMs were modulated *in vitro*, with the shortest degradation time being ca. 30 days (pH 7.4, 37 °C). Taken together, these studies demonstrate the high tuneability of this nanoparticulate platform based on CCL-PMs. This in turn will allow for the rational design of nanomedicinal products with anticipated therapeutic performances.

1. Introduction

Over the past three decades, nanoparticle technologies have shown great promise for the diagnosis and treatment of various diseases. The utilisation of nanoparticles can potentially improve the pharmacokinetic profile and the disposition of therapeutic agents in the body leading to enhanced efficacy and/or tolerability [1-3]. In particular, nanoparticles that exploit the “enhanced permeability and retention (EPR) effect” [4, 5] have been extensively investigated for the treatment of cancer and inflammatory diseases [6, 7]. To date, a few nanomedicinal products, such as Doxil® (a liposomal formulation of doxorubicin) [8] and Abraxane® (an albumin-based formulation of paclitaxel) [9] have been launched on the market and many more have nowadays entered different clinical development phases [10-16]. The increasing knowledge on the biological fates of nanomedicines [17] also facilitates the rational design and pharmaceutical development of the latter, ultimately yielding the anticipated therapeutic outcomes.

Among all pharmaceutical properties, particle size is a critical determinant of the circulation and biodistribution profiles of nanomedicines [18, 19]. To ensure circulation longevity, the size of a nanoparticle should be big enough to evade renal clearance and small enough to evade nonselective uptake by the mononuclear phagocyte system (MPS), leaving a size window between 6-200 nm [20, 21]. In particular, for tumour targeting a small particle size below 50 nm is generally desired to enable sufficient tumour accumulation via the EPR effect and (deep) tumour penetration [22, 23].

Next to particle size, the release profile of the entrapped drug from nanocarriers is also pivotal for the *in vivo* performance of nanomedicines [24]. Drug release kinetics are influenced by complex factors such as the physicochemical properties of the drug, the composition of the carrier system, the solubility of the drug in the matrix material and the release environment (e.g. temperature, pH) [25-27]. Actual control over the drug release rate *in vivo* is vital because either excessive drug release in the burst phase or retarded drug release kinetics could give rise to either toxic systemic drug levels or sub-therapeutic drug levels at the target site, respectively. Both will ultimately lead to poor therapeutic outcomes [28].

The degradation profile of the nanocarrier is another crucial attribute. After complete release of the payloads, nanocarriers ideally disintegrate and the formed degradation products are subsequently eliminated, thus preventing toxicities caused by their long-term residence in the body.

Collectively, given the importance of the above-mentioned attributes, a nanoparticulate platform with high adaptability is needed to enable fast development of nanomedicinal products with optimal pharmaceutical properties, eventually yielding excellent therapeutic outcomes.

In the last decades, polymer-based nanomedicines have received considerable attention due to their broad application potential. In particular, polymeric micelles (PMs) based on amphiphilic block copolymers have shown great promise as nanocarriers for therapeutic applications [13, 29-35]. In aqueous media, amphiphilic block copolymers comprising hydrophilic and hydrophobic units may self-assemble into micellar structures composed of a hydrophobic core stabilised by a hydrophilic shell. PMs are generally characterised by a small size (< 100 nm), which is dependent on the molecular weight and the composition of the block copolymer [36, 37]. However, following intravenous administration the *in vivo* stability of some of these PMs remains a great challenge as a result of extensive dilution and/or adsorption of block copolymers to plasma proteins [33, 38]. To stabilise PMs for *in vivo* applications, the hydrophobic blocks can be crosslinked [39-41], yielding core-cross-linked polymeric micelle (CCL-PMs). Further, drugs can be stably entrapped in the micellar core by means of transiently covalent conjugation to prevent their premature release from the PMs [39, 42, 43].

In the present study, we describe the expansion of a highly versatile platform based on CCL-PMs comprising methoxy poly(ethylene glycol)-*b*-poly[N-(2-hydroxypropyl) methacrylamide-lactate] (mPEG-*b*-pHPMAmLac_n) block copolymer. Specifically, it was aimed to obtain a series of CCL-PMs of varying sizes and degradation characteristics. In addition, a model drug, docetaxel (DTX), was covalently linked to the core of CCL-PMs via different hydrolytically sensitive linkers to establish the tuneability of the drug release profile.

2. Materials & Methods

2.1. Materials

Docetaxel (DTX) was obtained from Phyton Biotech GmbH (Ahrensburg, Germany). *N,N'*-dicyclohexylcarbodiimide (DCC), 4-dimethylaminopyridine (DMAP), 4-methoxyphenol, methacrylic anhydride, ammonium acetate, formic acid, Mukaiyama's reagent (2-chloro-1-methylpyridinium iodide), *N,N,N',N'*-tetramethylethylenediamine (TEMED), potassium peroxymonosulfate (Oxone), potassium persulfate (KPS), sodium sulfate (Na₂SO₄) and trifluoroacetic acid (TFA) were obtained from Sigma Aldrich (Zwijndrecht, The Netherlands). Acetonitrile (ACN), dichloromethane (DCM), diethyl ether (DEE), *N,N*-dimethylformamide (DMF) and tetrahydrofuran (THF) were purchased from Biosolve (Valkenswaard, The Netherlands). Absolute ethanol and triethylamine (TEA) were purchased from Merck (Darmstadt, Germany). The initiator (mPEG₅₀₀₀)₂-ABCPA was synthesised as described previously [44]. 2-(2-(Methacryloyloxy)ethylthio)acetic acid (referred

as “L1”), 2-(2-(methacryloyloxy)ethylsulfinyl)acetic acid (referred as “L2”) and 2-(2-(methacryloyloxy)ethylsulfonyl)acetic acid (referred as “L3”) were synthesised as described previously [42]. The other chemicals were used as received.

2.2. Synthesis of the block copolymers

2.2.1. Synthesis of block copolymers of varying molecular weights

Block copolymers containing a fixed hydrophilic block of methoxy poly(ethylene glycol) (mPEG, $M_n = 5000$) and a varying thermosensitive block composed of a random copolymer of N-(2-hydroxypropyl) methacrylamide monolactate (HPMAmLac₁) and N-(2-hydroxypropyl) methacrylamide dilactate (HPMAmLac₂) were synthesised by free radical polymerisation using (mPEG₅₀₀₀)₂-ABCPA as initiator, as described previously [38, 44]. The comonomer feed ratio HPMAmLac₁/Lac₂ was kept constant at 53/47 (mol/mol), unless specified otherwise. The feed molar ratio of monomer/initiator for the “standard block copolymer” was 150 and was varied between 20 and 300 to obtain a set of block copolymers of different molecular weights. To achieve this, the feed amount of total monomer was kept constant (0.7 g) while the feed amount of initiator was adjusted accordingly. In brief, HPMAmLac₁, HPMAmLac₂ and initiator were dissolved in ACN (450 mg of total monomer plus initiator per mL) in airtight glass vials. The reaction mixture was flushed with nitrogen for at least 10 min, heated to 70 °C and then stirred for 20-24 h. Next, the resulting block copolymer was precipitated by dropwise adding the mixture into an excess of DEE (18 mL per gram of polymer). The precipitate was filtered and dried in a vacuum oven overnight. The block copolymers were obtained as off-white solids and characterised using proton nuclear magnetic resonance (NMR) [39], gel permeation chromatography (GPC) and Ultraviolet-Visible (UV-Vis) spectroscopy as described in section 2.2.4.

2.2.2. Derivatisation of block copolymer with methacrylic acid

A fraction (5-20 mol%) of the lactate side chains of the synthesised block copolymer (feed ratio HPMAmLac₁/Lac₂ = 53/47 (mol/mol)) was derivatised with methacrylic acid [38] to obtain a methacrylic acid-derivatised block copolymer (referred as “MA-block copolymer”) (85-95% yield) with a critical micelle temperature (CMT) between 5 and 15 °C. The MA-block copolymers were characterised using NMR [39], GPC and UV-Vis spectroscopy as described in section 2.2.4.

2.2.3. Derivatisation of block copolymer with L2

A fraction (5-25 mol%) of the lactate side chains of the synthesised block copolymer (feed ratio HPMAmLac₁/Lac₂ = 30/70 or 53/47 (mol/mol)) was

derivatised with L2 to obtain a L2-derivatised block copolymer (referred as “L2-block copolymer”) (**Figure 1**). For those L2-block copolymers that were used for micelle formation, the comonomer composition was adjusted to $\text{HPMAmLac}_1/\text{Lac}_2 = 30/70$ (mol/mol) to allow for a relatively low CMT prior to derivatisation.

The carboxyl group of L2 was first activated to form a mixed anhydride 2-(2-(methacryloyloxy)ethylsulfinyl)acetic acid-pivaloyl (L2-Pv). In brief, L2 (0.46 mmol, 1 eq.) was dissolved in DCM (2.0 mL). Next, TEA (0.46 mmol, 1 eq.) was added and the reaction mixture was cooled to 0 °C. Thereafter, pivaloyl chloride (0.46 mmol, 1 eq.) was added and the mixture was stirred for 1 h at 0 °C to obtain L2-Pv, which was used for the next step without further purification or analysis. To derivatise x mol% (x = 5–25) of the lactate side groups with L2, block copolymer (1.50 g) was dissolved in THF (15 mL). Next, DMAP (0.03 g), L2-Pv (x% eq. compared to the terminal hydroxyl groups from lactate side groups of block copolymer) and TEA (1 eq. compared to L2-Pv) were added and the mixture was stirred at room temperature for 16 h. Thereafter, the reaction mixture was added dropwise to DEE (27 mL) to precipitate the L2-block copolymer. The precipitation, filtration and drying step were repeated once to obtain L2-block copolymer as an off-white solid (70–80% yield). The L2-block copolymers were characterised using NMR [43], GPC and UV-Vis spectroscopy as described in section 2.2.4. The percentage of hydroxyl end group derivatised with L2 as determined by NMR (**Figure S1**) was calculated using a similar approach as utilised for MA-block copolymers [39].

2.2.4. Characterisation of (derivatised) block copolymer by GPC and UV-Vis spectroscopy

The molecular weights and molecular weight distributions of the synthesised (derivatised) block polymers were determined by GPC essentially using a method reported previously [38], except that a PFG 5 µm Linear S column (Polymer Standards Service, Germany) was used instead of two PLgel 3 µm Mixed-D columns (Polymer Laboratories, UK).

The CMTs of the derivatised block copolymers in aqueous solutions were recorded on a UV-2450 spectrophotometer (Shimadzu, Japan). Prior to measurement, the block copolymers were dissolved overnight at 4 °C in ammonium acetate buffer (150 mM, pH 5.0) at a concentration of 2 mg/mL. The absorbance of the polymer solutions was read at 650 nm and at 0.2 °C intervals, while the solutions were heated in the thermostatic cells from 0 to 50 °C with a heating rate of 1 °C/min. The onset on the X-axis, obtained by extrapolation of the absorbance versus temperature curve to the baseline, was considered as the CMT of the (derivatised) block copolymer.

Figure 1. Derivatisation of block copolymer mPEG₅₀₀₀-*b*-pHPMAMLac_n with L2 (*p* and *m* are the numbers of HPMAMLac₁ and HPMAMLac₂ units present in the non-derivatised block copolymer, respectively; *r* and *s* are the numbers of non-derivatised and L2-derivatised HPMAMLac_n (*n*=1 or 2) units present in the derivatised block copolymer, respectively).

2.3. Synthesis and analysis of DTX derivatives

2.3.1. Synthesis of DTXL1

L1 was conjugated to the hydroxyl group at the C-2' position of DTX to obtain DTXL1 (**Figure 2**). In brief, L1 (24.75 mmol) was dissolved in DCM (1000 mL) and stirred at 750 rpm. Next, DMAP (59.41 mmol), DTX (24.75 mmol) and Mukaiyama's reagent (29.70 mmol) were added and the mixture was placed in a pre-heated oil bath and stirred at 40 °C for 45 min to obtain a yellow solution. Next, the mixture was cooled down to room temperature and water (450 mL) was added to yield a two-phase system. The aqueous layer was extracted with DCM (300 mL) and the combined organic layers were dried with Na₂SO₄, filtered and evaporated in vacuo to obtain a yellow oil. The resulting oil was purified by column chromatography (heptane/ethyl acetate (4/1 to 1/1)) to obtain DTXL1 as a white solid (71 % yield, > 95% purity).

2.3.2. Synthesis of DTXL2

L2 was conjugated to the hydroxyl group at the C-2' position of DTX to obtain DTXL2 (59 % yield, > 95% purity), using the same synthesis and purification methods as described in section 2.3.1 (**Figure 2**).

2.3.3. Synthesis of DTXL3

The sulfur atom in the linker segment of DTXL1 was oxidised to obtain DTXL3 (**Figure 2**). In brief, DTXL1 (17.10 mmol) was dissolved in ACN/water (60%/40% (v/v)) mixture (213 mL) and stirred at room temperature for 30 min to obtain a homogeneous solution. Thereafter, oxone (22.23 mmol) was added and the resulting mixture was stirred at room temperature for 2 d. Next, water (170 mL) was added to separate the layers. The organic layer was collected and the aqueous layer was extracted twice with ethyl acetate (200 mL). The combined organic layers were washed with water (100 mL), dried with Na_2SO_4 , filtered and evaporated in vacuo. The obtained solid was purified by column chromatography (heptane/ethyl acetate (3/1 to 1/3)) to obtain DTXL3 as a white solid (80% yield, > 95% purity).

2.3.4. Synthesis of DTX(L2)₂

Two L2 linkers were conjugated to the hydroxyl groups at the C-2' and C-7 positions of DTX, respectively, to obtain DTX(L2)₂ (**Figure 2**). In brief, DTX (2.5 mmol), L2 (5.0 mmol), Mukayama's reagent (6.20 mmol) and DMAP (12.4 mmol) were dissolved in DCM (83 mL) and stirred at 40 °C for 45 min. Next, the reaction mixture was washed with brine and water, and the organic layer was dried with MgSO_4 , filtered and evaporated in vacuo. The oily residue was purified using flash chromatography (ethyl acetate/n-hexane (9/1)) to obtain DTX(L2)₂ as an amorphous white solid (23% yield, > 90% purity).

2.3.5. Analysis of DTX derivatives

Proton NMR spectra of the DTX derivatives were recorded using a Gemini 300 MHz spectrometer (Varian Associates Inc. NMR Instruments, Palo Alto, CA). The ^1H NMR spectra of DTX derivatives were obtained in DMSO-d_6 solvent.

The molecular mass of DTX derivatives was determined using electrospray ionisation mass spectrometry (ESI-MS) on a Shimadzu liquid chromatography–mass spectrometry (LC-MS) QP8000 in positive ion mode. A X-Select CSH 3.5 μm C18 column (150 $\times$ 4.6 mm) (Waters, USA) was used with a gradient from 100% eluent A

Figure 2. Synthesis scheme of various docetaxel derivatives

(95% H₂O/5% ACN/0.1% TFA) to 100% eluent B (2% H₂O/98% ACN/0.1% TFA) in 18 min with a flow of 0.8 mL/min and UV-detection at 227 nm.

The purity of DTX derivatives was determined by ultra-performance liquid chromatography (UPLC) (Waters, USA) equipped with a UV-detector (TUV, Waters). An Acquity HSS T3 1.8 μ m column (50 $\times$ 2.1 mm) (Waters) was used for an isocratic run of 20 min (mobile phase: 0.1% formic acid in H₂O) with a flow of 0.7 mL/min and UV-detection at 227 nm. DTX derivative standards dissolved in a mixture of ACN/water (70%/30% (v/v)) were used to prepare calibration curves (linear between 0.5 and 100 μ g/mL).

2.4. Preparation of non-cross-linked polymeric micelles

Non-cross-linked polymeric micelles (NCL-PMs) were prepared using the fast heating method [45]. In brief, an ice-cold solution of derivatised (MA or L2) block copolymer (1.0 mL, 2.0 mg/mL) dissolved in ammonium acetate buffer (150 mM, pH 5.0) was rapidly heated to 60 °C while stirring vigorously for 1 min to form NCL-PMs.

2.5. Preparation of placebo CCL-PMs and DTX-entrapped CCL-PMs

CCL-PMs were prepared using the same method, irrespective of the type of block copolymer used. In brief, an ice-cold solution of derivatised (MA or L2) block copolymer (830 μ L, 24 mg/mL) was mixed with TEMED (25 μ L, 120 mg/mL), both dissolved in ammonium acetate buffer (150 mM, pH 5.0). Subsequently, absolute ethanol (100 μ L, for placebo CCL-PMs) or DTX derivative dissolved in absolute ethanol (100 μ L, 20 mg/mL DTX equiv., unless specified otherwise) was added, followed by rapid heating to 60 °C while stirring vigorously for 1 min to form PMs. The micellar dispersion was then transferred into a vial containing KPS (45 μ L, 30 mg/mL) dissolved in ammonium acetate buffer (150 mM, pH 5.0) at room temperature. The PMs were covalently stabilised by polymerisation of the methacrylate moieties on the block polymer in a N₂ atmosphere at room temperature for 1 h, yielding placebo or DTX-entrapped CCL-PMs. The resulting dispersion contained 20 mg/mL polymer, 1.35 mg/mL KPS, 3 mg/mL TEMED and 10% (v/v) ethanol in ammonium acetate buffer (150 mM, pH 5.0). In the case of DTX-entrapped CCL-PMs (DTXLx-CCL-PMs), the feed concentration of DTX equiv. was accordingly 2.0 mg/mL (unless specified otherwise). Next, the CCL-PMs dispersion was filtered using a 0.2 μ m regenerated cellulose membrane filter (Sartorius, CA) to remove potentially formed aggregates.

The DTXLx-CCL-PMs were purified using a KrosFlo Research III tangential

flow filtration (TFF) system equipped with modified polyethersulfone (mPES) MicroKros® filter modules (MWCO 500 kDa, surface area 20 cm²) to remove low molecular weight impurities. The purification was performed using a fed-batch approach and five washing volumes of ammonium acetate buffer (20 mM, pH 5.0, supplemented with 130 mM NaCl) were used. The concentration factor, i.e. the final product concentration relative to the initial concentration, was approximately 1.0.

2.6. Characterisation of CCL-PMs

2.6.1. Particle size distribution

The particle size of (drug-entrapped) CCL-PMs was measured by dynamic light scattering (DLS) using a Malvern ALV/CGS-3 Goniometer. The viscosity and refractive index of water at 25 °C were used for the measurements. DLS results are given as a z-average hydrodynamic diameter (Z_{ave}) and a polydispersity index (PDI).

2.6.2. Analysis of DTXLx-CCL-PMs by UPLC

The contents of non-entrapped DTX and DTX derivative in DTXLx-CCL-PMs were determined by UPLC. To this end, the micellar dispersion was diluted 10-fold with a mixture of ACN/water (70%/30% (v/v)) and next 7 µL of the resulting mixture was injected into UPLC equipped with a UV-detector (TUV, Waters). An Acquity HSS T3 1.8 µm column (50 × 2.1 mm) (Waters) was used for an isocratic run of 6 min (mobile phase: 50% H₂O/50% ACN/0.1% formic acid) with a flow of 0.8 mL/min and UV-detection at 227 nm. DTX and DTX derivative standards dissolved in ACN/water (70%/30% (v/v)) mixture were used to prepare calibration curves (linear between 0.5 and 100 µg/mL).

Considering the limited stability of DTX under physiological conditions [46], the total (entrapped plus non-entrapped) content of DTX in DTXLx-CCL-PMs was measured indirectly by quantifying the content of benzoic acid (the final degradation product of DTX) using the method reported previously [47]. The amount of entrapped DTX was then calculated as follows:

Amount of entrapped DTX = Amount of total DTX – Amount of non-entrapped DTX – Amount of DTX derivative (DTX equiv.).

The drug entrapment efficiency (EE) was calculated using the UPLC data as follows:

$$\%EE = \frac{\text{Amount of entrapped DTX}}{\text{Amount of DTX equiv. added}} \times 100\%$$

2.7. *In vitro* drug release from DTXLx-CCL-PMs

The *in vitro* release of DTX from DTXLx-CCL-PMs was measured in phosphate buffered saline (pH 7.4) at 37 °C. In brief, DTXLx-CCL-PMs were diluted 20-fold in phosphate buffer (100 mM, pH 7.4, supplemented with 15 mM NaCl) containing 1% (v/v) polysorbate 80 (to solubilise the released DTX). The mixture was incubated at 37 °C and samples were collected at different time points and analysed for released DTX and for 7-epi-DTX (the known epimer of DTX [48], formed through the epimerisation of the hydroxyl group at C-7) contents using UPLC. The concentrations of released DTX and 7-epi-DTX were determined by injecting 7 µL of the mixture into a UPLC system. An Acquity HSS T3 1.8 µm column (50 × 2.1 mm) (Waters) was used with a gradient from 100% eluent A (70% H₂O/30% ACN/0.1% formic acid) to 100% eluent B (10% H₂O/90% ACN/0.1% formic acid) in 11 min with a flow of 0.7 mL/min and UV-detection at 227 nm. DTX standards dissolved in ACN/water (70%/30% (v/v)) mixture were used to prepare a calibration curve (linear between 0.5 and 100 µg/mL) to determine the concentration of released DTX and of 7-epi-DTX. To calculate the percentage of actual DTX, only DTX and 7-epi-DTX (which together constitute ≥ 90% of the total peak area in the chromatogram) were taken into account and not the other degradation products of DTX that are generated in time under physiological conditions due to the hydrolytic instability of DTX [46]:

$$\% \text{ Actual DTX} = \frac{\text{Amount of DTX} + \text{Amount of 7-epi-DTX}}{\text{Amount of total DTX}} \times 100\%$$

2.8. *In vitro* degradation of placebo CCL-PMs

The degradation kinetics of placebo CCL-PMs composed of block copolymers derivatised with either methacrylic acid (5 or 10 mol% of the lactate side chain) or L2 (5 or 10 mol% of the lactate side chain) were studied *in vitro*. In brief, the placebo CCL-PMs were diluted 5-fold with phosphate buffer (100 mM, pH 7.4, supplemented with 15 mM NaCl) or borate buffer (100 mM, pH 9.4) and then incubated at 37 °C. The Z_{ave} and PDI of these incubated dispersions were monitored using DLS. In addition, the derived count rate (DCR, in kilo counts per second (kcps)) was also recorded during DLS measurements. The DLS measurements were terminated when the DCR decreased to ≤ 100 kcps.

3. Results and discussion

3.1. Synthesis and characterisation of block copolymers of different molecular weights

The size of block copolymer micelles is dependent upon the molecular weights of the hydrophobic and/or hydrophilic segments of the constituting block copolymers [49, 50]. CCL-PMs composed of standard mPEG₅₀₀₀-*b*-pHPMAmLac_n block copolymer derivatised with methacrylic acid generally have a hydrodynamic size of 65 nm. In the present study, it was aimed to expand the size range of CCL-PMs by modulating the molecular weight of the constituting block polymer. To achieve this, we kept the hydrophilic block length constant and varied the molecular weight of the thermosensitive pHPMAmLac_n block by altering the feed ratio of monomer (HPMAmLac_n)/initiator from 20 to 300 (mol/mol). Considering that the comonomer composition has a significant influence on the CMT of the corresponding block copolymer [51], the feed comonomer ratio was kept constant (HPMAmLac₁/Lac₂=53/47 (mol/mol)) for all polymerisations. Critical process parameters such as the feed amount of monomer, the type of solvent, the total concentration of monomer plus initiator as well as the polymerisation reaction time and temperature were kept constant. The characteristics of the obtained block copolymers are summarised in **Table 1**. Since the synthesis was highly reproducible for the standard block copolymer (**Table S1**), single batches of block copolymer were synthesised for each of the other molecular weights, which were considered representative.

The synthesised block copolymers had the same HPMAmLac₁/Lac₂ comonomer composition (within the experimental error), which corresponded well with the feed ratio (Table 1). As expected, a higher feed ratio of monomer/initiator gave rise to a longer thermosensitive pHPMAmLac_n block and thereby a higher molecular weight of the block copolymer (Table 1). In free radical polymerisation, the rate of polymerisation is proportional to the monomer concentration ($[M]$) and the inverse square root of initiator concentration ($[I]^{-0.5}$). Therefore under steady state conditions, the kinetic chain length and (given a fixed comonomer feed) the number average molecular weight (M_n) of the block copolymer scales with $[M_0][I_0]^{-0.5}$, i.e. the feed monomer concentration divided by the square root of the feed initiator concentration [52]. Interestingly, although the polymerisations of the present study were not performed under steady state conditions, the M_n of the obtained block copolymer as determined by NMR linearly scaled with $[M_0][I_0]^{-0.5}$ ($r^2 = 0.99$) (**Figure 3**). This correlation was also confirmed by the GPC results (M_n , $r^2 = 0.88$, Table 1). As shown in Table 1, the CMT of the obtained block copolymers decreased with increasing molecular weight. Clearly, at a fixed comonomer ratio, a longer pHPMAmLac_n block rendered this thermosensitive block more hydrophobic, leading to a lower CMT

[53]. Moreover, the yield of the obtained block copolymers decreased with increasing feed monomer/initiator (mol/mol) ratio (Table 1). Since the total concentration of monomer plus initiator was kept constant (i.e. 450 mg/mL), a higher feed monomer/initiator ratio also implied a higher monomer concentration in the reaction mixture. The latter likely rendered the polymerising solution more viscous, leading to less efficient polymerisation manifested as a lower yield [54].

Table 1. Characteristics of block copolymers of varying molecular weights

P	Feed monomer/initiator (mol/mol)	$[M_0]/[I_0]^{0.5}$ ($M^{0.5}$)	Mol% HPMAMLa ₂ (NMR)	# monomer units per block polymer	M_n (NMR) (kDa)	M_n (GPC) (kDa)	PD	CMT (°C)	Yield (%)
1	20	3.44	48	33	13	27	1.3	> 40	87
2	40	5.97	53	40	15	36	1.5	34	84
3	60	8.02	54	49	17	41	1.6	31	76
4	80	9.77	51	58	20	46	1.6	30	77
5	100	11.3	53	68	22	49	1.7	32	74
6	115	12.4	55	77	25	54	1.7	31	76
7	130	13.4	50	83	26	56	1.7	31	73
8	145	14.3	55	82	26	55	1.7	31	73
9	160	15.2	55	96	30	59	1.7	30	73
10	175	16.0	52	100	30	69	1.5	30	71
11	200	17.3	55	106	32	63	1.3	26	50
12	250	19.7	52	120	35	67	1.2	25	51
13	300	21.9	50	129	37	63	1.3	25	49

The synthesis scale and feed mol% HPMAMLa₂ were 0.8 ± 0.2 g and 47%, respectively, for all polymerisations. The feed concentration of monomer plus initiator was kept constant (i.e. 450 mg/mL). $[M_0]/[I_0]^{0.5}$ = the feed molar concentration of monomer divided by the square root of the feed molar concentration of initiator. The actual mol% HPMAMLa₂ and the number of monomer unit in the block copolymer were determined by ¹H NMR, which were used to calculate the M_n of the thermosensitive pHPMAMLa_n block. The M_n of the block copolymer as determined by ¹H NMR is the sum of the M_n of the thermosensitive block and the M_n of mPEG₅₀₀ (i.e. 5 kDa). The M_n and polydispersity (PD) of the block copolymer were determined by GPC using PEG calibration.

Figure 3. Linear correlation between the number average molecular weight of mPEG₅₀₀₀-*b*-pHPMAmLac_n block copolymer as determined by NMR and the $[M_0]/[I_0]^{0.5}$ (feed molar concentration of monomer divided by the square root of the feed molar concentration of initiator, $M^{0.5}$) ($r^2 = 0.99$)

The block copolymers listed in Table 1 were reacted with methacrylic anhydride to obtain their methacrylic acid-derivatised counterparts (MA-block copolymers; **Table 2**). Their batch-to-batch reproducibility was confirmed using the standard MA-block copolymer as a representative (**Table S2**). The NMR data demonstrate that the fraction of lactate groups derivatised with methacrylic acid (% M) was close to the feed amount. Similar to previous findings [39, 55], the derivatisation of terminal hydroxyl groups with methacrylic acid enhanced the hydrophobicity of the thermosensitive block, leading to a decreased CMT. Moreover, the decrease in CMT upon derivatisation (Δ CMT) statistically correlated with the actual % M ($p < 0.005$) (**Figure S2**), which is in good agreement with previous observations [38].

Table 2. Characterisation of MA-block copolymers of varying molecular weights and the resulting NCL-PMs

P	# monomer units per block polymer*	Aimed % M	% M	M_n (GPC) (kDa)	PD	CMT before derivatisation (°C)*	CMT after derivatisation (°C)	Δ CMT (°C)	NCL-PMs Z_{ave} (nm)	NCL-PMs PDI
1'	33	15	18	26	1.3	> 40	ND	ND	30	0.23
2'	40	14	17	33	1.5	34	10	24	36	0.13
3'	49	13	15	40	1.6	31	9	22	42	0.07
4'	58	12	14	47	1.6	30	9	21	46	0.05
5'	68	13	15	49	1.7	32	11	21	51	0.05
6'	77	13	16	53	1.7	31	11	20	55	0.01
7'	83	13	14	54	1.7	31	10	21	57	0.03
8'	82	13	18	55	1.7	31	10	21	58	0.01
9'	96	13	17	57	1.7	30	10	20	62	0.02
10'	100	13	15	67	1.6	30	9	21	63	0.02
11'	106	11	8	56	1.4	26	8	18	60	0.03
12'	120	11	11	59	1.4	25	8	17	67	0.01
13'	129	11	11	62	1.3	25	8	17	69	0.01

P1 - P13 from Table 1 were derivatised to obtain P1' - P13'. The M_n and PD of the block copolymers were determined by GPC using PEG calibration. The fraction (mol%) of lactate side chains derivatised with methacrylic acid (% M) was determined by ^1H NMR. The difference in CMT (Δ CMT) is the decrease of CMT after derivatisation. The aimed % M was calculated based on the correlation between Δ CMT and actual % M (ca. 2 °C per % M) [38] to allow for a resulting CMT between 5 and 15 °C. NCL-PMs were prepared from MA-block copolymers using the fast heating method (40 mg/mL for P1' - P4' and 2 mg/mL for P5' - P13') [45], without addition of ethanol. The Z_{ave} and PDI of NCL-PMs were measured by DLS. ND=not detected (the CMT of P1' could not be detected, likely due to the very small size of the formed NCL-PMs and thereby the insignificant absorbance at 650 nm).

*these data are also shown in Table 1.

Importantly, given a fixed comonomer composition, the Z_{ave} of the obtained NCL-PMs linearly scaled with the number of monomer units per block polymer ($r^2 = 0.94$) and thereby with the M_n of the MA-block polymer (determined by GPC, $r^2 = 0.93$) (**Figure 4**). This can be explained by the positive correlation between the end-to-end distance of the block copolymer and the number of monomer units in the polymer. As shown in Table 2, the hydrodynamic size of PMs composed of methacrylic acid-derivatised mPEG₅₀₀₀-*b*-pHPMAmLac_n block copolymer can be tuned in a well-controlled fashion (30-70 nm) by solely modulating the molecular weight of the thermosensitive block. The latter in turn can be tailor-made by adjusting the monomer/initiator feed ratio as shown in Table 1.

Figure 4. Linear correlation of Z-average hydrodynamic diameter of NCL-PMs ($r^2 = 0.93$) and CCL-PMs ($r^2 = 0.91$) with the number average molecular weight of methacrylic acid-derivatised mPEG₅₀₀₀-*b*-pHPMAmLac_n block polymer as determined by GPC

Earlier, Soga *et al.* reported that mPEG₅₀₀₀-*b*-pHPMAmLac₂ block copolymer containing a low-molecular-weight (3 kDa) thermosensitive block formed even larger NCL-PMs than did those comprising thermosensitive blocks of higher molecular weights (7 or 14 kDa) [53]. This is because the hydrophobic interactions between the extremely short thermosensitive blocks (3 kDa) are relatively weak, yielding a loose and hydrated micellar core with a large hydrodynamic size. In the present study, we synthesised block copolymers containing the same PEG block but a pHPMAmLac_n block of higher molecular weight (≥ 8 kDa). The strong hydrophobic interactions of these thermosensitive blocks resulted in a dense micellar core, as evidenced by the small hydrodynamic size of the PMs.

3.2. Preparation and characterisation of placebo and drug-entrapped CCL-PMs composed of MA-block copolymers of varying molecular weights

A series of placebo and drug-entrapped CCL-PMs were prepared using MA-block copolymers composed of a fixed PEG block and a thermosensitive block of varying molecular weights (Table 2). To attain the latter, the model drug DTX was derivatised with a methacrylated linker L3, which allowed DTX to be covalently attached to the core of CCL-PMs. The characteristics of placebo and DTX-entrapped CCL-PMs are summarised in **Table 3**.

Table 3. Characteristics of placebo and DTX-entrapped CCL-PMs prepared from MA-block copolymers composed of a fixed PEG block and a thermosensitive block of varying molecular weights

MA-block copolymer		Placebo CCL-PMs		DTX-entrapped CCL-PMs				
P [*]	M _n (GPC) (kDa) [#]	Z _{ave} (nm)	PDI	PM	Feed DTX equiv. concentration (mg/mL)	Z _{ave} (nm)	PDI	%EE
1 [*]	26	32; 33	0.16; 0.15	1	0.5	32; 34	0.17; 0.17	71; 72
				2	1	34; 36	0.15; 0.13	88; 82
2 [*]	33	38; 40	0.10; 0.09	3	0.5	38; 39	0.09; 0.10	76; 80
				4	1	40; 42	0.08; 0.08	85; 79
3 [*]	40	44; 45	0.05; 0.05	5	0.5	43; 45	0.05; 0.04	82; 79
				6	1	45; 47	0.07; 0.08	87; 82
4 [*]	47	43; 52	0.02; 0.04	7	0.5	50; 51	0.03; 0.05	85; 83
				8	1	53; 56	0.04; 0.09	79; 78
8 [*]	55	64; 65	0.03; 0.02	9	2	65; 68	0.03; 0.02	80; 84
11 [*]	56	74; 78	0.03; 0.03	10	2	77; 80	0.06; 0.04	80; 85
12 [*]	59	80; 82	0.06; 0.03	11	2	86; 89	0.05; 0.05	73; 77
13 [*]	62	83; 89	0.04; 0.02	12	2	91; 95	0.04; 0.06	82; 78

^{*} refers to Table 2. [#] these data are also shown in Table 2.

For placebo and DTX-entrapped CCL-PMs, the polymer feed concentration was 20 mg/mL and the characterisation results of each individual batch are presented (n=2).

Of note, both NCL-PMs (Table 2) and placebo CCL-PMs (Table 3) were prepared using the same batches of MA-block copolymers. Similar to the NCL-PMs, the hydrodynamic diameters of the obtained placebo CCL-PMs scaled with the molecular weight (M_n , determined by GPC) of MA-block polymers ($r^2 = 0.91$) (Table 3 and Figure 4). Compared to the non-cross-linked counterparts (Table 2), the placebo CCL-PMs had the same PDI values yet larger hydrodynamic diameters (Table 3 and Figure 4). The latter is likely ascribed to the swelling of micellar core caused by the addition of ethanol (10% v/v) during micelle formation [56].

MA-block copolymers with molecular weights lower than the standard block copolymer (i.e. P1'-P4', Table 2) formed placebo CCL-PMs between 32 and 52 nm in diameter. To prevent over-loading of these small-sized CCL-PMs with drug and to attain high drug entrapment efficiency, the corresponding feed drug/polymer ratios (w/w) were adjusted. Essentially, we reduced the drug feed concentration by 2 or 4-fold compared to the standard amount (i.e. 2 mg/mL DTX equiv.) while keeping the polymer feed concentration constant (PM 1-PM 8, Table 3). By doing so, consistently high drug entrapment efficiency of > 70% was attained for all CCL-PMs, regardless

of the micellar size. Remarkably, covalent drug entrapment did not significantly alter the size of these CCL-PMs while the size distribution remained consistently narrow (PM1-PM12, Table 3). These data convincingly demonstrate the tuneability of CCL-PMs with respect to particle size. Upon highly efficient drug entrapment, the obtained drug-entrapped CCL-PMs are still narrowly distributed, with a tuneable hydrodynamic size in the range of 30-100 nm.

3.3. Tuneable release of DTX from CCL-PMs

To obtain drug-entrapped CCL-PMs with tuneable drug release kinetics, DTX was covalently attached to the core of CCL-PMs via different hydrolysable ester linkages. First, we coupled a polymerisable linker that additionally contains a thioether (i.e. L1, L2 or L3) to DTX via the hydroxyl group in the C-2' position to generate various DTX derivatives (DTXL1, DTXL2 or DTXL3). Besides the linker type, the number of linkers coupled to DTX was also varied. To achieve this, two (identical) L2 linkers were conjugated to the hydroxyl groups at both C-2' and C-7 of DTX, yielding the derivative DTX(L2)₂ (Figure 2). The NMR spectra and LC-MS results of these DTX derivatives are shown in **Figure S3-S6**.

The DTX derivatives were entrapped in CCL-PMs composed of the standard MA-block copolymer, yielding narrowly distributed DTX-entrapped CCL-PMs (DTXLx-CCL-PMs, PDI < 0.1) with comparable hydrodynamic sizes (70 ± 3 nm) and high drug entrapment efficiency ($85 \pm 6\%$). These results demonstrate that key physicochemical properties of DTXLx-CCL-PMs, such as particle size (distribution) and drug entrapment efficiency, are not dictated by the type of linker used. Moreover, the *in vitro* release kinetics of DTX from the CCL-PMs were evaluated under physiological conditions (pH 7.4, 37 °C) (**Figure 5**). Importantly, we found that the hydrolysis of the thioether esters allowed DTX to be released following first-order kinetics ($r^2 > 0.95$) and the release rate of DTX decreased in the order of DTXL3 ($t_{1/2} = 1.34$ d, ca. 90% in 8 days) > DTXL2 ($t_{1/2} = 7.96$ d, ca. 50% in 8 days) > DTX(L2)₂ (ca. 15% in 8 days) > DTXL1 (ca. 10% in 8 days). The rather rapid hydrolysis of these thioether ester bonds can be explained by the strong electron-withdrawing effects of the thioethers in the linkers. These electron withdrawing groups reduce the electron density of the neighboring carbonyl bond of the ester groups and thereby accelerate their hydrolysis [42]. Among the linkers, the oxidation degrees of sulfur in the thioethers and thereby their electron withdrawing abilities decrease in the order of L3 > L2 > L1. Accordingly, the hydrolysis rate of the neighboring ester bond and thereby the release kinetics of the attached compound also follows this order, as previously demonstrated by Crielaard *et al.* who coupled dexamethasone to CCL-PMs via the same ester linkages [42]. Compared to dexamethasone (e.g. $t_{1/2} = 18.4$ d for L2) [42], DTX (e.g. $t_{1/2} = 7.96$ d for L2) was however released from the CCL-

PMs at a faster rate under physiological conditions. The divergent release rates are attributed to the different electron withdrawing effects of the drug molecules on the hydrolysable ester group.

Besides the linker type, the number of linkers that are coupled to the drug molecule also affected the drug release kinetics of DTXLx-CCL-PMs. As expected, DTX covalently linked to CCL-PMs via two L2 linkers was released from the CCL-PMs at a substantially slower rate (ca. 15% in 8 days) than that coupled to CCL-PMs via a single L2 linker (ca. 50% in 8 days). Clearly, the necessity for hydrolysis of both sulfoxide ester bonds prior to DTX release leads to a slower drug release profile. The data obtained with the various constructs convincingly show that drug release kinetics can be dominantly controlled by the type of linker and can be further fine-tuned by varying the number of linkers via which the drug is coupled to the core of CCL-PMs.

Figure 5. *In vitro* release of DTX from CCL-PMs under physiological conditions (pH 7.4, 37 °C). Data are expressed as the mean $\pm$ SD (n=3).

3.4. Degradation characteristics of placebo CCL-PMs

CCL-PMs composed of mPEG-*b*-pHPMAmLac_n block copolymer are biodegradable [39, 57, 58]. Given the importance of the carrier degradation profile, in the present study it was aimed to explore the degradation characteristics of CCL-PMs by varying the type of crosslinker and the crosslinking density in the micellar core. Thereto, different fractions (5 or 10 mol%) of the terminal hydroxyl groups of the lactate side chains were esterified using either methacrylic anhydride (referred as “5% MA” or “10% MA”) or L2 (referred as “5% L2” or “10% L2”), which provided methacrylate groups on the thermosensitive blocks for subsequent crosslinking.

Similar as for MA derivatisation [38], the Δ CMT of block copolymer linearly scaled with the extent of L2 derivatisation ($p = 0.004$, $r^2 = 0.99$) (**Figure S7**). However, compared to the former (1-2 °C per mol% derivatisation), L2 derivatisation has a lower impact on the Δ CMT (0.5-1 °C per mol% derivatisation) likely due to its relatively high hydrophilicity ($\log P = -0.78$ for L2 versus $\log P = 0.73$ for methacrylic acid, calculated using ChemDraw) and thereby lower hydrophobicity of the thermosensitive block upon derivatisation. For the preparation of PMs, it was aimed to obtain a derivatised block copolymer with a CMT between 5 and 15 °C. This is because a CMT within this temperature range allows the block polymers to dissolve as unimers in aqueous media at temperatures slightly above 0 °C and to form PMs at room temperature. To achieve that, a block copolymer with a relatively low CMT is preferred prior to L2 derivatisation. Considering the effect of comonomer composition on the CMT of a block copolymer [51], we increased the feed ratio of HPMAM_{Lac₂}/Lac₁ to 70/30 (mol/mol) and kept the other critical process parameters of polymerisation constant, yielding block copolymers with similar molecular weights yet substantially lower CMTs (P3-P4, **Table 4**). As anticipated, for all derivatisations, the actual extent (mol %) of lactate side chains derivatised as determined experimentally was close to feed. Compared to the non-cross-linked counterparts, placebo CCL-PMs composed of the above mentioned block copolymers had slightly larger sizes (Table 4) likely due to the addition of ethanol (10% v/v) during micelle formation [53].

Table 4. Characteristics of derivatised block copolymers that formed placebo CCL-PMs with divergent degradation characteristics

Block copolymer before derivatisation				Block copolymer after derivatisation								Placebo CCL-PMs	
P	Mol% HPMAM-Lac ₂	CMT (°C)	M _n (NMR) (kDa)	Aimed % D	% D	M _n (GPC) (kDa)	PD (GPC)	CMT (°C)	Δ CMT (°C)	NCL-PMs Z _{ave} (nm)	NCL-PMs PDI	CCL-PMs Z _{ave} (nm)	CCL-PMs PDI
1	66	17	20	5; with MA	3	46	1.4	7	10	52	0.07	60	0.03
2	52	26	20	10; with MA	8	52	1.4	11	15	57	0.01	64	0.03
3	72	13	21	5; with L2	3	49	1.4	8	5	62	0.06	71	0.08
4	72	11	21	10; with L2	11	50	1.4	5	6	62	0.04	71	0.07

The feed molar ratio of monomer/initiator was 150 for all synthesised block polymers. The mol% HPMAM_{Lac₂}, M_n of the non-derivatised block copolymer and the fraction (mol%) of lactate side chains derivatised (% D) were determined by ¹H NMR. The M_n and PD of the derivatised block copolymer were determined by GPC using PEG calibration. The difference in CMT (Δ CMT) is the decrease in the CMT of block copolymer after derivatisation. NCL-PMs were prepared using the fast heating method (2 mg/mL polymer in feed) without addition of ethanol [45] and CCL-PMs were prepared (20 mg/mL polymer in feed) with the addition of ethanol.

Interestingly, CCL-PMs composed of L2-block copolymers exhibited larger hydrodynamic sizes (ca. 10 nm) than those composed of the MA-derivatised counterparts (Table 4). This is because compared to the latter, the more hydrophilic L2-block copolymers formed less dense micellar cores, yielding larger hydrodynamic sizes. The obtained placebo CCL-PMs composed of MA- or L2-derivatised block copolymers (Table 4) were monitored under stressed (pH 9.4, 37 °C) (**Figure 6**) and physiological (pH 7.4, 37 °C) (**Figure 7**) conditions by means of DLS and their degradation characteristics were evaluated in terms of Z_{ave} , PDI and absolute light scattering intensity.

Figure 6. Degradation characteristics of placebo CCL-PMs under stressed conditions (pH 9.4, 37 °C). (A) Z-average hydrodynamic diameter; (B) polydispersity index and (C) derived count rate. Data are expressed as the mean $\pm$ SD (n=3).

Under stressed conditions (pH 9.4, 37 °C), the hydrodynamic sizes of CCL-PMs increased at first and then decreased (Figure 6A). This changing pattern of size can be explained as follows. The degradation of CCL-PMs takes places in two major steps [38]. Initially, the non-modified lactate side groups are hydrolysed relatively rapidly, finally resulting in a CMT above 37 °C and subsequent hydration and then swelling of the micellar core, manifested as an increase in size. In particular, the hydrolysis of the dilactate side groups is faster than that of the monolactate groups owing to the so-called back-biting mechanism [59]. The hydrolysis of the lactate groups derivatised with the crosslinker is expected to take place at a much slower rate via a process called random chain scission [38, 59]. Accordingly, the CCL-PMs may first increase in size due to swelling of the micellar core, but they can only fully disintegrate after all the crosslinks are hydrolytically cleaved [38]. As observed for all CCL-PMs, the PDI values continuously increased (Figure 6B), which is inherently associated with the degradation of particles. Furthermore, the absolute light scattering (reflected by derived count rate, DCR) in DLS measurements also decreased under stressed conditions (Figure 6C). According to the Rayleigh scattering law, in a fixed volume the intensity of light scattered by colloidal particles is proportional to the number and to the sixth power of the diameter (Rayleigh's approximation) of the particles. Meanwhile, the scattering intensity is also proportional to the square of the refractive index increment (dn/dc ; change in refractive index of the solvent with respect to change in solute concentration) of the dispersion [60]. Accordingly, based on the change of Z_{ave} and DCR, several stages of the degradation process can be distinguished. Initially, the rapid swelling of the micellar core resulted in a decrease in dn/dc and a moderate increase in size (Figure 6A). Compared to the latter, the reduction in dn/dc is likely more dominant, giving rise to a rapid decrease in the magnitude of light scattered. The dominant impact of dn/dc on scattering intensity was also observed previously with protein aggregates [61]. The next stage of degradation was characterised by a gradual decrease in the hydrodynamic size and scattering intensity (Figure 6C). These changes point to the substantial degradation of the CCL-PMs (into smaller polymeric network fragments/particles).

Under stressed conditions, the degradation rate of the CCL-PMs decreased in the order of: 5% L2 > 10% L2 > 5% MA > 10% MA (Figure 6). Obviously, a lower crosslinking density leads to a faster carrier degradation rate as fewer crosslinks need to be hydrolysed prior to particle disintegration. Importantly, compared to MA, derivatisation of the block copolymer with L2 provided the resulting CCL-PMs with significantly faster degradation kinetics. Presumably, the sulfoxide ester in the crosslink is more hydrolytically sensitive than the methacrylate ester due to the presence of neighboring thioether as a strong electron withdrawing group. Under both stressed and physiological conditions, a bi/multi-phasic decrease of DCR

was observed for all CCL-PMs (Figure 6C and 7C), which points to the divergent hydrolysis kinetics of these various ester groups in the derivatised block copolymers. The relative hydrolysis rates of these ester bonds are depicted in **Figure 8**.

Figure 7. Degradation characteristics of placebo CCL-PMs under physiological conditions (pH 7.4, 37 °C). (A) Z-average hydrodynamic diameter; (B) polydispersity index and (C) derived count rate. Data are expressed as the mean $\pm$ SD (n=3).

Figure 8. Chemical structure of mPEG-*b*-PHPMAMlac_n block copolymer partially derivatised with (A) methacrylic acid or (B) L2. In the derivatised block copolymer the ester groups that are sensitive to hydrolysis are highlighted and their relative hydrolysis rates are ranked in the order of 1 > 2 > 3 (> 4).

The trends observed under stressed conditions were confirmed at physiological conditions (Figure 7). Compared to the former, hydrolysis of these ester groups was significantly slowed down at pH 7.4 due to the reduced hydroxyl ion concentration [62]. Nonetheless, under physiological conditions (pH 7.4) the degradation rate of the CCL-PMs also decreased following the order of 5% L2 > 10% L2 > 5% MA > 10% MA, as supported by DLS data (Figure 7). Based on the change of DCR, the degradation time of these CCL-PMs under both conditions is obtained (**Table 5**). Although the hydrolysis of esters is expected to follow pseudo-first-order in the concentration of hydroxide ion [63], a 100-fold difference in the degradation time was not observed between physiological (pH 7.4) and stressed (pH 9.4) conditions. This is because in the present study, the time needed to obtain a DCR ≤ 100 kcps was used as the surrogate for carrier degradation time, which may deviate from the absolute value.

Table 5. Degradation time of various placebo CCL-PMs under stressed (pH 9.4, 37 °C) or physiological (pH 7.4, 37 °C) conditions based on the change of derived count rate

Type of derivatisation	Aimed % D	Stressed conditions	Physiological conditions
MA	5	8-11 d	100-200 d*
MA	10	27-34 d	300-400 d*
L2	5	ca. 2 d	27-34 d
L2	10	ca. 2 d	27-34 d

The time needed for DCR to reach ≤ 100 kcps is considered as the degradation time. % D = the fraction (mol%) of lactate side chains derivatised.*estimated by extrapolating the DCR curve to 100 kcps at linear scale.

The present study demonstrates the divergent degradation profiles of CCL-PMs in aqueous buffers resulted from chemical ester hydrolysis. However, for translation to the *in vivo* setting, additional factors should be taken into consideration. For example, the degradation of CCL-PMs can be catalysed and thus accelerated due to the presence of enzymes in biological fluids. This contribution will be limited in the initial phases. This is because the core of CCL-PMs is anticipated to be rather dense and therefore enzymes are likely not able to penetrate into the micellar core to catalyse the hydrolytic degradation of ester bonds. However, as the micellar core swells and the carrier disintegrates into smaller particles, enzymatic hydrolysis can take place [64], which may accelerate the degradation of CCL-PMs.

4. Conclusions

In the present study we demonstrate that the key pharmaceutical properties of (drug-entrapped) CCL-PMs such as particle size, drug release kinetics and carrier degradation characteristics can be independently tailored in a well-controlled fashion. This nanoparticulate platform based on CCL-PMs provides high flexibility to rationally design nanomedicines with divergent pharmaceutical properties for manifold therapeutic applications.

Acknowledgements

This work was supported by Cristal Therapeutics.

Abbreviations

% D	The fraction of lactate side chains derivatised
% M	The fraction of lactate side chains derivatised with methacrylic acid
ACN	Acetonitrile
CCL-PMs	Core-cross-linked polymeric micelles
CMT	Critical micelle temperature
DCC	<i>N,N'</i> -dicyclohexylcarbodiimide
DCM	Dichloromethane
DCR	Derived count rate
DEE	Diethyl ether
DLS	Dynamic light scattering
DMAP	4-Dimethylaminopyridine
DMF	<i>N,N</i> -dimethylformamide
dn/dc	Refractive index increment
DTX	Docetaxel
DTXLx-CCL-PMs	Docetaxel-entrapped core-cross-linked polymeric micelles
EE	Entrapment efficiency
EPR	Enhanced permeability and retention
GPC	Gel permeation chromatography
HPMAmLac ₁	<i>N</i> -(2-hydroxypropyl) methacrylamide monolactate
HPMAmLac ₂	<i>N</i> -(2-hydroxypropyl) methacrylamide dilactate
kcps	Kilo counts per second
KPS	Potassium persulfate
L1	2-(2-(Methacryloyloxy)ethylthio)acetic acid
L2	2-(2-(methacryloyloxy)ethylsulfinyl)acetic acid
L3	2-(2-(Methacryloyloxy)ethylsulfonyl)acetic acid
M_n	Number average molecular weight
mPEG- <i>b</i> -pHPMAmLac _n	Methoxy poly(ethylene glycol)- <i>b</i> -poly[<i>N</i> -(2-hydroxypropyl) methacrylamide-lactate]
mPES	Modified polyethersulfone
MPS	Mononuclear phagocyte system
Mukaiyama's reagent	2-Chloro-1-methylpyridinium iodide
Na ₂ SO ₄	Sodium sulfate
NCL-PMs	Non-cross-linked polymeric micelles
NMR	Nuclear magnetic resonance
Oxone	Potassium peroxymonosulfate
PD	Polydispersity
PDI	Polydispersity index
PMs	Polymeric micelles
TEA	Triethylamine
TEMED	<i>N,N,N',N'</i> -tetramethylethylenediamine
TFA	Trifluoroacetic acid
TFF	Tangential flow filtration
THF	Tetrahydrofuran
UPLC	Ultra-performance liquid chromatography
UV-Vis	Ultraviolet-Visible
Z_{ave}	<i>Z</i> -average hydrodynamic diameter
Δ CMT	The decrease in the critical micelle temperature of block copolymer after derivatisation.

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Supplementary information

Table S1. Characteristics of block copolymers of the same composition

Mol% HPMAmLac ₂	M _n of block copolymer (kDa) (NMR)	M _w of block copolymer (kDa) (GPC)	PD	CMT (°C)	Yield (%)
51 ± 3	23 ± 1	95 ± 16	1.5 ± 0.2	29 ± 1	85 ± 3

The synthesis scale was 55 ± 2 g, the feed molar ratio of monomer/initiator was 150 and the feed mol% HPMAmLac₂ was 47%, in all cases. Data are expressed as the mean ± SD (n=6).

Table S2. Characteristics of MA-block copolymers of the same composition

% M	M _n of block copolymer (kDa) (GPC)	PD	CMT (°C)	NCL-PMs Z _{ave} (nm)	NCL-PMs PDI
13 ± 1	89 ± 15	1.5 ± 0.2	10 ± 1	65 ± 4	0.02 ± 0.02

The feed molar ratio of monomer/initiator was 150, the feed mol% HPMAmLac₂ was 47% and the yield was 90 ± 1 %, in all cases. Data are expressed as the mean ± SD (n=6).

Figure S1. ^1H NMR spectrum of block copolymer $\text{mPEG}_{5000}\text{-}b\text{-pHPMAmLac}_n$ partially derivatised with L2 (dissolved in DMSO-d_6). The extent of derivatisation (mol%) is calculated as: $I(\text{CH}_2)/[I(\text{CH}_2) + 2 \times I(\text{lacOH})] \times 100\%$, where $I(\text{CH}_2)$ is the integral for the protons from the double bond of methacrylate moiety at 5.8 and 6.1 ppm and $I(\text{lacOH})$ is the sum of integrals for the protons from the terminal hydroxyl groups of HPMAmLac_1 and HPMAmLac_2 at 5.3 and 5.5 ppm, respectively. In this example, the extent of derivatisation (mol%) is calculated as: $(5.64 + 7.66)/(5.64 + 7.66 + 2 \times 55.32) \times 100\% = 10.7\%$

Figure S2. Correlation between the extent of derivatisation (mol %) of the lactate side chain in $\text{mPEG}_{5000}\text{-}b\text{-pHPMAmLac}_n$ block copolymer with methacrylic acid and the decrease in the CMT of block copolymer ($r^2 = 0.60$). Various batches of $\text{mPEG-}b\text{-pHPMAmLac}_n$ block copolymers of varying molecular weights were derivatised with methacrylic acid to various extents. The comonomer composition in the block copolymer was $\text{HPMAmLac}_1/\text{Lac}_2 = 53/47$ (mol/mol).

A

B

Figure S3. Characterisation of DTXL1. (A) ^1H NMR spectrum and (B) LC-MS result.

Figure S4. Characterisation of DTXL2. (A) ^1H NMR spectrum and (B) LC-MS result.

Figure S6. Characterisation of DTX(L2)₂. (A) ¹H NMR spectrum and (B) LC-MS result.

Figure S7. Linear correlation between the extent of derivatisation (mol %) of the lactate side chains in mPEG₅₀₀₀-*b*-pHPMAmLac_n block copolymer with L2 and the decrease in the CMT of block copolymer ($r^2 = 0.99$). The same batch of mPEG-*b*-pHPMAmLac_n block copolymer was derivatised with L2 to various extents. The comonomer composition in the block copolymer was HPMAMlac₁/Lac₂=53/47 (mol/mol). The M_n of the block copolymer as determined by NMR was 22 kDa. Prior to derivatisation, the CMT of the block copolymer was 33 °C.

Chapter 3

Complete regression of breast tumours with a single dose of docetaxel-entrapped core-cross-linked polymeric micelles

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Abstract

Treatment with chemotherapy such as docetaxel (DTX) is associated with significant toxicity and tumour recurrence. In this study, we developed DTX-entrapped core-cross-linked polymeric micelles (DTX-CCL-PMs, 66 nm size) by covalently conjugating DTX to CCL-PMs via a hydrolysable ester bond. The covalent conjugation allowed for sustained release of DTX under physiological conditions *in vitro*. *In vivo*, DTX-CCL-PMs demonstrated superior therapeutic efficacy in mice bearing MDA-MB-231 tumour xenografts as compared to the marketed formulation of DTX (Taxotere®). Strikingly, a single intravenous injection of DTX-CCL-PMs enabled complete regression of both small (~150 mm³) and established (~550 mm³) tumours, leading to 100% survival of the animals. These remarkable antitumour effects of DTX-CCL-PMs are attributed to its enhanced tumour accumulation and anti-stromal activity. Furthermore, DTX-CCL-PMs exhibited superior tolerability in healthy rats as compared to Taxotere. These preclinical data strongly support clinical translation of this novel nanomedicinal product for the treatment of cancer.

1. Introduction

Despite many marketed anti-cancer agents, treatment of solid tumours still represents a major medical challenge. Conventional chemotherapeutics suffer from a narrow therapeutic index as a result of poor pharmacokinetic and tissue distribution profiles. Besides that, biological barriers at the tumour site such as abnormal blood supply, abundant tumour stroma and high intratumoural pressure limit intratumoural drug penetration, leading to suboptimal therapeutic drug levels [1, 2]. To improve the therapeutic index of chemotherapeutics, nanoparticulate systems offer a set of tools to achieve enhanced intratumoural drug accumulation, sustained intratumoural drug release and reduced side effects [3-5].

Compared to normal tissues, tumour tissues generally have hyperpermeable vasculature and poor lymphatic drainage, which allow extravasation and greater retention of nanoscale medicines in tumours, the phenomenon known as the Enhanced Permeability and Retention (EPR) effect [6]. By exploiting the EPR effect, nanoparticles can preferentially localise in tumours and enhance local drug concentration [7-9]. A few passively targeted anti-cancer nanomedicines such as Doxil® (liposomal doxorubicin) and DaunoXome® (liposomal daunorubicin) are already in the market [10] and others, such as polymeric micelles (e.g. NK105 for paclitaxel delivery) and polymer conjugates (e.g. Opaxio™ for paclitaxel delivery), are in advanced clinical trials [11-13]. Although the currently marketed nanomedicines have shown benefits in subsiding the side effects, a gain at the level of antitumour activity has only marginally been achieved [12, 14-16]. Also in preclinical studies with nanomedicines, complete regression of solid tumours has hardly been reported. The latter shortcoming is likely attributed to a poor EPR effect and/or insufficient drug release from the extravasated nanoparticles, leading to sub-therapeutic drug levels. Moreover, the delivery of anti-cancer agents can also be significantly limited by the physical barrier of stroma in tumour tissues [17]. Tumour stroma (including cancer-associated fibroblasts, immune cells and extracellular matrix) is the supporting tissue adjacent to tumour cells, which plays a pivotal role in tumour growth and progression [18]. Elimination of activated stroma has been considered as a potential approach to anti-cancer therapy [17, 18]. Altogether, the development of a nanomedicine with efficient tumour accumulation, sufficient intratumoural drug release and anti-stromal activities is very likely mandatory for achieving optimal antitumour activity.

Docetaxel (DTX), a potent anti-mitotic chemotherapeutic agent, acts by binding to microtubules and thereby interfering with cell division. DTX is approved for the treatment of locally advanced or metastatic breast cancer, gastric cancer, hormone-refractory prostate cancer and non-small cell lung cancer [19-21]. In spite of its wide clinical use, serious side effects are often observed in patients such as acute hypersensitivity reactions, cumulative fluid retention, neurotoxicity, febrile

neutropenia, myalgia, nasolacrimal duct stenosis and asthenia [22, 23]. Several nanosized vehicles have been developed in recent years to improve the therapeutic index of DTX, including polymeric nanoparticles (NPs) [24], drug-polymer conjugates [25], polymeric micelles [26], lipid-based nanocarriers [27] and inorganic NPs [28]. Many of these nanoparticulate systems demonstrated superior antitumour activity compared to the marketed formulation in preclinical models, yet complete tumour regression was rarely reported and most of them were not (fully) evaluated for their tolerability profiles.

Core-cross-linked polymeric micelles (CCL-PMs) have shown prolonged circulation kinetics upon intravenous (i.v.) administration and enhanced tumour accumulation in various tumour models [29-31]. In the present study, we developed CCL-PMs composed of methoxy poly(ethylene glycol)-*b*-poly[N-(2-hydroxypropyl) methacrylamide-lactate] (mPEG-*b*-pHPMAMLac_n) block copolymers to deliver DTX to tumours after i.v. administration. To assure sufficient drug release from the extravasated CCL-PMs, we conjugated DTX covalently to CCL-PMs via a hydrolysable ester linker to allow controlled drug release [32]. In the present study, the antitumour effects of DTX-CCL-PMs and Taxotere were compared after multi-dose or a single-dose i.v. administration at various doses to tumour-bearing mice. Furthermore, to obtain the safety profile of DTX-CCL-PMs for future clinical translation, the pharmacokinetics (PK) and tolerability profile of DTX-CCL-PMs were examined in healthy rats.

2. Materials and methods

2.1. Materials

Docetaxel (DTX) was obtained from Phyton Biotech GmbH (Ahrensburg, Germany). *N,N'*-dicyclohexylcarbodiimide (DCC), 4-dimethylaminopyridine (DMAP), 4-methoxyphenol, methacrylic anhydride, ammonium acetate, formic acid, Mukaiyama's reagent (2-chloro-1-methylpyridinium iodide), potassium peroxymonosulfate (oxone), potassium persulfate (KPS), lactic acid, *N,N,N',N'*-tetramethylethylenediamine (TEMED) and trifluoroacetic acid (TFA) were obtained from Sigma Aldrich (Zwijndrecht, The Netherlands). Dichloromethane (DCM), *N,N*-dimethylformamide (DMF) and acetonitrile (ACN) were purchased from Biosolve (Valkenswaard, The Netherlands). Absolute ethanol and triethylamine (TEA) were purchased from Merck (Darmstadt, Germany). The initiator (mPEG₅₀₀₀)₂-ABCPA was synthesised as described previously [33]. 2-(2-(Methacryloyloxy)ethylthio)acetic acid (linker) was synthesised as described previously [32]. Taxotere® was purchased from Sanofi-Aventis (Berlin, Germany). The other chemicals were used as received.

2.2. Preparation of docetaxel-entrapped core-cross-linked polymeric micelles

First, DTX-derivative (DTXL) was synthesised in a two-step procedure, as shown in **Figure 1A**. The detailed synthesis, purification and analysis of DTXL are described in supplementary methods. A methacrylated block copolymer containing methoxy poly(ethylene glycol) (mPEG, $M_n = 5000$) as hydrophilic block and a random copolymer of N-(2-hydroxypropyl) methacrylamide monolactate (HPMAMLac₁) and N-(2-hydroxypropyl) methacrylamide dilactate (HPMAMLac₂) as thermosensitive block was synthesised as described previously [29, 34]. Docetaxel-entrapped core-cross-linked polymeric micelles (DTX-CCL-PMs) were prepared essentially using the fast heating method [35]. In brief, an ice-cold aqueous solution of methacrylated mPEG-*b*-pHPMAMLac_n block copolymer (830 μ L, 24 mg/mL) was mixed with TEMED (25 μ L, 120 mg/mL) dissolved in ammonium acetate buffer (150 mM, pH 5). Subsequently, DTXL (100 μ L, 20 mg/mL DTX equivalents, dissolved in ethanol) was added, followed by rapid heating to 60 °C while stirring vigorously for 1 min to form polymeric micelles. The micellar dispersion was then transferred into a vial containing KPS (45 μ L, 30 mg/mL) dissolved in ammonium acetate buffer (150 mM, pH 5). The polymeric micelles were covalently stabilised by crosslinking the methacrylate moieties in DTXL and block polymer in a N₂ atmosphere for 1 h at RT, to obtain DTX-CCL-PMs. The final feed concentrations of block copolymer and DTXL (DTX equivalents) were 20 and 2 mg/mL, respectively. Next, the DTX-CCL-PMs dispersion was filtered through a 0.2 μ m cellulose membrane filter to remove potential aggregates. DTX-CCL-PMs dispersions were purified and concentrated for 10 times using a KrosFlo Research IIⁱ tangential flow filtration (TFF) system equipped with modified polyethersulfone (mPES) MicroKros[®] filter modules (MWCO 500 kDa). Ammonium acetate buffer (20 mM, pH 5) containing 130 mM NaCl was used as the washing buffer for TFF and referred to as “vehicle” in the following sections.

2.3. Characterisation of DTX-CCL-PMs by DLS, TEM and UPLC

The size of DTX-CCL-PMs was measured by dynamic light scattering (DLS) using a Malvern ALV/CGS-3 Goniometer. DLS results are given as a z-average hydrodynamic diameter (Z_{ave}) and a polydispersity index (PDI).

Transmission electron microscopy (TEM) analysis of DTX-CCL-PMs was conducted using a Philips Tecnai 12 microscope equipped with a Biotwin lens and a LaB6 filament, operated at 120 kV acceleration voltage. Glow discharged grids (copper 200 mesh grid with a carbon-coated thin polymer film, Formvar on top) were used for sample preparation and 2% uranyl acetate (w/v) was used as a negative stain. Images were captured with a SIS Megaview II CCD camera and processed with AnalySIS software.

The contents of free DTX, free DTXL, total DTX and polymer in DTX-CCL-PMs dispersions were determined by ultra-performance liquid chromatography (UPLC) as described in supplementary methods. The drug entrapment efficiency (EE) and drug loading (DL) were calculated using the UPLC data as follows:

$$\%EE = \frac{\text{Amount of drug entrapped}}{\text{Amount of drug added}} \times 100\%$$

$$\%DL = \frac{\text{Amount of drug entrapped}}{\text{Amount of drug entrapped} + \text{Amount of polymer}} \times 100\%$$

The amount of drug entrapped was calculated as: amount of drug entrapped = amount of total DTX content – amount of free DTX – amount of free DTXL (DTX equivalents).

3

2.4. *In vitro* docetaxel release from docetaxel-entrapped core-cross-linked polymeric micelles

The *in vitro* release of DTX from DTX-CCL-PMs was measured in phosphate buffer (100 mM, pH 7.4) containing 15 mM NaCl, whole rat blood and whole human blood at 37°C, respectively. DTX-CCL-PMs were incubated at 37 °C in different matrices and the samples were collected at different time points and analysed for released DTX content using UPLC. In brief, DTX-CCL-PMs were diluted in phosphate buffer (100 mM, pH 7.4) containing 15 mM NaCl and 1% polysorbate 80 (v/v). The concentration of released DTX was determined by injecting 7 µL of the mixture into a UPLC system (Waters, USA) equipped with an ultraviolet/visible light detector (TUV, Waters). An Acquity HSS T3 1.8 µm column (50 × 2.1 mm) (Waters) was used with a gradient from 100% eluent A (70% H₂O/30% ACN/0.1% formic acid) to 100% B (10% H₂O/90% ACN/0.1% formic acid) in 11 min with a flow of 0.7 mL/min and UV-detection at 227 nm. DTX standards dissolved in ACN were used to prepare a calibration curve (linear between 0.5 and 110 µg/mL). In the case of whole blood, rat or human whole blood was first incubated at 37 °C for 10 min. Next, blood (85 µL) was spiked with DTX-CCL-PMs (15 µL) and incubated at 37 °C for various lengths of time. After incubation, water (100 µL) was added to the mixture, followed by ACN (600 µL). The reaction mixture was vortexed for 30 s and centrifuged at 10,000 × g for 5 min at 20 °C. Thereafter, the supernatant (500 µL) was added to water (100 µL) and 7 µL of the resulting mixture was injected into the UPLC system. An Acquity HSS T3 1.8 µm column (50 × 2.1 mm) (Waters) was used with an isocratic run of 3.5 min (mobile phase: 55% H₂O/45% ACN/0.1% formic acid) with a flow

of 0.7 mL/min and UV-detection at 227 nm. Only DTX and 7-epi-DTX (the major degradation product of DTX [36, 37]) were taken into account for the calculation of the percentage release of DTX, so not the other degradation products of DTX [38]:

$$\% \text{Release of DTX} = \frac{\text{Amount of DTX} + \text{Amount of 7-epi-DTX}}{\text{Amount of total DTX}} \times 100\%$$

2.5. Efficacy studies in MDA-MB-231 xenografts

All animal experiments were approved by the local ethical committee. All animals were housed in a temperature-controlled room ($21 \pm 3^\circ\text{C}$), with $55 \pm 15\%$ relative humidity, and a photoperiod of 12/12 h. Female NCr nu/nu mice (8-12 week old, Charles River) were used to induce the MDA-MB-231 breast tumour model. Tap-water and pelleted rodent food (SM R/M-Z from SSNIFF® Spezialdiäten GmbH, Germany) were provided to the animals. To induce tumours, 5×10^6 MDA-MB-231 human breast tumour cells were subcutaneously implanted in the mammary fat pad of the mice. When tumours attained $\sim 150 \text{ mm}^3$ size (small, early stage) or $\sim 550 \text{ mm}^3$ size (established, late-stage), mice received either a single i.v. injection or multiple injections (weekly i.v. bolus injections for 3 weeks) of either Taxotere, DTX-CCL-PMs or vehicle in the tail vein. The details of the doses, injections and duration of the experiment are specified in the figure (captions). Tumour volume was analysed by caliper measurement biweekly. Animals were monitored individually. The measurement was terminated when a tumour volume of 1500 mm^3 was attained. In the case of animals exiting the study prematurely, the tumour volume data were carried forward until the endpoint (i.e. when $\leq 50\%$ of the animals remained in the study), which was the point when data plotting stopped. % Survival was calculated using a cut-off tumour volume of 1500 mm^3 as a surrogate for mortality.

2.6. Effect of the DTX-CCL-PMs on the tumour stromal proteins

In the MDA-MB-231 tumour model, when the mean tumour volume reached $\sim 150 \text{ mm}^3$, a single i.v. injection of Taxotere (30 mg DTX/kg), DTX-CCL-PMs (125 mg DTX/kg) or vehicle was administered. Tumours were isolated from mice 4 days after the injections. The collected tumours were frozen at -80°C till analysis for biomarkers for tumour stroma using Western Blot analyses, as described in the supplementary methods.

2.7. Tumour accumulation of DTX-CCL-PMs in MDA-MB-231 xenografts

In the MDA-MB-231 tumour model, when the mean tumour volume reached $\sim 150 \text{ mm}^3$, mice received a single dose i.v. bolus injection of Taxotere (30 mg/kg) or DTX-CCL-PMs (30 mg/kg). Tumours were collected on day 2 or day 4 post-administration and homogenised to determine total and released DTX contents using HPLC-MS/MS (see supplementary methods).

2.8. Pharmacokinetics and tolerability studies in rats

Charles River Crl:CD (SD) rats were used for the *in vivo* studies. For pharmacokinetic studies, rats were randomly divided into groups of six (three female and three male). A single i.v. bolus injection of DTX-CCL-PMs was given into the tail vein of rats at escalating doses of 1.5, 7.5 or 24 mg/kg. Blood samples ($\sim 200 \mu\text{L}$) were collected at different time points in EDTA vials and meanwhile systemic tolerance was observed. The content of total DTX in rat whole blood was determined using HPLC-MS/MS (see supplementary methods). Pharmacokinetic evaluation of blood data was performed using WinNonlin, version 6.3 (Pharsight Corporation, Mountain View, CA, USA).

For tolerability studies, acute toxicity and 5-day repeated dose toxicity studies were performed in healthy rats. For the acute toxicity study, a single i.v. bolus injection of DTX-CCL-PMs was administered at a dose of 7.5 mg/kg or 24 mg/kg and clinical observations were recorded systemically for 2 weeks. In addition, the change of body weight and food consumption were also monitored. For 5-day repeated dose toxicity study, an i.v. injection of DTX-CCL-PMs (9.7 mg/kg/day) or Taxotere (6.7 mg/kg/day) was administered into the tail vein of rats daily for five consecutive days. Animals were observed for any signs of behavioural changes, reaction to treatment and illness before and after each dosing. On test day 6, the animals were sacrificed, dissected and inspected microscopically. The size and weight of these organs, as well as any abnormalities in the appearance of these organs were recorded. In addition, clinical haematological parameters and serum biochemistry parameters in these animals were analysed.

3. Results

3.1. Synthesis and purification of DTX-CCL-PMs

DTX-CCL-PMs were prepared in three main steps: (i) derivatisation of DTX; (ii) synthesis of methacrylated block copolymer and (iii) preparation of DTX-CCL-PMs. The methacrylated DTX derivative (DTXL) was synthesised in a two-step reaction (Figure 1A). First, DTX was esterified at its C-2' hydroxyl group with a methacrylated linker containing a sulfide ester. Next, the sulfide bond was oxidised to a sulfone to obtain DTXL. The synthesised DTXL was purified by column chromatography and obtained as a white solid with high purity (> 95%). The identity and purity of the compound were confirmed by ¹H NMR, LCMS-UV and UPLC-UV (Figure S1). Methacrylated block copolymer composed of a hydrophilic mPEG block and a random block of pHPMAmLac₁/Lac₂ was prepared via radical polymerisation (75% yield) and its characteristics were in good agreement with previous data [30] (Table S1). DTXL was covalently linked to CCL-PMs upon polymerisation of the methacrylate moieties in DTXL as well as in the polymer lactate side chain to obtain DTX-CCL-PMs as an opalescent dispersion. By means of tangential flow filtration (TFF), DTX-CCL-PMs were purified and concentrated to 20 mg DTX equiv. per mL. The mean particle size and polydispersity index (PDI) of DTX-CCL-PMs as determined by dynamic light scattering were 66 nm and < 0.1, respectively (Table S2), which are typical for CCL-PMs prepared from this type of block copolymer [32]. A transmission electron microscope (TEM) image showed spherical morphology and confirmed the homogenous size distribution of DTX-CCL-PMs (Figure 1C). DTX entrapment efficiency and loading were ca. 75% (w/w) and ca. 12% (w/w) respectively.

3.2. *In vitro* DTX release from DTX-CCL-PMs

Hydrolysis of the ester bond linking DTX to the CCL-PMs allows native DTX to be released under physiological conditions (pH 7.4, 37 °C) following first-order kinetics (Figure 1D). Due to the degradation of DTX itself [36], the *in vitro* drug release did not reach 100% (Figure S2). In addition to PBS (pH 7.4), the drug release profile of DTX-CCL-PMs was also evaluated in fresh rat and human blood, in which similar drug release kinetics was observed (Figure 1D). Accordingly, DTX release kinetics is likely solely dependent on chemical hydrolysis of the ester linkage and is not influenced by e.g. enzymes present in biological fluids, enabling a predictable drug release profile *in vivo*.

Figure 1. Synthesis and characterisation of DTX-CCL-PMs. (A) Synthesis scheme of DTX-derivative (DTXL), linker =2-(2-(methacryloyloxy)ethylthio)acetic acid; (B) Particle size distribution of DTX-CCL-PMs as determined by dynamic light scattering; (C) Transmission electron microscopical image of DTX-CCL-PMs and (D) Representative *in vitro* release of DTX and 7-epi-DTX (a major degradation product of DTX) from DTX-CCL-PMs in PBS (pH 7.4), rat blood and human blood at 37 °C. The first measurement time point was at 1 h after the onset of incubation at 37 °C.

3.3. Dose-dependent effect of DTX-CCL-PMs on breast tumour growth

To establish the therapeutic efficacy, the MDA-MB-231 human tumour xenograft model was used as an established *in vivo* model for breast cancer [39].

3.3.1. Multiple dose study

The therapeutic efficacies of Taxotere and DTX-CCL-PMs were first assessed in MDA-MB-231 xenografts following three weekly i.v. injections in nude mice at a dose of 30 mg DTX/kg (referred as 30 mg/kg later), i.e. the maximum tolerated dose (MTD) of Taxotere in nude mice upon weekly i.v. administrations [40]. As shown in **Figure S3A**, both Taxotere and DTX-CCL-PMs inhibited tumour growth effectively.

However, Taxotere induced a significant ($P<0.05$) loss in body weight as compared to vehicle group while treatment with DTX-CCL-PMs did not induce any body weight loss (**Figure S3B**).

3.3.2. Single dose studies

Since the multiple dose study with DTX-CCL-PMs demonstrated high therapeutic efficacy and good tolerability in terms of body weight loss, studies with different but single doses were carried out to find the best therapeutic outcome. When a tumour size of 150-200 mm³ was attained, mice were treated with a single i.v. injection of equivalent dose of DTX (30 or 60 mg/kg) in Taxotere or DTX-CCL-PMs. As shown in **Figure 2A**, at the dose of 30 mg/kg, both Taxotere and DTX-CCL-PMs exhibited comparable antitumour activity till day 51 post-administration. After that, animals from the Taxotere-treated group had to be sacrificed due to the attainment of humane end point (i.e. larger tumour volumes than allowed) in $\geq 50\%$ animals. On the other hand, at a dose of 60 mg/kg, DTX-CCL-PMs significantly inhibited the tumour growth as opposed to Taxotere which did not show additional benefit compared to the 30 mg/kg dose, reaching the endpoint at day 54 (**Figure 2A**). Remarkably, by virtue of the substantial tumour regression, 100% survival was achieved with a single dose of DTX-CCL-PMs (60 mg/kg) over the 79-day period of study (**Figure 2B**). Regarding tolerability, a single i.v. injection of DTX-CCL-PMs did not result in body weight loss at both doses. In contrast, significant body weight loss was observed at day 5 and 9 with Taxotere treatments ($P<0.05$), indicating acute toxicity of Taxotere at these doses (**Figure S4**). Yet, there was no benefit with Taxotere in inhibition of tumour growth after an increase of the dose from 30 to 60 mg/kg.

Figure 2. Antitumour effect of DTX-CCL-PMs at a single dose of 30 and 60 mg DTX/kg. (A) Tumour growth curve and (B) % survival of mice bearing MDA-MB-231 xenografts after a single i.v. injection of Taxotere or DTX-CCL-PMs at equivalent doses (30 and 60 mg DTX/kg). The vehicle group received ammonium acetate buffer (20 mM, pH 5) containing 130 mM NaCl. The duration of the study was 79 days. Data are expressed as the mean $\pm$ SEM ($n=8$).

3.4. A single dose of DTX-CCL-PMs suppresses tumours completely

Although a single dose of DTX-CCL-PMs at 60 mg/kg markedly inhibited tumour growth, mice were not completely cured. As this dose was well tolerated, we examined the antitumour activity of DTX-CCL-PMs at a higher dose of 125 mg/kg. For Taxotere, a dose of 125 mg/kg was not approved by the local experimental animal committee given its known single dose MTD (i.e. 98 mg/kg) [41]. Considering the lack of benefit in antitumour effects and yet acute toxicity with Taxotere after doubling the dose to 60 mg/kg, a single dose of 30 mg/kg was selected for assessing comparative efficacy. Interestingly, we found that a single dose of DTX-CCL-PMs at 125 mg/kg completely abolished tumour growth in mice bearing tumours of 150–200 mm³ size with a 100% tumour-free survival after the 62-day period of study (**Figure 3A and 3B**).

One essential point in preclinical evaluation is that tumours are generally treated at early stage (i.e. small tumours that may not represent the clinical situation), which may overestimate the potency of a new anti-cancer therapy. Taking this aspect into account, we also assessed DTX-CCL-PMs in mice bearing established tumours of approximately 550 mm³ size. As shown in **Figure 3**, Taxotere exhibited only a moderate antitumour effect in both early and advanced tumour models. Remarkably, a single dose of DTX-CCL-PMs (125 mg/kg) induced complete regression of

established tumours leading to 100% tumour-free survival of these mice for 62 days (Figure 3C and 3D). Moreover, no significant loss in body weight was observed in both treatment groups (Figure S5).

Figure 3. Antitumour effect of DTX-CCL-PMs (125 mg DTX/kg) in early and established MDA-MB-231 xenografts tumours. (A) Tumour growth curve and (B) % survival of tumour-bearing mice after a single i.v. injection of Taxotere (30 mg DTX/kg), DTX-CCL-PMs (125 mg DTX/kg) or vehicle (when tumours attained ~150 mm³ size, depicted as day 1). The vehicle group received ammonium acetate buffer (20 mM, pH 5) containing 130 mM NaCl. (C) Tumour growth curve and (D) % survival of tumour-bearing mice after a single i.v. injection of Taxotere (30 mg DTX/kg) or DTX-CCL-PMs (125 mg DTX/kg) (when tumours attained ~550 mm³ size, depicted as day 10). The duration of the study was 62 days. Data are expressed as the mean $\pm$ SEM (n=10).

3.5. Intratumoural effect of DTX-CCL-PMs

To investigate the intratumoural mechanisms for the tumour growth inhibition, we set up an experiment to study the intratumoural effect of the DTX-CCL-PMs within short duration of 4 days. A single dose of Taxotere (30 mg/kg) or DTX-CCL-PMs (125 mg/kg) was injected intravenously into mice bearing tumours of approximately 150-200 mm³ size. After 4 days, tumours were isolated and analysed

for biomarkers for tumour stroma with Western Blot analyses. The data revealed that treatment with DTX-CCL-PMs significantly reduced tumour stroma markers, as shown by the reduction in the protein expression of NG2 (a pericyte marker), α -SMA (a pericyte and cancer-associated fibroblast marker) and collagen-1 (a major extracellular matrix protein) (**Figure 4**). In addition, there was also a clear reduction in β -tubulin expression (a marker for microtubules) after the treatment with DTX-CCL-PMs. In contrast, treatment with Taxotere showed no significant effects on any of these markers. These data suggest that the antitumour effects of DTX-CCL-PMs are not only caused by the direct inhibitory effects on tumour cells but also by anti-stromal effects.

Figure 4. Intratumoural effect of DTX-CCL-PM on tumour stroma. Western blot analyses in tumour samples collected 4 days after a single i.v. injection of Taxotere (30 mg DTX/kg), DTX-CCL-PMs (125 mg DTX/kg) or vehicle. The vehicle group received ammonium acetate buffer (20 mM, pH 5) containing 130 mM NaCl. (A) Treatment scheme; (B) Protein bands of Western blot and (C) Semi-quantitative analyses of the bands. Data are expressed as the mean $\pm$ SEM (n=3). *P<0.05 versus vehicle, #P<0.05 versus Taxotere.

3.6. Tumour accumulation of DTX-CCL-PMs in mice

To characterise the tumour distribution of DTX-CCL-PMs in mice, the intratumoural levels of released DTX and total DTX (released plus entrapped) in tumour-bearing mice were measured at 2 and 4 days after a single i.v. administration of DTX-CCL-PMs or Taxotere at equivalent dose of 30 mg/kg. As illustrated in **Figure 5A**, a single i.v. administration of DTX-CCL-PMs (30 mg/kg) provided a 20-fold (2 days, $P<0.01$) and 59-fold (4 days, $P<0.001$) higher total DTX level as compared to Taxotere (30 mg/kg). In addition to the significantly enhanced total DTX levels, 2-fold (2 days) and 4-fold (4 days, $P<0.05$) higher released DTX levels were found in mice treated with DTX-CCL-PMs. Having expressed as the percentage of injected dose (%ID) in tumour, DTX-CCL-PMs rendered 5-fold ($P<0.05$) higher released DTX levels and 77-fold ($P<0.001$) higher total DTX levels in tumour as compared to Taxotere 4 days after the onset of treatment.

3.7. Pharmacokinetic studies in healthy rats

In the present study, the superior efficacy of DTX-CCL-PMs as well as enhanced tumour accumulation was demonstrated in tumour-bearing mice. To continue the development of DTX-CCL-PMs towards clinical evaluation, the PK and tolerability profile were evaluated in healthy rats (as required by regulatory authorities).

PK studies with a single i.v. administration of DTX-CCL-PMs were conducted in healthy rats at the escalating doses (**Figure 5B**). The PK evaluation at various doses is given in **Table 1**. These studies demonstrated that DTX-CCL-PMs had an elimination half-life of 15.9 ± 0.7 h and the extrapolated AUC from zero to infinity ($AUC_{0-\infty}$) at different doses linearly correlated with the administered dose of DTX-CCL-PMs (**Figure 5C**, $R^2=0.997$).

Table 1. Pharmacokinetic evaluation for total docetaxel levels in whole blood after a single i.v. administration of DTX-CCL-PMs to female rats at different doses (n=6)

	DTX-CCL-PMs 1.5 mg/kg	DTX-CCL-PMs 7.5 mg/kg	DTX-CCL-PMs 24 mg/kg
C_0 ($\mu\text{g/mL}$)	12.6	114.2	364.6
$t_{1/2}$ (h)	15.1	16.2	16.4
$AUC_{0-\infty}$ ($\mu\text{g}\cdot\text{h/mL}$)	229.8	1948.1	5808.9
$AUC_{0-\infty}/\text{Dose}$ ($\text{g}\cdot\text{h/mL}$)	158.5	266.9	240.0
V_z (mL/kg)	137.2	60.0	67.9
V_{ss} (mL/kg)	129.5	56.1	62.3

The values are calculated by PK analysis using a non-compartmental model.

C_0 = concentration at $t=0$, extrapolated, $t_{1/2}$ = elimination half-life, $AUC_{0-\infty}$ = extrapolated area under the curve from zero to infinity, V_z = volume of distribution and V_{ss} = volume of distribution at steady state

A **B** **C**

Figure 5. Pharmacokinetics and tumour accumulation of DTX-CCL-PMs. (A) Intratumoural levels of released DTX and total DTX (released + entrapped) after a single i.v. injection of Taxotere (30 mg DTX/kg) or DTX-CCL-PMs (30 mg DTX/kg) in mice bearing MDA-MB-231 xenografts. Data are expressed as the mean $\pm$ SEM (n=3), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (B) Total DTX (released + entrapped) levels in blood after a single i.v. injection of DTX-CCL-PMs (7.5 mg DTX/kg) in healthy female rats. Data are expressed as the mean $\pm$ SEM (n=6) and (C) Correlation between the injected dose and $AUC_{0-\infty}$ for DTX-CCL-PMs in healthy rats after a single i.v. injection of DTX-CCL-PMs at 1.5, 7.5 and 24 mg DTX/kg, respectively ($R^2 = 0.997$).

3.8. Tolerability studies in healthy rats

To investigate the potential toxicity of DTX-CCL-PMs, both acute and 5-day repeated dose toxicities were examined in healthy rats. With respect to acute toxicity, a single i.v. administration of DTX-CCL-PMs at 7.5 mg/kg or 24 mg/kg was well tolerated albeit a slightly reduced (transient) motility for both male and female animals at 24 mg/kg. To establish repeated dose toxicity, DTX-CCL-PMs or Taxotere was administered intravenously to male and female rats daily for 5 consecutive days. Taxotere was administered intravenously at a dose of 6.7 mg/kg/day (i.e. 40 mg/m²), the dose often used for toxicity evaluation e.g. by Burstein *et al.* [42] whereas a 45% higher dose of DTX-CCL-PMs (9.7 mg/kg/day) was administered to the animals considering the superior tolerability of the DTX-CCL-PM as demonstrated in the acute toxicity study.

Although no mortality occurred, DTX-related target organ toxicities were observed in both groups as reflected by food consumption, diarrhea, size and weight of thymus as well as weight of spleen (**Table S3**). However, compared to Taxotere, these toxicities were substantially reduced in animals that received DTX-CCL-PMs despite a 45% higher dose given. In addition, hematology and serum biochemistry parameters were also examined (**Table S4** and **Table S5**). Compared to DTX-CCL-PMs, the hematological changes such as panleukopenia, thrombocytopenia and reduction in reticulocytes were significantly higher in rats that received Taxotere.

4. Discussion

Long-circulating nanoparticles such as polymeric micelles are exploited with the aim to improve solubility, stability, pharmacokinetics and tumour accumulation of drugs by means of their capacity to encapsulate and target drugs to tumours via the EPR effect [3, 43-45]. However, low stability of micelles in circulation and uncontrolled drug release rate remain critical issues [29, 44]. As demonstrated in the present study, covalent conjugation of docetaxel (DTX) to CCL-PMs not only provided small-sized (66 nm) and stable micellar nanoparticles but also enabled prolonged systemic circulation with enhanced tumour accumulation and sustained release of DTX. Convincingly, treatment with a single administration of DTX-CCL-PMs led to complete regression of the human xenograft MDA-MB-231 breast tumours in mice. This remarkable antitumour efficacy was confirmed by the significantly enhanced tumour accumulation of targeted DTX, attributed to the prolonged systemic circulation of DTX-CCL-PMs and the EPR effect [29].

The synthesis of DTXL is straightforward. Although there are four hydroxyl groups in DTX, the methacrylated linker was selectively conjugated to DTX at its C-2' hydroxyl group, the most amenable and sterically available group for structural modifications [46, 47]. Such selective conjugation was also demonstrated in the work

of Liu *et al.* in which PEG was selectively conjugated to DTX at the 2'-hydroxyl position [41]. Importantly, the manufacturing of DTX-CCL-PMs is a well-controlled step with confirmed scalability (up to multi-liters under Good Manufacturing Practices conditions), excellent batch-to-batch reproducibility and tailorable concentration (e.g. 5-20 mg DTX equiv. per mL) (data not shown). Drug release from polymeric micelles is generally dependent on the degradation of the polymers and/or diffusion of the drug from the micelles, which leads to uncontrolled release of the encapsulated drug [48]. However, as shown in this study, a hydrolysis-sensitive covalent linkage of DTX to the CCL-PMs resulted in sustained release of the drug under physiological conditions (Figure 1D). As indicated in Figure 1D, the release of DTX from DTX-CCL-PMs is solely dependent on ester hydrolysis. Such hydrolysis from the CCL-PMs was also reported by Crielaard and coworkers with dexamethasone [32]. Compared to dexamethasone with the same derivatised unit ($t_{1/2} = 8.9 \pm 0.1$ days), DTX was released from the CCL-PMs at a much faster rate ($t_{1/2} = 1.5 \pm 0.3$ days). Ester hydrolysis kinetics is influenced by the electron-density of the surrounding moieties of the ester bond. Unlike dexamethasone, the derivatisation of DTX occurred at a secondary hydroxyl group, which gave rise to a reduced electron density at 2' carbon and therefore faster hydrolysis kinetics under physiological conditions.

With respect to the tumour accumulation, a single i.v. administration of DTX-CCL-PMs (30 mg/kg) rendered not only significantly greater total DTX levels but also higher released DTX levels in tumours as compared to Taxotere administration at the equivalent dose (Figure 5A). The enhanced released DTX levels in tumour could be ascribed to the release of DTX from the CCL-PMs within the tumour microenvironment and possibly the accumulation of native DTX released from the circulating DTX-CCL-PMs. High tumour accumulation of DTX-CCL-PMs and intratumoural release of DTX obviously attributed to the strong antitumour effect of DTX-CCL-PMs.

At the reported MTD of DTX, i.e. 30 mg/kg (weekly injections) [40], DTX-CCL-PMs and Taxotere showed similar tumour inhibitory effects after three weekly i.v. injections. However, DTX-CCL-PMs had a clear benefit in terms of body weight in comparison to Taxotere (Figure S3B). The absence of body weight loss in mice that received DTX-CCL-PMs was most likely attributed to the covalent entrapment of DTX within the CCL-PMs and thereby the lower 'active' DTX concentration in systemic circulation and lower exposure of normal tissues as compared to Taxotere.

Based on the high efficacy and good tolerability of DTX-CCL-PMs after multiple dosing, single dose studies were carried out to determine the therapeutic superiority over the marketed formulation. Compared to Taxotere, a clear gain in therapeutic efficacy with an increased dose of DTX-CCL-PMs (from 30 to 60 mg/kg) underlined the benefit of the targeted DTX. The lack of the benefit with Taxotere can be

explained by the short plasma half-life and thereby no substantial gain in the tumour accumulation with the increase of the dose. Importantly, a further increase in the dose of DTX-CCL-PMs to 125 mg/kg induced complete regression of the tumours leading to 100% tumour-free survival. These antitumour effects were not only limited to the early stage tumours but also observed with the late-stage (established) tumours, which were completely suppressed with a single dose of DTX-CCL-PMs (Figure 3). Complete regression of established tumours is highly challenging and rarely achieved in preclinical studies. Huang *et al.* reported profound regression of MDA-MB-231 tumours after multiple injections of DTX-loaded self-assembled nanoparticles, yet complete tumour regression was not achieved [24]. Similarly, substantial tumour regression was observed in the same breast cancer xenografts, although three i.v. injections of NC-6301 (polymeric micelles of DTX) at a 4-day interval were required [49]. In the present study, the remarkable therapeutic effects already obtained with a single dose of DTX-CCL-PMs highlight the potent antitumour activity of DTX-CCL-PMs and its potential for clinical application.

Since DTX is an antimitotic drug, it is primarily assumed to display its antitumour activity through tumour cell growth inhibition. However, interestingly we found that treatment with DTX-CCL-PMs also led to the reduction in tumour stromal components such as pericytes (α -SMA, NG2), fibroblasts (α -SMA) and extracellular matrix (collagen-1) at 96 h after a single dose administration (Figure 4). In the tumour microenvironment, fibroblastic cells produce excessive extracellular matrix such as collagen, which induces tumour rigidity, less perfusion and increase in interstitial fluid pressure [50]. In addition, pericytes support endothelial cells and thereby the maturation of blood vessels during angiogenesis leading to enhanced nutrition supply to tumours. Inhibition of these components is highly essential to achieve complete regression of tumours. In contrast to DTX-CCL-PMs, treatment with Taxotere showed only a mild reduction in pericyte marker (NG2). These data corroborate with the recent findings of Murakami and coworkers who showed a reduction in collagen and α -SMA in orthotopic MDA-MB-231 tumour model after the treatment with a DTX-conjugate nanoparticle formulation (PEGylated acetylated carboxymethylcellulose-docetaxel conjugate) named Cellax [51]. Our data suggest that the observed antitumour effects of DTX-CCL-PMs are at least partially mediated through the depletion of tumour stroma in addition to the direct effect on tumour cells.

In addition to the studies in tumour-bearing mice, preclinical PK and tolerability studies were carried out in healthy rats as part of the clinical translation program of DTX-CCL-PMs. In the PK studies, the blood levels in healthy rats revealed that DTX-CCL-PMs remained in the circulation for extended periods of time ($t_{1/2}$ = 16.2 h) and total DTX was detected in blood up to 7 days after a single dose of

DTX-CCL-PMs (7.5 mg/kg) (Figure 5B). Importantly, this demonstrates that DTX remained entrapped in PMs for several days due to the transiently stable covalent linkage. With respect to tolerability, DTX-CCL-PMs at 9.7 mg/kg was much better tolerated by rats as compared to a lower dose of Taxotere (6.7 mg/kg) (Table S3-S5). The superior tolerability of DTX-CCL-PMs is likely attributed to the blood circulation profile of the intact nanoparticles and thereby the absence of high DTX blood levels and significantly improved volume of distribution at steady state (0.06 L/kg) as compared to Taxotere (4 L/kg) [52]. It is of interest that major DTX dose limiting toxicities observed in the clinic such as diarrhea, pan-leukopenia and effects on immunologically related tissues occurred at a lesser severity and/or incidence in the DTX-CCL-PMs treated rats as compared to animals that received Taxotere. Together with other assays as required by regulatory authorities, these preliminary results of toxicology evaluation advocate the clinical translation of DTX-CCL-PMs.

5. Conclusions

In conclusion, this study demonstrates the development of a novel docetaxel-containing nanomedicine of which a single dose can regress both early and established human xenograft tumours completely, providing 100% tumour-free survival to these animals. Importantly, DTX-CCL-PMs were well tolerated by animals at the examined doses, showing superior tolerability to the marketed Taxotere formulation. Altogether, the improved therapeutic index of DTX-CCL-PMs and straightforward manufacturability strongly support its clinical development.

Acknowledgments

This work was supported by Cristal Therapeutics. The authors are thankful to Dr. Anne Vroon and J.B. van den Dikkenberg for their contribution to our work.

Abbreviations

DTX	Docetaxel
ACN	Acetonitrile
AUC _{0-∞}	Extrapolated area under the curve from zero to infinity
CCL-PMs	Core-cross-linked polymeric micelles
DCC	<i>N,N'</i> -dicyclohexylcarbodiimide
DCM	Dichloromethane
DL	Drug loading
DLS	Dynamic light scattering
DMAP	4-Dimethylaminopyridine
DMF	<i>N,N</i> -dimethylformamide
DTX-CCL-PMs	Docetaxel-entrapped core-cross-linked polymeric micelles
EE	Entrapment efficiency
EPR	Enhanced permeability and retention
i.v.	Intravenous
KPS	Potassium persulfate
mPEG- <i>b</i> -pHPMAmLac _n	Methoxy poly(ethylene glycol)- <i>b</i> -poly[N-(2-hydroxypropyl) methacrylamide-lactate]
mPES	Modified polyethersulfone
MTD	Maximum tolerated dose
Mukaiyama's reagent	2-Chloro-1-methylpyridinium iodide
NPs	Nanoparticles
Oxone	Potassium peroxymonosulfate
PDI	Polydispersity index
PK	Pharmacokinetics
t _½	Elimination half-life
TEA	Triethylamine
TEM	Transmission electron microscopy
TEMED	<i>N,N,N',N'</i> -tetramethylethylenediamine
TFA	Trifluoroacetic acid
TFF	Tangential flow filtration
UPLC	Ultra-performance liquid chromatography
V _{ss}	Volume of distribution at steady-state
V _z	Volume of distribution
Z _{ave}	Z-average hydrodynamic diameter

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Appendix A : Supplementary methods

Synthesis and purification of DTX-derivative (DTXL)

DTXL was synthesised in a two-step procedure. In brief, 2-(2-(methacryloyloxy)ethylthio)acetic acid (linker) (6.19 mmol) was dissolved in DCM (100 mL). Next, DMAP (14.85 mmol), DTX (6.19 mmol) and Mukaiyama's reagent (7.43 mmol) were added and the mixture was placed in a pre-heated oil bath (40 °C) and stirred for 1 h. Thereafter, the mixture was cooled to room temperature and washed with water (150 mL), yielding a two phase system. The organic layer was separated and the aqueous layer was extracted with DCM (100 mL). Next, the combined organic layers were dried with Na₂SO₄ and evaporated in vacuo to obtain a yellow oil. The resulting oil was purified by column chromatography using heptane/ethyl acetate (4/1 to 1/1) and the pooled fractions were evaporated in vacuo to obtain compound 1 as a white solid.

Next, compound 1 (4.39 mmol) was dissolved in ACN/water (60%/40% (v/v)) mixture (100 mL) by stirring at room temperature for 10 min. Then oxone (7.89 mmol) was added and the mixture was stirred at room temperature for 2 d. Thereafter, water (100 mL) and DCM (250 mL) were added to the reaction mixture, yielding a two phase system. The organic layer was separated and the aqueous layer was extracted twice with DCM (2 × 100mL). The combined organic layers were washed with water (100 mL), dried with Na₂SO₄ and evaporated in vacuo to obtain a white solid. The obtained solid was purified by column chromatography using heptane/ethyl acetate (3/1 to 1/3) to obtain a white solid. Next, the product was dissolved in ACN (100 mL) to which water (150 mL) was added. The resulting product was freeze-dried to obtain DTXL (81% yield).

Analysis of DTXL

Nuclear magnetic resonance (NMR) characterisation of DTXL was recorded using a Gemini 300 MHz spectrometer (Varian Associates Inc. NMR Instruments, Palo Alto, CA). DMSO-d₆ was used as solvent.

The molecular mass of DTXL was determined using electrospray ionisation mass spectrometry (ESI-MS) on a Shimadzu liquid chromatography–mass spectrometry (LC-MS) QP8000 in positive ion mode. A X-Select CSH 3.5 µm C18 column (150 × 4.6 mm) (Waters, USA) was used with a gradient from 100% eluent A (95% H₂O/5% ACN/0.1% TFA) to 100% eluent B (2% H₂O/98% ACN/0.1% TFA) in 18 min with a flow of 0.8 mL/min and UV-detection at 227 nm.

The purity of DTXL was determined by UPLC (Waters, USA). An Acquity HSS T3 1.8 μm column (50×2.1 mm) (Waters) was used for an isocratic run of 20 min (mobile phase: 0.1% formic acid in H_2O) with a flow of 0.7 mL/min and UV-detection at 227 nm. DTXL standards dissolved in ACN/water (70%/30% (v/v)) were used to prepare a calibration curve (linear between 0.5 and 100 $\mu\text{g/mL}$).

Characterisation of (methacrylated) block copolymer

The (methacrylated) block copolymer was characterised using various methods. In brief, the percentage of HPMAM Lac_1 monomer (mol% HPMAM Lac_1), the number average molecular weight (M_n) of the pHPMAM Lac_n block of the non-methacrylated block copolymer and the percentage of methacrylation (% M) were determined by ^1H NMR analysis [1]. The weight average molecular weight (M_w) and polydispersity (PD) of the (methacrylated) block copolymer were determined by gel permeation chromatography (GPC), essentially as described previously [1, 2]. The critical micelle temperature (CMT) of the methacrylated block copolymer was determined using Ultraviolet/Visible (UV-Vis) spectrophotometer [3].

Analysis of DTX-CCL-PMs by UPLC

To determine free DTX and DTXL contents, DTXL-CCL-PMs were diluted 10 times with ACN/water (70%/30% (v/v)) mixture, of which 7 μL was injected into UPLC (Waters, USA), equipped with an ultraviolet/visible light detector (TUV, Waters). DTX standards and DTXL standards dissolved in ACN/water (70%/30% (v/v)) mixture were used to prepare calibration curves. The total DTX content in DTX-CCL-PMs was measured indirectly by quantifying the content of benzoic acid (one stable final degradation product of DTX) using UPLC. In brief, DTX-CCL-PMs (80 μL) and 6 M NaOH (200 μL) were added to a 10 mL volumetric flask, to which water (220 μL) was added. The resulting mixture was incubated at 60 $^\circ\text{C}$ for 24 h. Next, 6 M formic acid (250 μL) was added and the volume was filled up to 10 mL with water. The concentration of benzoic acid was determined by injecting 7 μL of the mixture into UPLC. Benzoic acid standards dissolved in water were used to prepare a calibration curve. The mass conversion ratio of DTX/benzoic acid is 6.62. An Acquity HSS T3 1.8 μm column (50×2.1 mm) (Waters) was used for an isocratic run of 6 min with a flow of 0.8 mL/min. The mobile phase used for the determination of free DTX and DTXL content was 50% H_2O /50% ACN/0.1% formic acid and the UV-detection was set at 227 nm. The mobile phase used for the determination of total DTX content was 97.3% H_2O /2.7% ACN/0.1% formic acid and the UV-detection was set at 230 nm.

The polymer content in DTX-CCL-PMs was measured by quantifying the content of lactic acid (a degradation product of block copolymer) using UPLC, based

on a method reported previously [4]. In brief, DTX-CCL-PMs (200 μ L) was added to 6 M NaOH (200 μ L), followed by incubation at 60 °C for 2 h. Next, 6 M HCl (400 μ L) was added and the resulting solution was diluted 10 times with 10 mM KH_2PO_4 pH 2.5 solution prior to injection. An Acquity HSS T3 1.8 μ m column (150 $\times$ 2.1 mm) (Waters) was used for an isocratic run of 2.5 min (mobile phase: 10 mM KH_2PO_4 pH 2.5) with a flow of 0.85 mL/min and UV-detection at 210 nm. Lactic acid standards dissolved in the mobile phase were used to prepare a calibration curve (linear between 5 and 400 μ g/mL). The corresponding polymer content was calculated as:

Amount of polymer = amount of lactic acid $\times$ (M+5000)/ [90.08 $\times$ (m + n)], where M is the M_n of the thermosensitive block pHPMAmLac_n; m and n are the units of HPMAMlac₁ and HPMAMlac₂ in the block copolymer, respectively. The molecular weight of lactic acid is 90.08 g/mol.

Western blot analysis

Frozen tumour tissues (10 mg) were homogenised on ice in cold radio-immunoprecipitation assay (RIPA) buffer (Pierce) supplemented with protease inhibitors (Roche) using a tissue homogeniser. Next, samples of 20 μ g of protein were loaded on 4-12% NuPAGE Bis-Tris gels (Life technologies) and separated using 1D SDS-PAGE. Separated proteins were transferred to PVDF membrane using semi-dry western blot apparatus (Invitrogen) as per manufacturer's instructions. The membranes were developed according to standard protocols using primary and secondary antibodies. The protein bands were visualised using ECL Plus Western Blotting Detection Reagent (Amersham Biosciences) and photographed using FluorChem M MultiPlex Western Blot Imager. The bands were quantified using ImageJ software. The antibodies used are described as follows:

Primary Antibody	Source	Dilution
Rabbit monoclonal α -SMA (EPR5368)	Millipore	1:1000
Goat polyclonal anti-collagen I	Southern Biotech	1:200
Rabbit polyclonal NG2 (H-300)	Santa Cruz	1:200
Mouse monoclonal β 3-Tubulin (TU-20)	Santa Cruz	1:200
Mouse monoclonal β -actin	Sigma	1:5000
Secondary Antibody	Source	Dilution
Polyclonal Goat anti-rabbit IgG	DAKO	1:2000
Polyclonal Goat anti-mouse IgG	DAKO	1:2000
Polyclonal Rabbit anti-Goat IgG	DAKO	1:100

Determination of released and total docetaxel contents in tumours

To determine the total DTX contents in tumours, mouse tumour samples (~ 50 mg) were weighed and to which phosphate buffer (0.3 M, pH 7.4) (150 µL) was added. Next, tumour samples were homogenised using a Berthin homogeniser at 5000 rpm for 3 × 20 min and the resulting homogenate was incubated at 37 °C for 6 d to assure full release of DTX from the micelles. To determine the released DTX contents in tumours, mouse tumour samples (~ 50 mg) were weighed and to which ammonium acetate buffer (0.5 M, pH 5) (150 µL) was added. Next, tumour samples were homogenised using a Berthin homogeniser at 5000 rpm for 3 × 20 min and the resulting homogenate was stored at -20 °C till analysis.

The released DTX and total DTX contents were quantified by HPLC-MS/MS, comprised of an API 4000 triple quad mass spectrometer (AB SCIEX, USA) and a Shimadzu Co-Sense system. A Eclipse XDB 5 µm C8 column (50 × 2.1 mm) was used (mobile phase A: 0.1% formic acid; mobile phase B: methanol).

Determination of total docetaxel contents in rat whole blood

Water (20 µL) was added to rat blood (10 µL) and mixed by vortexing. Next, K₂HPO₄ buffer (0.5M, pH 7.4) (500 µL) was added and the resulting mixture was vortexed and incubated at 37 °C for 48 h to assure full release of DTX. After incubation, internal standard (docetaxel d9) was added, followed by vortexing for 30 s. Thereafter, the resulting mixture (100 µL) was added to ACN (400 µL) and mixed by vortexing for 30 s. The solvent layer (300 µL) was diluted with water (200 µL) and analysed by HPLC-MS/MS, comprised of an API 4000 triple quad mass spectrometer (Applied Biosystems, UK) and a Shimadzu prominence HPLC system. An Acquity HSS T3 1.8 µm C18 column (50 × 2.1 mm) (Waters) was used (mobile phase: 15% H₂O/85% ACN/0.1% formic acid) with a flow of 0.5 mL/min.

Supplementary references

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Appendix B Supplementary data

Table S1. Characteristics of (methacrylated) mPEG₅₀₀₀-*b*-pHPMAmLac_n block copolymer

Mol%	M _n of pHPMAmLac _n	% M	M _w	PD (M _w /M _n)	CMT
HPMAmLac ₁	(kg/mol) (NMR)		(kg/mol) (GPC)	(GPC)	(°C)
49	17.6	14	82.1	1.45	9

% M= the percentage of methacrylation; PD = polydispersity; CMT= critical micelle temperature

Table S2. Pharmaceutical characterisation of DTX-CCL-PMs

	Z _{ave} (nm)	PDI	EE (%)	DL (%)	Free DTX (%)	Free DTXL (%)
DTX-CCL-PMs	66 ± 1	0.08 ± 0.01	75 ± 5	12 ± 1	< 2	< 1

Z_{ave} = z-average hydrodynamic diameter; PDI = polydispersity index; EE = (drug) entrapment efficiency; DL= drug loading. Data are expressed as the mean ± SD (n=3).

Table S3. Significant biological differences in healthy rats on test day 6 following five daily i.v. injections of 9.7 mg/kg/day DTX-CCL-PMs or 6.7 mg/kg/day Taxotere

	Taxotere 6.7 mg/kg/day		DTX-CCL-PMs 9.7 mg/kg/day	
	Male	Female	Male	Female
Diarrhea	Grade 2-3 5/5	Grade 2-3 5/5	Grade ≤1 2/5	Grade ≤1 2/5
Food consumption reduction	57%	55%	46%	48%
Decrement in thymus size	5/5	4/5	3/5	2/5
Absolute thymus weight (g)	0.106 ± 0.029	0.112 ± 0.018	0.178 ± 0.042* (41% [#])	0.166 ± 0.048* (48% [#])
Absolute spleen weight (g)	0.166 ± 0.041	0.174 ± 0.011	0.204 ± 0.047 (11% [#])	0.202 ± 0.043 (22% [#])
White blood cell count (10⁹/L blood)	1.22 ± 0.30	1.46 ± 0.30	1.80 ± 0.35* (48% [#])	2.10 ± 0.44* (44% [#])

#greater compared to the Taxotere group; *statistically significant at p ≤ 0.05.

Data are expressed as the mean ± SD (n=5).

Table S4. The clinical haematology parameters detected in healthy rats on test day 6 following five daily i.v. injections of 9.7 mg/kg/day DTX-CCL-PMs or 6.7 mg/kg/day Taxotere

	DTX-CCL-PMs 9.7 mg/kg/day		Taxotere 6.7 mg/kg/day	
	Male	Female	Male	Female
WBC (10 ⁹ /L blood)*	1.80 ± 0.35	2.10 ± 0.44	1.22 ± 0.30	1.46 ± 0.30
Neut (abs.) (10 ⁹ /L blood)	0.376 ± 0.234	0.204 ± 0.34	0.058 ± 0.033	0.224 ± 0.248
Lym (abs.) (10 ⁹ /L blood)	1.330 ± 0.408	1.832 ± 0.442	1.116 ± 0.255	1.194 ± 0.388
Mono (abs.) (10 ⁹ /L blood)	0.046 ± 0.027	0.026 ± 0.005	0.010 ± 0.007	0.012 ± 0.011
Eos (abs.) (10 ⁹ /L blood)	0.010 ± 0.007	0.008 ± 0.004	0.004 ± 0.005	0.018 ± 0.013
LUC (abs.) (10 ⁹ /L blood)	0.002 ± 0.004	0.008 ± 0.004	0.002 ± 0.004	0.000 ± 0.000
Baso (rel.) (%)	0.76	0.50	0.48	0.18
Baso (abs.) (10 ⁹ /L blood)	0.012 ± 0.008	0.008 ± 0.008	0.004 ± 0.005	0.002 ± 0.004

Baso=basophilic granulocytes, Eos=eosinophilic granulocytes, Mono=monocytes, LUC=large unstained cells, Lym=lymphocytes, Neut=neutrophilic granulocytes, WBC=leucocytes (white blood cells). Data are expressed as the mean ± SD (n=5). # these data are the same as exhibited in Table S4. Apart from WBC, these groups showed no clinically significant difference.

Table S5. The clinical serum biochemistry parameters detected in healthy rats on test day 6 following five daily i.v. injections of 9.7 mg/kg/day DTX-CCL-PMs or 6.7 mg/kg/day Taxotere

	DTX-CCL-PMs 9.7 mg/kg/day		Taxotere 6.7 mg/kg/day	
	Male	Female	Male	Female
Albumin (g/L plasma)	29.12 ± 1.42	29.28 ± 1.72	27.02 ± 1.23	28.48 ± 1.08
Globulin (g/L plasma)	16.08 ± 1.26	15.92 ± 1.04	13.78 ± 1.63	14.12 ± 0.63
Albumin/Globulin Ratio (by subtraction)	1.818 ± 0.142	1.840 ± 0.081	1.982 ± 0.218	2.020 ± 0.083
Bilirubin (μmol/L plasma)	3.02 ± 0.67	2.34 ± 0.42	2.74 ± 1.08	1.60 ± 0.37
Creatinine (mmol/L plasma)	29.4 ± 0.9	28.2 ± 2.2	27.2 ± 1.1	26.6 ± 2.3
Protein (g/L plasma)	45.2 ± 2.2	45.2 ± 2.6	40.8 ± 2.4	42.6 ± 1.5
Calcium (mmol/L plasma)	2.472 ± 0.078	2.538 ± 0.056	2.334 ± 0.079	2.460 ± 0.068
Sodium (mmol/L plasma)	138.2 ± 1.3	136.8 ± 0.8	134.8 ± 1.5	135.4 ± 2.3
aP (U/L plasma)	193.2 ± 48.2	149.0 ± 37.8	130.0 ± 28.4	76.6 ± 13.2

Data are expressed as the mean ± SD (n=5). These groups showed no clinically significant difference.

Figure S1. Characterisation of DTX-derivative (DTXL). (A) Purity (>95%, determined by UPLC); (B) ^1H NMR spectrum and (C) LC-MS result (observed $m/z = 1043.4$ $[\text{M} + \text{NH}_4]^+$).

Figure S2. UPLC chromatogram of DTX-CCL-PMs after incubated in PBS containing 1% polysorbate 80 (v/v) at 37 °C for 8 days.

Figure S3. Multi-dose effect of DTX-CCL-PMs on MDA-MB-231 xenograft tumour model. (A) Tumour growth curve and (B) Change of body weight (%) of mice bearing MDA-MB-231 xenografts after 3 weekly i.v. bolus injections of Taxotere (30 mg DTX/kg), DTX-CCL-PMs (30 mg DTX/kg) or vehicle (7.5% ET) (i.e. 7.5% ethanol + 7.5% polysorbate 80 in 5% dextrose in water). The duration of the study was 49 days. Data are expressed as the mean $\pm$ SEM (n=6). *P<0.01 versus DTX-CCL-PMs, unpaired t test.

Figure S4. Change of body weight (%) of mice bearing small MDA-MB-231 xenografts after a single i.v. injection of Taxotere or DTX-CCL-PMs at (A) 30 mg DTX/kg and (B) 60 mg DTX/kg, respectively. The vehicle group received ammonium acetate buffer (20mM, pH 5) containing 130 mM NaCl. The duration of the study was 79 days. Data are expressed as the mean $\pm$ SEM (n=8). Data obtained in (A) and (B) were from the same experiment.

Figure S5. Change of body weight (%) of mice bearing (A) small or (B) established MDA-MB-231 xenografts after a single i.v. injection of Taxotere (30 mg DTX/kg) or DTX-CCL-PMs (125 mg DTX/kg). The vehicle group received ammonium acetate buffer (20mM, pH 5) containing 130 mM NaCl. The duration of the study was 62 days. Data are expressed as the mean $\pm$ SEM (n=10).

Chapter 4

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.

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 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 stabilise 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-lactate (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 linkers 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-NHC₂H₅, molecular mass 1209.5 Da) and the internal standard for leuprolide (pGlu-His-Trp-Ser-Tyr-Ala-Leu-Arg-Pro-NHC₂H₅) were obtained from Bachem AG (Bubendorf, Switzerland). Testosterone-17 β (4-androsten-17 β -ol-3-one) and internal standard testosterone-17 β -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), *N,N,N',N'*-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 (HPMAmLac₁) and *N*-(2-hydroxypropyl) methacrylamide dilactate (HPMAmLac₂) as well as the initiator (mPEG₅₀₀₀)₂-ABCPA were synthesised 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)ethylthio)acetic acid (L1) was synthesised as described previously [24] (**Figure 1A**). Next, leuprolide was conjugated to L1 as illustrated in **Figure 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 μ m OBD 30 $\times$ 50 mm column; eluent A: 95% H₂O/ 5% ACN/ 0.1% formic acid; eluent B: 5% H₂O/ 95% ACN/ 0.1% formic acid) and freeze-dried to obtain leuprolide-L1 (LeuL1) as fluffy white powder.

Figure 1. Synthesis of leuprolide-L1. (A) Synthesis scheme of L1 [24] and (B) Conjugation of L1 to leuprolide.

2.2.2. Synthesis of leuprolide-L2

2-(2-(Methacryloyloxy)ethylsulfanyl)acetic acid (L2) was synthesised essentially as previously described with minor modifications [24] (**Figure 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 H₂O (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 Na₂SO₄ 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 polymerisation) 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. (**Figure 2B**).

Figure 2. Synthesis of leuprolide-L2. (A) Synthesis scheme of L2 and (B) Conjugation of L2 to leuprolide.

2.2.3. Analysis of leuprolide derivatives

The molecular mass of leuprolide derivatives was determined using electrospray ionisation 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 $\times$ 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 ($\text{M}+\text{H}$)⁺ and m/z 1412 ($\text{M}+\text{H}$)⁺, respectively.

The synthesised 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 $\text{DMSO}-d_6$ as solvent. For each leuprolide derivative, the numbering scheme of the linker is given in supplementary **Figure S1** 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 methoxy poly(ethylene glycol) (mPEG, $M_n = 5000$) and a thermosensitive block composed a random copolymer of HPMAmLac₁ and HPMAmLac₂ (**Figure 3**) was synthesised. The feed molar ratio of HPMAmLac₁/HPMAmLac₂ was 53/47. The block copolymer was prepared by free radical polymerisation using $(\text{mPEG}_{5000})_2$ -ABCPA as initiator (molar ratio of monomer: initiator was 150:1) as described previously [20, 32]. Subsequently, 13 mol% of the lactate side chains was derivatised with methacrylate groups upon reaction with methacrylic anhydride according to a protocol reported previously [20]. The obtained block copolymer was characterised using methods described elsewhere [23, 33].

2.4. Preparation of leuprolide-entrapped CCL-PMs

Polymeric micelles were formed using the fast heating method [34]. In brief, an ice-cold solution of methacrylated mPEG -*b*-pHPMAmLac_n block copolymer (830 μL , 24 mg/mL) was mixed with TEMED (25 μL , 120 mg/mL) dissolved in ammonium acetate 150 mM pH 5.0 buffer. Subsequently, LeuL1 or LeuL2 (100 μL , 20 mg/mL leuprolide equivalents, dissolved in 50/50 (v/v) ethanol/water mixture) was added, followed by rapid heating to 60 $^\circ\text{C}$ while stirring vigorously for 1 min to form polymeric micelles. The micellar dispersion was then transferred into a

vial containing KPS (45 μ L, 30 mg/mL). The polymeric micelles were covalently stabilised by crosslinking the methacrylate moieties of both the leuprolide derivatives as well as the polymers under a N_2 atmosphere for 1 h at room temperature, to obtain either LeuL1-entrapped CCL-PMs (LeuL1 CCL-PMs) or LeuL2-entrapped CCL-PMs (LeuL2 CCL-PMs) (Figure 3). The final concentrations of block copolymer and leuprolide derivatives (leuprolide equivalents) were 20 and 2 mg/mL, respectively. Next, the LeuL1 CCL-PMs and LeuL2 CCL-PMs dispersions were filtered using 0.2 μ m cellulose membrane filters to remove large particles/aggregates.

For the *in vivo* study, LeuL1 CCL-PMs were purified and concentrated 5 times using a KrosFlo Research Iii tangential flow filtration (TFF) System equipped with modified polyethersulfone (mPES) MicroKros® filter modules (MWCO 500 kDa). Ammonium acetate 20 mM pH 5.0 buffer containing 130 mM NaCl was used as the washing buffer and referred to as “vehicle” in the following sections.

2.5. Particle size distribution

The size of LeuL1 and LeuL2 CCL-PMs was measured by dynamic light scattering (DLS) using a Malvern ALV/CGS-3 Goniometer. DLS results are given as a z-average hydrodynamic diameter (Z_{ave}) and a polydispersity index (PDI).

2.6. Transmission electron microscopy (TEM)

Transmission electron microscopy (TEM) analysis of the different micellar dispersions was conducted using a Philips Tecnai 12 microscope equipped with a Biotwin-lens and a LaB6 filament, operated at 120 kV acceleration voltage. Glow discharged grids (copper 200 mesh grid with a carbon-coated thin polymer film, Formvar on top) were used for sample preparation and 2% uranyl acetate (w/v) was used as a negative stain. Images were captured with a SIS Megaview II CCD camera and processed with AnalySIS software.

Figure 3. Synthesis scheme of leuprolide-entrapped CCL-PMs

2.7. Determination of free and total leuprolide contents

The concentrations of free leuprolide, free leuprolide-L1 or leuprolide-L2 in the micellar dispersions were determined by ultra-performance liquid chromatography (UPLC) (Waters, USA) using an Acquity BEH C18 1.7 μm column (50×2.1 mm) (Waters). A gradient from 100% eluent A (95% H_2O / 5% ACN/ 0.1% formic acid) to 100% B (10% H_2O / 90% ACN/ 0.1% formic acid) was used at a flow of 0.25 mg/mL. The injection volume was 7 μL and the runtime was 5 min. The determination was performed using an ultraviolet/visible light detector (TUV, Waters) set at 210 nm. Leuprolide dissolved in water was used for calibration. Prior to injection, LeuL1 CCL-PMs or LeuL2 CCL-PMs were diluted 10-fold in ammonium acetate 150 mM pH 5.0 buffer and stored at 5 $^\circ\text{C}$ to minimise additional hydrolysis.

The concentration of total (entrapped and released) leuprolide in the micellar dispersions was determined by measurement of released leuprolide upon hydrolysis of the ester bonds in borate 100 mM pH 9.4 buffer supplemented with NaCl to

isotonicity at 37 °C. The release was considered complete when a plateau in leuprolide concentration was reached. The concentration of total leuprolide was measured by UPLC using the same analytical method mentioned above.

The amount of peptide entrapped is calculated as follows: amount of peptide entrapped = amount of total leuprolide content – amount of free leuprolide – amount of free leuprolide derivative (leuprolide equiv.).

The peptide entrapment efficiency (EE) and peptide loading (PL) were calculated using the UPLC data as follows:

$$\%EE = \frac{\text{Amount of peptide entrapped}}{\text{Amount of peptide added}} \times 100\%$$

$$\%PL = \frac{\text{Amount of peptide entrapped}}{\text{Amount of (peptide entrapped + polymer added)}} \times 100\%$$

2.8. *In vitro* leuprolide release from CCL-PMs

The *in vitro* release of leuprolide from CCL-PMs was measured in phosphate buffered saline pH 7.4 at 37°C. Briefly, LeuL1 CCL-PMs or LeuL2 CCL-PMs dispersions (prepared using the methods described in section 2.4) were diluted 40-fold in phosphate 100 mM pH 7.4 buffer supplemented with 15 mM NaCl (max. 50 µg/mL leuprolide equiv.). The concentration of released leuprolide was determined by UPLC using the analytical method mentioned in section 2.7. The percentage of leuprolide release is calculated as:

$$\% \text{Release of leuprolide} = \frac{\text{Amount of released leuprolide}}{\text{Amount of total leuprolide}} \times 100\%$$

2.9. Pharmacokinetics

Animal experiments were conducted in compliance with the national regulations and approved by the local ethical committee for animal experimentation. Fifteen male Sprague Dawley rats (Charles River Deutschland, Germany) weighing ~300 g were randomly divided into five groups of three rats. All animals were housed in a temperature-controlled room (21 ± 3°C), with 55 ± 15% relative humidity and a photoperiod of 12/12 h. Tap-water and pelleted rodent food (SM R/M-Z from SSNIFF® Spezialdiäten GmbH, Germany) were provided to the animals.

Leuprolide dissolved in ammonium acetate 20 mM pH 5.0 buffer also containing 130 mM NaCl (referred to as “soluble leuprolide”) was injected i.v. into the tail vein of the rats at a dose of 0.10 mg/kg. We selected LeuL1 CCL-PMs for the *in vivo* studies considering the peptide release profile as explained in the result and discussion section. Dispersions of LeuL1 CCL-PMs were injected i.v. into the tail vein of the rats at doses of 0.13, 1.3 and 13 mg/kg leuprolide, respectively. As a control group, the vehicle itself was injected i.v. into the rats. Blood samples (approximately 0.5 mL per sampling time point) were serially collected in tubes containing K₃-EDTA as anticoagulant from the vena cava via a cannula which was inserted into the jugular vein. Within 30 min after sampling, the blood samples were centrifuged and the plasma samples were stored at -75 °C until analysis. The concentrations of released and total (released plus entrapped) leuprolide as well as endogenous testosterone-17 β in plasma were determined using the methods described in the following section.

2.10. Determination of released leuprolide, total leuprolide and endogenous testosterone-17 β concentrations in rat plasma

Released and total (released plus entrapped) leuprolide as well as endogenous testosterone-17 β concentrations in rat plasma were determined by LC-MS. Prior to the extraction procedure of the samples, the internal standards pGlu-His-Trp-Ser-Tyr-Ala-Leu-Arg-Pro-NHC₂H₅ and testosterone-17 β -d₃ were added to 100 μ L of rat plasma for the quantification of leuprolide and testosterone-17 β , respectively. For the determination of released leuprolide and testosterone-17 β concentrations, the rat plasma samples were diluted 1:1 with ammonium acetate 150 mM pH 5.0 buffer. For the determination of total leuprolide concentration, rat plasma samples were 1:1 diluted with borate 100 mM pH 9.4 buffer supplemented with NaCl to isotonicity and incubated at 37 °C for 48 h to ensure full release of leuprolide. Next, ice-cold acetonitrile (2 $\times$ 200 μ L) was added stepwise to the plasma, followed by brief vortexing. The samples were subsequently centrifuged (12000 $\times$ g) for 8 min and the supernatants were evaporated in a SpeedVac until dryness. Next, the residue was dissolved in 40 μ L of solvent (50% ACN in water with 0.1% formic acid).

The samples were subsequently analysed by LC-MS using a Dionex Ultimate 3000 RSLC system equipped with a NCS-3500RS binary nanoLC pump and VWD-3400RS variable wavelength detector (Dionex Softron GmbH, Germering, Germany). Chromatographic separation was achieved on an Eclipse Plus C18 column (1.8 μ m, 50 $\times$ 2.1 mm) from Agilent (Waldbronn, Germany) at a flow rate of 0.3 mL/min. The mobile phase consisted of eluent A (90% H₂O/ 10% ACN/ 0.1% formic acid (v/v)/ 2 mM ammonium acetate) and eluent B (10% H₂O/ 90% ACN/ 0.1% formic acid (v/v)/ 2 mM ammonium acetate) and the injection volume was 10 μ L. Mass spectrometric

detection was performed on an Agilent 6540 Q-TOF Accurate Mass spectrometer (Agilent Technologies, Santa Clara, CA), which operated in the positive ion mode using a Jet Stream electrospray ionisation (ESI) source. The masses used to identify and quantify the analytes were m/z 605.3300 $(M+2H)^{2+}$ for leuprolide, m/z 584.3065 $(M+2H)^{2+}$ for internal standard leuprolide (pGlu-His-Trp-Ser-Tyr-Ala-Leu-Arg-Pro-NHC₂H₅), m/z 289.2162 $(M+H)^+$ for testosterone-17 β and m/z 292.2350 $(M+H)^+$ for testosterone-17 β -d₃.

2.11. Statistical analysis

Pharmacokinetic data are presented as the mean $\pm$ SD of 3 rats per group unless otherwise noted. Pharmacokinetic parameters were calculated using Multifit pharmacokinetic software (University of Groningen, The Netherlands) by a two compartment nonlinear model as described previously [35]. Statistical significance was analysed using two-tailed unpaired Student's t-test. A p -value < 0.05 was considered statistically significant. The linear correlation between AUC $_{\infty}$ and administered leuprolide dose was tested using an unpaired two-tailed Student's t-test.

3. Results and discussion

3.1. Synthesis and purification of leuprolide derivatives

L1 or L2 was conjugated to leuprolide through DCC/DMAP mediated esterification of the carboxyl group of the linker and the hydroxyl group of serine residue of the peptide, thereby yielding methacrylated leuprolide derivatives containing either a sulfide (LeuL1) or a sulfoxide (LeuL2) ester.

LeuL1 and LeuL2 were purified by prep-HPLC and obtained in 42% and 27% yields, respectively. As evidenced by LC-MS analysis, only one linker molecule was conjugated to leuprolide as peaks for LeuL1 and LeuL2 were observed at m/z 1394 $(M+H)^+$ and m/z 1410 $(M+H)^+$, respectively (**Figure S2**). As shown in Table S1, the ^1H chemical shifts of the serine residue in leuprolide derivatives significantly differed from those observed in leuprolide [36], indicating that the linker was conjugated at the hydroxyl group of serine residue. Evidence for the covalent attachment follows directly from the observed ^1H - ^1H NOE contacts going from serine sidechain protons to methylene (H1) protons in the linker, as well as the presence of long-range ^1H - ^{13}C couplings between serine beta protons and the adjacent C=O carbonyl carbon (C8) of the linker. To mention, the ^1H chemical shifts of tyrosine residue in leuprolide derivatives were in good agreement with those observed in leuprolide [36], suggesting that the tyrosine hydroxyl group was not modified in the conjugation

reaction. This selective conjugation also corroborates the findings of Sundaram *et al.* where the carboxyl group of docetaxel hemiglutarate was selectively conjugated to GnRH agonist deslorelin via the serine hydroxyl group of the peptide (instead of the phenolic hydroxyl group in tyrosine) [37].

3.2. Synthesis and characterisation of mPEG₅₀₀₀-*b*-pHPMAmLac_n block copolymer

A thermosensitive block copolymer composed of a random block of pHPMAmLac₁/Lac₂ and a hydrophilic mPEG block was prepared via radical polymerisation and obtained in good yield (~85%). The critical micelle temperature (CMT) of the synthesised block copolymer was 28 °C. The number average molecular weight (M_n) of the block copolymer as determined by NMR was ca. 20 kg/mol (**Table 1**). Gel permeation chromatography (GPC) analysis showed that the block copolymer had a weight average molecular weight (M_w) of ca. 32 kg/mol with a polydispersity of 2.5, which is normal for this type of free radical polymerisation [21]. In a subsequent step, 12 mol% of the lactate groups of the mPEG-*b*-pHPMAmLac_n block copolymer was derivatised with methacrylate groups and the CMT of the methacrylated polymer was 10 °C, which is in good agreement with previous data [21]. The Z-average hydrodynamic diameter and PDI of polymeric micelles composed of the methacrylated block copolymer (2 mg/mL) were 65 nm and 0.04, respectively, which are in line with previous data [33].

Table 1. Characteristics of (methacrylated) mEPG₅₀₀₀-*b*-pHPMAmLac_n block copolymer

M_w (Kg/mol)	PD (M_w/M_n)	M_n of pHPMAmLac _n (Kg/mol)	M %	CMT (°C)
31.6	2.5	15.0	12	10

The M_w and polydispersity (PD) of the (methacrylated) block copolymers were determined by GPC; the M_n of the thermosensitive block of the block copolymer and percentage of methacrylation (M %) were determined by ¹H NMR analysis; the critical micelle temperature (CMT) of the methacrylated block copolymer was determined by Ultraviolet-Visible (UV/Vis) spectrophotometer, essentially as described previously [38].

3.3. Preparation and Characterisation of leuprolide-entrapped CCL-PMs

LeuL1 and LeuL2 were covalently entrapped in CCL-PMs to obtain LeuL1 CCL-PMs and LeuL2 CCL-PMs, respectively. The z-average hydrodynamic diameters of these CCL-PMs were about 70 nm, with a low PDI (<0.1) (**Table 2, Figure 4A**). This particle size is typical for CCL-PMs prepared from this type of block copolymer [24]. The morphology of the LeuL1 CCL-PMs and LeuL2 CCL-PMs was spherical, as demonstrated by TEM analysis (**Figure 4B**). After purification and concentration by TFF, the mean particle size and PDI of LeuL1 CCL-PMs remained the same. The leuprolide entrapment efficiency was 35% and 40% for LeuL1 and LeuL2 respectively, while free leuprolide, free LeuL1 or LeuL2 content was less than 1%. Considering the high hydrophilicity of LeuL1 and LeuL2 (calculated (c) Log P=1.08 and 0.15 respectively, calculated using Chemdraw), the entrapment efficiency is surprisingly high, likely due to the covalent linkage that is formed between the peptide and the CCL-PMs. A similar drug entrapment efficiency was also found with doxorubicin covalently entrapped in CCL-PMs [23].

Table 2. Characterisation of LeuL1 CCL-PMs and LeuL2 CCL-PMs

	Z_{ave} (nm)	PDI	EE (%)	PL (%)	Free leuprolide (%)	Free leuprolide derivative (%)
LeuL1 CCL-PMs	74 ± 7	0.03 ± 0.02	35 ± 3	3.3 ± 0.3	$<1\%$	$<1\%$
LeuL2 CCL-PMs	68 ± 1	0.02 ± 0.01	40 ± 8	3.9 ± 0.7	$<1\%$	$<1\%$

The z-average hydrodynamic diameter (Z_{ave}) and polydispersity index (PDI) of LeuL1 CCL-PMs and LeuL2 CCL-PMs were analysed using DLS. The percentage of peptide entrapment efficiency (% EE), peptide loading (% PL) as well as the percentage of free leuprolide and leuprolide derivative in LeuL1 CCL-PMs and LeuL2 CCL-PMs were determined by UPLC analysis. Data are expressed as the mean $\pm$ SD of 2~3 different batches.

Figure 4. Particle size distribution and morphology of LeuL1 CCL-PMs and LeuL2 CCL-PMs. (A) Hydrodynamic size distribution and (B) TEM image.

3.4. *In vitro* leuprolide release from leuprolide-entrapped CCL-PMs

The hydrolysis of the ester bonds linking leuprolide to CCL-PMs allows leuprolide to be released in time. The peptide release profiles of LeuL1 CCL-PMs and LeuL2 CCL-PMs were evaluated under physiological conditions (pH 7.4, 37 °C). **Figure 5** shows that leuprolide was released from the CCL-PMs in a sustained fashion, following first-order kinetics ($R^2 > 0.99$ and $R^2 > 0.96$ for LeuL1 CCL-PMs and LeuL2 CCL-PMs, respectively). Importantly, the release of leuprolide from CCL-PMs containing sulfide-ester linked leuprolide (LeuL1, $t_{1/2} = 3.7 \pm 0.2$ days) was much slower than that from CCL-PMs containing the sulfoxide-ester linked leuprolide (LeuL2, $t_{1/2} = 0.9 \pm 0.1$ day). Compared to LeuL1, LeuL2 has a higher degree of oxidation of sulfur and therefore a reduced electron density at the carbonyl group of the ester bond. This in turn results in a faster hydrolysis of the ester bond in sulfoxide ester and thereby faster release of leuprolide from LeuL2 CCL-PMs. These data demonstrate that the release kinetics of leuprolide can be tailored by employing linkers containing thioether esters with different degrees of oxidation (i.e. a sulfide (L1) or sulfoxide (L2) ester), as previously reported by Crielaard *et al.* for dexamethasone [24].

Figure 5. Release of leuprolide from core-cross-linked polymeric micelles with leuprolide covalently linked to the core via two different hydrolysable linkers at 37 °C, pH 7.4. Data are expressed as the mean $\pm$ SD of 2~3 batches.

Leuprolide is a peptide hormone that exerts its therapeutic effect through the suppression of luteinizing hormone (LH) and follicle stimulating hormone (FSH). Thus sustained leuprolide levels are desired in the bloodstream. To achieve this, the linkage that renders the slowest leuprolide release kinetics should be employed. Besides, given a polymeric micelle system with prolonged circulation kinetics, the release rate of peptide should be slow enough to exploit the long residence of the carrier system as to obtain steady drug plasma levels. Therefore, in our study the micellar dispersion with the slowest peptide release kinetics (i.e. LeuL1 CCL-PMs) was selected for *in vivo* evaluation.

3.5. Pharmacokinetic profile of LeuL1 CCL-PMs

The pharmacokinetics of leuprolide formulated in either free form or CCL-PMs were evaluated in a rat model. Plasma was obtained at various time points after a single i.v. administration of vehicle, soluble leuprolide and different doses of LeuL1 CCL-PMs. Plasma samples were analysed to determine released and total (released plus entrapped) leuprolide concentrations. The plasma-disappearance curves of released and total leuprolide are shown in **Figure 6** and the calculated pharmacokinetic parameters for LeuL1 CCL-PMs are depicted in **Table 3**.

As expected, rats treated with vehicle alone (without peptide) showed leuprolide levels below the limit of detection (LOD) (i.e. 0.1 ng/mL) at all the time points. We found that soluble leuprolide (0.1 mg/kg) was rapidly eliminated from the circulation and the plasma levels were detectable only for 2 hours post-administration. Due to the limited time points, pharmacokinetic parameters for soluble leuprolide could not be calculated and are therefore not included in Table 3.

In contrast, LeuL1 CCL-PMs (0.13 mg/kg) drastically extended the blood residence time of released leuprolide ($t_{1/2} = 14.4$ h), which was detected in blood for 3 days (Figure 6A). This is attributed to the prolonged blood residence of the CCL-PMs [39, 40] and covalent peptide conjugation. Accordingly, the AUC_{∞} of LeuL1 CCL-PMs (26,158 h*ng/ mL) was 178-fold higher than the predicted AUC_{∞} value of soluble leuprolide (147 h*ng/ mL). Compared to soluble leuprolide, the i.v. administration of LeuL1 CCL-PMs gave rise to a five-fold lower peak of leuprolide plasma concentration, potentially precluding peak-related side effects. Importantly, the administration of LeuL1 CCL-PMs led to a 2-fold higher plasma AUC_{∞} of released leuprolide than did soluble leuprolide. The systemic exposure to soluble leuprolide was essentially due to the high peak concentration while sustained plasma levels of released leuprolide was attained with LeuL1 CCL-PM for 3 days (Figure 6A). As shown with other (similar) systems [20, 39, 41, 42], the small hydrodynamic size and stealth properties provided by PEG chains allow polymeric micelles to escape mononuclear phagocyte system (MPS) recognition and subsequent clearance, leading to prolonged circulation time. It may be assumed that while CCL-PMs are circulating in blood, hydrolysis of the sulfide ester occurs, giving rise to the release of leuprolide in the bloodstream in a sustained manner. It is also possible that (part of) leuprolide is released from the micelles upon uptake by macrophages (e.g. in the liver) and subsequently re-enters the systemic circulation as released leuprolide.

Figure 6. Leuprolide plasma levels after single i.v. administration of leuprolide solution (0.10 mg/kg) or LeuL1 CCL-PMs (0.13 mg/kg, 1.3 mg/kg and 13 mg/kg, respectively) in rats. (A) Released leuprolide plasma levels and (B) Total leuprolide plasma levels. Data are expressed as the mean $\pm$ SD (n=3).

Table 3. Pharmacokinetic data of total (released plus entrapped) leuprolide in plasma following i.v. administration of LeuL1 CCL-PMs (0.13 mg/kg, 1.3 mg/kg and 13 mg/kg, respectively) in rats. Data are presented as the mean $\pm$ standard error (n=3).

	LeuL1 CCL-PMs 0.13 mg/kg	LeuL1 CCL-PMs 1.3 mg/kg	LeuL1 CCL-PMs 13 mg/kg
$t_{1/2}$ (h)	18.6 $\pm$ 29.9	22.6 $\pm$ 11.0	26.6 $\pm$ 4.0
AUC ∞ (h*ng/ mL)	26,158 $\pm$ 7843	268,877 $\pm$ 61,414	2,666,680 $\pm$ 338,457
CL (mL/kg/h)	5 $\pm$ 1	5 $\pm$ 1	5 $\pm$ 1
V_{ss} (mL/ kg)	113 $\pm$ 67	143 $\pm$ 38	187 $\pm$ 10
MRT (h)	23 $\pm$ 19	30 $\pm$ 13	38 $\pm$ 6

$t_{1/2}$ = Half-life of elimination; AUC ∞ = Area under the plasma concentration-time curve; CL = Clearance; V_{ss} = Steady-state volume of distribution; MRT = Mean residence time.

4

As shown in Figure 6, the plasma-disappearance curves of released and total leuprolide followed the same pattern after i.v. administration of LeuL1 CCL-PMs at various doses. These data suggest that the leuprolide levels in plasma are dictated by the residence of LeuL1 CCL-PMs in circulation. Moreover, the AUC ∞ of both released and total leuprolide plasma levels are correlated linearly with the administered dose of LeuL1 CCL-PMs (released leuprolide level: $P=0.008$, $R^2 > 0.999$; total leuprolide level: $P < 0.001$, $R^2 > 0.999$) (**Figure 7**). The linear correlation between AUC ∞ and the administration dose of LeuL1 CCL-PMs implies a linear bioavailability at a dose between 0.13 and 13 mg/kg. These data demonstrate that the plasma leuprolide levels on demand can be achieved by adjusting the dose proportionally.

As the total leuprolide plasma levels are mainly contributed by the intact LeuL1 CCL-PMs, the observed half-life of elimination ($t_{1/2}$) of total leuprolide plasma level reflects the circulation half-life of LeuL1 CCL-PMs. Plasma disappearance of LeuL1-CCL-PMs showed log-linear pharmacokinetics for all doses, with a half-life of elimination of 22.6 ± 4.0 h, indicating dosage-independence of blood clearance of LeuL1 CCL-PMs. This phenomenon has also been observed in PEGylated liposomes [43].

Figure 7. AUC ∞ after i.v. administration of LeuL1 CCL-PMs at three different doses (0.13 mg/kg, 1.3 mg/kg and 13 mg/kg) in rats. (A) Released leuprolide plasma levels; (B) Total leuprolide plasma levels. These data are from the same experiment as that in Figure 6. Data are expressed as the mean $\pm$ SD (n=3).

3.6. Testosterone levels in plasma

Both animal and human studies have demonstrated that the administration of leuprolide induces a marked release of hormones LH and FSH in the circulation, which subsequently stimulates an initial and temporary boost in testosterone levels in males [44-47]. Therefore, to establish that leuprolide is released from LeuL1 CCL-PMs in its biologically active form, plasma testosterone levels were determined. As shown in **Figure 8**, plasma testosterone levels increased immediately after i.v. administration of soluble leuprolide. Likewise, an increase of testosterone levels was also observed after i.v. administration of LeuL1 CCL-PMs at different doses. The surge of plasma testosterone levels clearly demonstrates that leuprolide is released from LeuL1 CCL-PMs in its biologically active form. The continuous stimulation of the pituitary suppresses the hypophyseal-gonadal axis (possibly through the process of down-regulation of pituitary receptors for GnRH and desensitisation of the pituitary gonadotropins) and in turn the plasma testosterone levels [48], as observed in all leuprolide-treated groups. However, there was a clear difference between the duration of the effect. The plasma testosterone levels decreased by >150-fold in 24 h after i.v. injection of soluble leuprolide (0.1 mg/kg) whereas it only dropped by ca. 10-fold in 24 h after i.v. administration of LeuL1 CCL-PMs (0.13 mg/kg). Between 48 h and 120 h, the testosterone levels decreased to ≤ 0.5 ng/mL in both cases. Surprisingly, as shown in Figure 8, testosterone peak levels were not dose-dependent yet within the same order of magnitude for all leuprolide-treated groups, indicating that a plateau-level of testosterone surge was reached already at 1.3 mg/kg dose level. The biological response pattern may be attributed to the saturation and down-regulation

of the pituitary GnRH receptors by the analogue or some unknown phenomena [49]. Nonetheless, compared to soluble leuprolide, the plasma testosterone levels decreased at a substantially lower rate when formulated in LeuL1 CCL-PMs, owing to the steady and long-lasting effect of released leuprolide on plasma testosterone levels. As the aim of testosterone level determination was to examine the bioactivity of the released leuprolide rather than long-term efficacy, testosterone levels were only monitored until 120 h. In the course of the study, no clinically relevant signs were found in animals treated with LeuL1-CCL-PMs (supplementary **Table S4**).

The present study reports on a novel strategy to covalently link peptides to CCL-PMs via different hydrolysable ester linkages. The small-sized and long-circulating polymeric micelle system allows sustained release of the biologically active peptide into the systemic circulation for several days following intravenous administration. Commercial products containing leuprolide such as Eligard® (gel-like depot) and Lupron depot® (microspheres) have also demonstrated good efficacy *in vivo* after subcutaneous or intramuscular administration [50, 51]. However, the strategy we propose enables the nanoformulation to be absorbed directly in the systemic circulation following intravenous administration and allows for higher efficiency for reaching various target sites (e.g. the systemic circulation for leuprolide). Furthermore, considering the good tolerability of this nanoformulation, we believe this novel strategy has a great potential for the delivery of therapeutic peptides, especially when local administration is not feasible (e.g. due to unwanted immunogenicity induced by the absorption of peptides from the injection site).

Figure 8. Testosterone and released leuprolide plasma levels in rats between 0 and 24 h post single i.v. injection of leuprolide solution (0.10 mg/kg) or LeuL1 CCL-PMs (0.13 mg/kg, 1.3 mg/kg and 13 mg/kg, respectively). The blue dashed line indicates the average basal plasma testosterone level in male Sprague-Dawley rats [52]. Released leuprolide levels (grey line) have been shown in Figure 6A. Data are expressed as the mean $\pm$ SD ($n=3$).

To mention, we selected leuprolide as the model peptide and developed this delivery strategy for broad applications. This reported technology may be used to entrap not only leuprolide, but also other (hydrophobic) peptides in the micellar core to achieve higher entrapment efficiency. The use of hydrolysable linkers may allow tuneable peptide release profile at various disease sites (e.g. inflammatory and tumour tissues). Owing to the enhanced permeability and retention (EPR) effect [53], CCL-PMs can preferentially localise into tumour and inflammatory tissues, as demonstrated in mouse melanoma model and arthritis models [24, 39]. Thus these CCL-PMs may be employed for not only sustained release of therapeutic peptides in blood, but also targeting of anticancer or anti-inflammatory peptides to tumours and inflammatory sites. Altogether, these key features encourage application of this polymeric micelle system for the sustained release of a range of therapeutic peptides.

Acknowledgements

This work was supported by Cristal Therapeutics. The authors are thankful to J.B. van den Dikkenberg and H.W. Hilbers for their contribution to our work.

Abbreviations

ACN	Acetonitrile
AUC	Area under the plasma concentration-time curve
CCL-PMs	Core-cross-linked polymeric micelles
CL	Clearance
CMT	Critical micelle temperature
DCC	<i>N,N'</i> -dicyclohexylcarbodiimide
DLS	Dynamic light scattering
DMAP	4-Dimethylaminopyridine
DMF	<i>N,N</i> -dimethylformamide
EE	Entrapment efficiency
EPR	Enhanced permeability and retention
ESI-MS	Electrospray ionisation mass spectrometry
FSH	Follicle stimulating hormone
GnRH	Gonadotropin releasing hormone
GPC	Gel permeation chromatography
HPMAmLac ₁	<i>N</i> -(2-hydroxypropyl) methacrylamide monolactate
HPMAmLac ₂	<i>N</i> -(2-hydroxypropyl) methacrylamide dilactate
i.m.	Intramuscular
i.v.	Intravenous
KPS	Potassium persulfate
L1	2-(2-(Methacryloyloxy)ethylthio)acetic acid
L2	2-(2-(methacryloyloxy)ethylsulfinyl)acetic acid
LC-MS	Liquid chromatography-mass spectrometry
LeuL1	Leuprolide-L1
LeuL2	Leuprolide-L2
LH	Luteinizing hormone
LOD	Limit of detection
mPES	Modified polyethersulfone
MPS	Mononuclear phagocyte system
MRT	Mean residence time
NMR	Nuclear magnetic resonance
PD	Polydispersity
PDI	Polydispersity index
PEG	Poly(ethylene glycol)
pHPMAmLac _n	Poly(<i>N</i> -(2-hydroxypropyl) methacrylamide-lactate
PL	Peptide loading
PLGA	Poly(D,L-lactide-co-glycolide)
s.c.	Subcutaneous
<i>t</i> _{1/2}	Half-life of elimination
TEA	Triethylamine
TEM	Transmission electron microscopy
TEMED	<i>N,N,N',N'</i> -tetramethylethylenediamine
TFA	Trifluoroacetic acid
TFF	Tangential flow filtration
UPLC	Ultra-performance liquid chromatography
V _{ss}	Steady-state volume of distribution
Z _{ave}	Z-average hydrodynamic diameter

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Appendix: Supplementary data

Figure S1. Numbering scheme of atoms in (A) the linker part in leuprolide-L1 and (B) the linker part in leuprolide-L2

Figure S2. LC-MS results of leuprolide derivatives. (A) Leuprolide-L1 and (B) Leuprolide-L2. The mass of leuprolide-L1 and leuprolide-L2 were observed at m/z 1394 ($M+H$)⁺ and m/z 1410 ($M+H$)⁺, respectively.

Table S1. ¹H chemical shifts (ppm) of the peptide part in leuprolide-L1 and leuprolide-L2 at 298K in DMSO-d₆

	Residue	Leuprolide #				Leuprolide-L1				Leuprolide-L2			
		NH	αH	βH	others	NH	αH	βH	others	NH	αH	βH	others
1	pGlu	7.76	3.97	1.73	γH: 2.14; 2.09	7.77	3.98	2.14; 1.73	γH: 2.06; 2.00	7.77	3.98	2.15; 1.74	γH: 2.07; 2.01
2	His	8.01	4.43	2.89; 2.77	ε1H: 7.49, δ2H: 6.75	7.95	4.44	2.90; 2.81	ε1H: 7.47, δ2H: 6.79	7.88 7.92	4.42 4.43	2.89; 2.90; 2.82	ε1H: 7.47, δ2H: 6.78 ε1H: 7.47, δ2H: 6.78
3	Trp	8.18	4.54	3.16; 2.97	δ1H: 7.11, ε3H: 7.56, ζ3H: 6.90, η2H: 7.02, ζ2H: 7.29, ε1H: 10.80	8.20	4.55	3.20; 2.99	δ1H: 7.13, ε3H: 7.56, ζ3H: 6.94, η2H: 7.04, ζ2H: 7.31, ε1H: 10.81	8.21 8.17	4.57 4.56	3.24; 2.97 3.21; 2.98	δ1H: 7.12, ε3H: 7.57, ζ3H: 6.94, η2H: 7.04, ζ2H: 7.30, ε1H: 10.80 δ1H: 7.12, ε3H: 7.57, ζ3H: 6.94, η2H: 7.04, ζ2H: 7.30, ε1H: 10.80
4	Ser	8.43	4.33	3.58; 3.47		8.89	4.61	4.23; 4.16		8.99 8.91	4.68 4.66	4.29; 4.26 4.28; 4.25	
5	Tyr	8.10	4.38	2.89; 2.73	δH: 6.99, εH: 6.62	8.06	4.43	2.82; 2.74	δH: 6.97, εH: 6.61	8.10	4.44	2.82; 2.75	δH: 6.98, εH: 6.62
6	D-Leu	8.14	4.21	1.40; 1.34	δH: 0.79; 0.75	8.15	4.23	1.35	γH: 1.26, δH: 0.79; 0.74	8.17	4.24	1.35	γH: 1.27, δH: 0.79; 0.74
7	Leu	8.08	4.27	1.55; 1.44	δH: 0.85; 0.79	8.00	4.30	1.46; 1.42	γH: 1.55, δH: 0.85; 0.79	8.03	4.30	1.44	γH: 1.55, δH: 0.85; 0.80
8	Arg	8.08	4.46	1.72; 1.59	γH: 1.54, δH: 3.04, εH: 8.67	8.14	4.45	1.72; 1.59	γH: 1.54, δH: 3.08; 3.04, εH: 8.45	8.13	4.45	1.73; 1.59	γH: 1.54, δH: 3.08; 3.04, εH: 8.37
9	Pro	--	4.19	1.99; 1.89	γH: 1.75, δH: 3.62; 3.54	--	4.20	1.99; 1.73	γH: 1.90; 1.80, δH: 3.63; 3.54	--	4.20	2.00; 1.73	γH: 1.90; 1.80, δH: 3.63; 3.54
10	NHEt	7.78	3.01	0.96		7.80	3.06; 2.99	0.97		7.80	3.06; 2.99	0.97	

The ¹H chemical shifts of the residues in leuprolide are obtained from the work of Laimou *et al.* [1]. As leuprolide-L2 contains a sulfoxide group, different chemical shifts for the two possible diastereoisomers are presented (applicable when pairs of signals could be resolved by small differences in chemical shift).

[1] D. Laimou, M. Katsara, M.T. Matsoukas, V. Apostolopoulos, A. Troganis, T. Tselios, Structural elucidation of Leuprolide and its analogues in solution: insight into their bioactive conformation, *Amino Acids*, 39 (2010) 1147-1160.

Table S2. Chemical shifts (ppm) of leuprolide-L1 at 298K in DMSO-d₆. ¹H and ¹³C shifts are given rel. to TMS at zero ppm. ¹⁵N chemical shifts are given rel. to neat nitromethane-d₃ at zero ppm.

Conformer 1 (Pro9 trans - 88.5% populated)				Conformer 2 (Pro9 cis - 11.5% populated)*			
Group	Atom	Nuclei	Shift	Group	Atom	Nuclei	Shift
Linker	C1	¹³ C	32.58				
	C2	¹³ C	30.24				
	C3	¹³ C	63.00				
	C4	¹³ C	125.92				
	C5	¹³ C	17.87				
	C6	¹³ C	135.59				
	C7	¹³ C	166.27				
	C8	¹³ C	169.72				
	H1#	¹ H	3.35				
	H2#	¹ H	2.85				
	H3#	¹ H	4.24				
	H4a	¹ H	6.02				
	H4b	¹ H	5.67				
	H5#	¹ H	1.86				
pGlu1	C	¹³ C	172.10				
	CA	¹³ C	55.39				
	CB	¹³ C	25.10				
	CD	¹³ C	177.37				
	CG	¹³ C	28.99				
	H	¹ H	7.77				
	HA	¹ H	3.98				
	HB1	¹ H	2.14				
	HB2	¹ H	1.73				
	HG1	¹ H	2.06				
	HG2	¹ H	2.00				
	N	¹⁵ N	-259.61				

H2	C	^{13}C	170.99	
	CA	^{13}C	52.71	
	CB	^{13}C	29.58	
	CD2	^{13}C	missing peak in ^{13}C - ^1H HSQC	
	CE1	^{13}C	134.67	
	H	^1H	7.95	
	HA	^1H	4.44	
	HB1	^1H	2.90	
	HB2	^1H	2.81	
	HD2	^1H	6.79	
	HE1	^1H	7.47	
	N	^{15}N	-263.56	
W3	C	^{13}C	171.80	
	CA	^{13}C	53.60	
	CB	^{13}C	27.16	
	CD1	^{13}C	123.49	
	CD2	^{13}C	127.22	
	CE2	^{13}C	135.95	
	CE3	^{13}C	118.25	
	CG	^{13}C	109.83	
	CH2	^{13}C	120.74	
	CZ2	^{13}C	111.15	
	CZ3	^{13}C	118.13	
	H	^1H	8.20	
	HA	^1H	4.55	
	HB1	^1H	3.20	
	HB2	^1H	2.99	
	HD1	^1H	7.13	
	HE1	^1H	10.81	
	HE3	^1H	7.56	
	HH2	^1H	7.04	
	HZ2	^1H	7.31	
	HZ3	^1H	6.94	
	N	^{15}N	-261.65	
	NE1	^{15}N	-249.10	

S4	C	¹³ C	168.26	S4b			
	CA	¹³ C	51.66		CA	13C	51.59
	CB	¹³ C	63.71		CB	13C	63.64
	H	¹ H	8.89				
	HA	¹ H	4.61		HA	1H	4.65
	HB1	¹ H	4.23		HB1	1H	4.23
	HB2	¹ H	4.16		HB2	1H	4.17
	N	¹ H	missing peak in ¹⁵ N- ¹ H HSQC				
Y5	C	¹³ C	170.38	Y5b			
	CA	¹³ C	54.82		CA	13C	54.82
	CB	¹³ C	36.80		CB	13C	36.84
	CD	¹³ C	129.97				
	CE	¹³ C	114.79				
	CG	¹³ C	127.02				
	CZ	¹³ C	155.86				
	H	¹ H	8.06		H	1H	8.09
	HA	¹ H	4.43		HA	1H	4.43
	HB1	¹ H	2.82		HB1	1H	2.81
	HB2	¹ H	2.74		HB2	1H	2.75
	HD#	¹ H	6.97		HD#	1H	6.97
	HE#	¹ H	6.61				
	HO	¹ H	9.27				
	N	¹⁵ N	-260.64		N	15N	-260.32
dL6	C	¹³ C	171.62	dL6b			
	CA	¹³ C	51.07		CA	13C	51.26
	CB	¹³ C	40.85		CB	13C	40.69
	CD1	¹³ C	22.92				
	CD2	¹³ C	21.40				
	CG	¹³ C	23.78				
	H	¹ H	8.15		H	1H	8.19
	HA	¹ H	4.23		HA	1H	4.19
	HB#	¹ H	1.35		HB#	1H	1.35
	HD1#	¹ H	0.79		HD1#	1H	0.78
	HD2#	¹ H	0.74		HD2#	1H	0.73
	HG	¹ H	1.26		HG	1H	1.26
	N	¹⁵ N	-259.64		N	15N	-259.04

L7	C	¹³ C	171.78	L7b	CA	¹³ C	50.61
	CA	¹³ C	50.61		CB	¹³ C	40.29
	CB	¹³ C	40.55		H	¹ H	8.05
	CD1	¹³ C	23.08		HA	¹ H	4.30
	CD2	¹³ C	21.22		HB#	¹ H	1.46
	CG	¹³ C	23.99		HD1#	¹ H	0.85
	H	¹ H	8.00		HD2#	¹ H	0.79
	HA	¹ H	4.30		HG	¹ H	1.55
	HB#	¹ H	1.44		N	¹⁵ N	-262.02
	HB1	¹ H	1.46				
	HB2	¹ H	1.42				
	HD1#	¹ H	0.85				
	HD2#	¹ H	0.79				
	HG	¹ H	1.55				
	N	¹⁵ N	-262.21				
R8	C	¹³ C	169.58	R8b	CA	¹³ C	50.05
	CA	¹³ C	50.05		CB	¹³ C	29.03
	CB	¹³ C	27.90		CD	¹³ C	40.24
	CD	¹³ C	40.43		CG	¹³ C	24.44
	CG	¹³ C	24.27		H	¹ H	7.93
	CZ	¹³ C	157.14		HA	¹ H	4.28
	H	¹ H	8.14		HB1	¹ H	1.64
	HA	¹ H	4.45		HB2	¹ H	1.53
	HB1	¹ H	1.72		HD#	¹ H	3.05
	HB2	¹ H	1.59		HE	¹ H	8.74
	HD1	¹ H	3.08		HG#	¹ H	1.46
	HD2	¹ H	3.04		HN2	¹ H	7.48
	HE	¹ H	8.45		N	¹⁵ N	-262.92
	HG#	¹ H	1.54		NE	¹⁵ N	-295.54
	HN2	¹ H	7.53				
	N	¹⁵ N	-261.91				
	NE	¹⁵ N	-295.43				
	NH2	¹⁵ N	-305.46				

P9	C	¹³ C	171.10	P9b	CA	¹³ C	59.43
	CA	¹³ C	59.60		CB	¹³ C	31.30
	CB	¹³ C	29.21		CD	¹³ C	46.46
	CD	¹³ C	46.65		CG	¹³ C	21.80
	CG	¹³ C	24.40		HA	¹ H	4.37
	HA	¹ H	4.20		HB1	¹ H	2.10
	HB1	¹ H	1.99		HB2	¹ H	2.00
	HB2	¹ H	1.73		HD1	¹ H	3.44
	HD1	¹ H	3.63		HD2	¹ H	3.34
	HD2	¹ H	3.54		HG#	¹ H	1.73
	HG1	¹ H	1.90				
	HG2	¹ H	1.80				
NHEt10	CA	¹³ C	33.23	NHEt10b	CA	¹³ C	33.59
	CB	¹³ C	14.57		CB	¹³ C	14.44
	H	¹ H	7.80		H	¹ H	8.26
	HA1	¹ H	3.06		HA1	¹ H	3.10
	HA2	¹ H	2.99		HA2	¹ H	3.04
	HB#	¹ H	0.97		HB#	¹ H	1.00
	N	¹⁵ N	-264.14		N	¹⁵ N	-261.30

¹H chemical shifts of part of the groups are also displayed in Table S1.

Table S3. Chemical shifts (ppm) of leuprolide-L2 at 298K in DMSO-d₆. ¹H and ¹³C shifts are given rel. to TMS at zero ppm. ¹⁵N chemical shifts are given rel. to neat nitromethane-d₃ at zero ppm.

Diastereoisomer P1 (ca. 43.5% populated with Pro9 trans)				Diastereoisomer P2 (ca. 43.5% populated with Pro9 trans)				Conformer 3 (hydrolysed linkage of P3 or Pro9 cis - ca. 13% populated)*			
Sulfoxide S=O (R or S)				Sulfoxide S=O (S or R)							
Group	Atom	Nuc	Shift	Group	Atom	Nuc	Shift	Group	Atom	Nuc	Shift
Linkp1	C1	¹³ C	55.29	Linkp2	C1	¹³ C	55.22	Linkp3	C1	¹³ C	60.58
	C2	¹³ C	50.06		C2	¹³ C	50.03		C2	¹³ C	49.66
	C3	¹³ C	57.39		C3	¹³ C	57.39		C3	¹³ C	57.61
	C4	¹³ C	126.29		C4	¹³ C	126.27		C4	¹³ C	126.09
	C5	¹³ C	17.82		C5	¹³ C	17.78				
	C6	¹³ C	135.39		C6	¹³ C	135.32				
	C7	¹³ C	166.12		C7	¹³ C	166.08		C7	¹³ C	166.23
	C8	¹³ C	165.58		C8	¹³ C	165.66		C8	¹³ C	167.37
	H1a	¹ H	4.08		H1a	¹ H	4.07		H1a	¹ H	3.52
	H1b	¹ H	3.86		H1b	¹ H	3.83		H1b	¹ H	3.46
	H2a	¹ H	3.27		H2a	¹ H	3.27		H2a	¹ H	3.26
	H2b	¹ H	3.24		H2b	¹ H	3.23		H2b	¹ H	2.97
	H3a	¹ H	4.52		H3a	¹ H	4.52		H3a	¹ H	4.48
	H3b	¹ H	4.41		H3b	¹ H	4.41		H3b	¹ H	4.39
	H4a	¹ H	6.05		H4a	¹ H	6.03		H4a	¹ H	6.06
	H4b	¹ H	5.71		H4b	¹ H	5.68		H4b	¹ H	5.70
	H5#	¹ H	1.88		H5#	¹ H	1.85				
pGlu1	C	¹³ C	172.10								
	CA	¹³ C	55.40								
	CB	¹³ C	25.11								
	CD	¹³ C	177.39								
	CG	¹³ C	28.98								
	H	¹ H	7.77								
	HA	¹ H	3.98								
	HB1	¹ H	2.15								
	HB2	¹ H	1.74								
	HG1	¹ H	2.07								
	HG2	¹ H	2.01								
	N	¹⁵ N	-259.60								

H2p1	C	¹³ C	170.81	H2p2	C	¹³ C	170.81	H2p3			
	CA	¹³ C	52.61		CA	¹³ C	52.62				
	CB	¹³ C	29.53		CB	¹³ C	29.53				
	CD2	¹³ C	missing in ¹³ C - ¹ H HSQC		CD2	¹³ C	missing in ¹³ C - ¹ H HSQC				
	CE1	¹³ C	134.66		CE1	¹³ C	134.66				
	H	¹ H	7.88		H	¹ H	7.92		H	¹ H	7.98
	HA	¹ H	4.42		HA	¹ H	4.43		HA	¹ H	4.43
	HB1	¹ H	2.89		HB1	¹ H	2.90		HB1	¹ H	2.89
	HB2	¹ H	2.83		HB2	¹ H	2.82		HB2	¹ H	2.78
	HD2	¹ H	6.78		HD2	¹ H	6.78				
	HE1	¹ H	7.47		HE1	¹ H	7.47				
	N	¹⁵ N	-264.02		N	¹⁵ N	-263.81		N	¹⁵ N	-263.39
W3p1	C	¹³ C	171.80	W3p2	C	¹³ C	171.80				
	CA	¹³ C	53.49		CA	¹³ C	53.51				
	CB	¹³ C	27.10		CB	¹³ C	27.14				
	CD1	¹³ C	123.48		CD1	¹³ C	123.48				
	CD2	¹³ C	127.24		CD2	¹³ C	127.24				
	CE2	¹³ C	135.94		CE2	¹³ C	135.94				
	CE3	¹³ C	118.24		CE3	¹³ C	118.24				
	CG	¹³ C	109.88		CG	¹³ C	109.88				
	CH2	¹³ C	120.74		CH2	¹³ C	120.74				
	CZ2	¹³ C	111.16		CZ2	¹³ C	111.16				
	CZ3	¹³ C	118.12		CZ3	¹³ C	118.12				
	H	¹ H	8.21		H	¹ H	8.17				
	HA	¹ H	4.57		HA	¹ H	4.56				
	HB1	¹ H	3.24		HB1	¹ H	3.21				
	HB2	¹ H	2.97		HB2	¹ H	2.98				
	HD1	¹ H	7.12		HD1	¹ H	7.12				
	HE1	¹ H	10.80		HE1	¹ H	10.80				
	HE3	¹ H	7.57		HE3	¹ H	7.57				
	HH2	¹ H	7.04		HH2	¹ H	7.04				
	HZ2	¹ H	7.30		HZ2	¹ H	7.30				
	HZ3	¹ H	6.94		HZ3	¹ H	6.94				
	N	¹⁵ N	-261.80		N	¹⁵ N	-261.88				
	NE1	¹⁵ N	-249.11		NE1	¹⁵ N	-249.11				

S4p1	C	¹³ C	168.19	S4p2	C	¹³ C	168.19	S4p3			
	CA	¹³ C	51.43		CA	¹³ C	51.45		CA	¹³ C	51.34
	CB	¹³ C	64.08		CB	¹³ C	64.08		CB	¹³ C	63.89
	H	¹ H	8.99		H	¹ H	8.91		H	¹ H	8.16
	HA	¹ H	4.68		HA	¹ H	4.66		HA	¹ H	4.72
	HB1	¹ H	4.29		HB1	¹ H	4.28		HB#	¹ H	4.27
	HB2	¹ H	4.26		HB2	¹ H	4.25				
	N	¹⁵ N	missing in ¹⁵ N- ¹ H HSQC		N	¹⁵ N	missing in ¹⁵ N- ¹ H HSQC				
Y5	C	¹³ C	170.37					Y5b			
	CA	¹³ C	54.79								
	CB	¹³ C	36.83								
	CD	¹³ C	129.97								
	CE	¹³ C	114.80								
	CG	¹³ C	127.02								
	CZ	¹³ C	155.84								
	H	¹ H	8.10						H	¹ H	8.15
	HA	¹ H	4.44						HA	¹ H	4.46
	HB1	¹ H	2.82						HB1	¹ H	2.80
	HB2	¹ H	2.75						HB2	¹ H	2.77
	HD#	¹ H	6.98								
	HE#	¹ H	6.62								
	HO	¹ H	9.26								
	N	¹⁵ N	-260.49								

dL6	C	¹³ C	171.63			dL6b			
	CA	¹³ C	51.07				CA	¹³ C	51.37
	CB	¹³ C	40.89				CB	¹³ C	40.61
	CD1	¹³ C	22.91						
	CD2	¹³ C	21.40						
	CG	¹³ C	23.82						
	H	¹ H	8.17				H	¹ H	8.23
	HA	¹ H	4.24				HA	¹ H	4.18
	HB#	¹ H	1.35				HB#	¹ H	1.35
	HD1#	¹ H	0.79				HD1#	¹ H	0.79
	HD2#	¹ H	0.74				HD2#	¹ H	0.73
	HG	¹ H	1.27				HG	¹ H	1.27
	N	¹⁵ N	-259.59				N	¹⁵ N	-258.41
L7	C	¹³ C	171.79			L7b			
	CA	¹³ C	50.62				CA	¹³ C	50.61
	CB	¹³ C	40.58				CB	¹³ C	40.21
	CD1	¹³ C	23.09						
	CD2	¹³ C	21.19						
	CG	¹³ C	24.01						
	H	¹ H	8.03				H	¹ H	8.13
	HA	¹ H	4.30				HA	¹ H	4.29
	HB#	¹ H	1.44				HB#	¹ H	1.47
	HD1#	¹ H	0.85				HD1#	¹ H	0.85
	HD2#	¹ H	0.80				HD2#	¹ H	0.79
	HG	¹ H	1.55						
	N	¹⁵ N	-262.14						

R8	C	¹³ C	169.61		R8b	CA	¹³ C	50.02
	CA	¹³ C	50.05			CB	¹³ C	29.03
	CB	¹³ C	27.91			CD	¹³ C	40.24
	CD	¹³ C	40.43			CG	¹³ C	24.48
	CG	¹³ C	24.28					
	CZ	¹³ C	157.08			H	¹ H	7.92
	H	¹ H	8.13			HA	¹ H	4.29
	HA	¹ H	4.45			HB1	¹ H	1.65
	HB1	¹ H	1.73			HB2	¹ H	1.54
	HB2	¹ H	1.59			HD#	¹ H	3.06
	HD1	¹ H	3.08					
	HD2	¹ H	3.04			HE	¹ H	8.68
	HE	¹ H	8.37			HG#	¹ H	1.46
	HG#	¹ H	1.54			HN2	¹ H	7.53
	HN2	¹ H	7.49			N	¹⁵ N	-263.13
	N	¹⁵ N	-261.88			NE	¹⁵ N	-295.50
P9	NE	¹⁵ N	-295.40		P9b	C	¹³ C	170.49
	NH2	¹⁵ N	-305.58			CA	¹³ C	59.45
						CB	¹³ C	31.33
						CD	¹³ C	46.46
						CG	¹³ C	21.79
						HA	¹ H	4.37
						HB1	¹ H	2.10
						HB2	¹ H	2.00
						HD1	¹ H	3.43
						HD2	¹ H	3.35
						HG#	¹ H	1.73

NHEt10	CA	¹³ C	33.24	NHEt10b	CA	¹³ C	33.59
	CB	¹³ C	14.56		CB	¹³ C	14.44
	H	¹ H	7.80		H	¹ H	8.24
	HA1	¹ H	3.06		HA1	¹ H	3.10
	HA2	¹ H	2.99		HA2	¹ H	3.04
	HB#	¹ H	0.97		HB#	¹ H	1.00
	N	¹⁵ N	-264.11		N	¹⁵ N	-261.37

*only shifts that are distinctly different from conformer P1/P2 are given.

¹H chemical shifts of part of the groups are also displayed in Table S1.

Table S4. Toxicological evaluation of LeuL1-CCL-PMs

Group	Animal #	Clinical signs
Vehicle	1	none (day 1-day 5)
	2	none (day 1-day 5)
	3	none (day 1-day 5)
Soluble leuprolide (0.10 mg/kg)	4	none (day 1)
	5	none (day 1)
	6	none (day 1)
LeuL1-CCL-PMs (0.13 mg/kg)	7	Scabs* on both forelegs (day 4, day5)
	8	none (day 1-day 5)
	9	none (day 1-day 5)
LeuL1-CCL-PMs (1.3 mg/kg)	10	none (day 1-day 5)
	11	none (day 1-day 5)
	12	none (day 1-day 5)
LeuL1-CCL-PMs (13 mg/kg)	13	none (day 1-day 5)
	14	none (day 1-day 5)
	15	none (day 1-day 5)

Animals that received a single i.v. injection of soluble leuprolide (0.10 mg/kg) were monitored for 1 day and then sacrificed, whereas animals treated with LeuL1 CCL-PMs (0.13 mg/kg, 1.3 mg/kg and 13 mg/kg, respectively) or vehicle (i.e. ammonium acetate 20 mM pH 5.0 buffer containing 130 mM NaCl) were monitored for 5 days before sacrificed.

*these side effects have also been reported by patients who received leuprolide containing product such as Lupron depot®.

Chapter 5

High systemic availability of core-cross-linked polymeric micelles after subcutaneous administration

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Submitted for publication

Abstract

Covalent entrapment of drug molecules within core-cross-linked polymeric micelles (CCL-PMs) represents an attractive approach to improve their *in vivo* disposition and thereby enhance their therapeutic index. Although the intravenous (i.v.) route of administration is most commonly employed for nanomedicinal products, subcutaneous (s.c.) administration could be more attractive as it offers the possibility of self-administration and thereby may reduce healthcare costs. The aim of this work was to assess the pharmacokinetic profile and systemic availability of CCL-PMs containing a corticosteroid or a taxane following s.c. injection. In the present study, dexamethasone (DMS) was derivatised with three different linkers which allowed for covalent attachment of this drug to the core of CCL-PMs. The obtained DMS-containing CCL-PMs exhibited varying drug release kinetics *in vitro*. Remarkably, a single dose of DMS-containing CCL-PMs resulted in high systemic availability of about 30% following s.c. injection into the flank of healthy mice, as evidenced by an area under the plasma concentration-time curve (AUC) between 26-37% relative to the AUC attained following i.v. injection. In all cases, plasma levels of total (entrapped plus released) DMS were detected for at least 4 days post s.c. administration and the type of linker did not alter the pharmacokinetic profile of these nanomedicines. Next to DMS, we covalently attached the drug paclitaxel (PTX) to the core of CCL-PMs. Similarly, high systemic availability of about 40% was attained following s.c. administration of the obtained PTX-containing CCL-PMs as compared to i.v. injection and PTX (entrapped plus released) was detected in the blood for at least 4 days. Importantly, the systemic availability of s.c. administered drug-containing CCL-PMs is substantially higher than that of other nanoformulations as reported in the literature (e.g. 3% in rodents). These results demonstrate that s.c. administration is a promising route to attain high systemic availability of CCL-PMs, enabling a potentially more patient-friendly and cost-effective treatment approach than the i.v. route.

1. Introduction

The development of nanoparticulate technologies has brought new solutions for the diagnosis and treatment of various diseases [1-6]. Nanoparticulate nanomedicines have the potential to enhance the solubility and stability of poorly-soluble drugs, to provide control over their pharmacokinetics (PK), tissue distribution and release profile and thereby to improve their therapeutic index. Nanoparticles for therapeutic applications are typically characterised by a small size (< 200 nm) and prolonged circulation properties. Owing to the enhanced permeability and retention (EPR) effect [7, 8], nanoparticles can preferably localise in pathological areas such as tumours and sites of inflammation, potentially leading to superior efficacy while side effects are reduced due to a favorably altered biodistribution profile [9-11].

To date, a few nanomedicinal products are already on the market and many more are currently in clinical development phases [12, 13]. In particular, polymeric micelles (PMs) composed of methoxy poly(ethylene glycol)-*b*-poly[N-(2-hydroxypropyl) methacrylamide-lactate] (mPEG-*b*-pHPMAmLac_n) block copolymers have received considerable attention [14, 15]. These PMs possess a core-shell structure in which the hydrophobic core enables facile entrapment of (hydrophobic) drugs while the hydrophilic shell sterically stabilises the PMs. However, many micellar systems as such are not stable *in vivo* due to the extensive dilution in the vascular compartment and/or adsorption of the block polymer to plasma proteins (e.g. albumin and lipoproteins) [16, 17]. To prevent disintegration following intravenous (i.v.) administration, PMs are stabilised by cross-linking the block copolymers in the micellar core [16, 18, 19]. The cross-linking provides the PMs with prolonged circulation kinetics and thereby boosts the potential to target tumours and sites of inflammation via the EPR effect [15, 19]. To avoid premature drug leakage, drug molecules are covalently attached to the core of PMs via hydrolysable linkages, yielding a stabilised micellar system with controllable drug release kinetics [20, 21]. Previously, nanomedicines based on core-cross-linked polymeric micelles (CCL-PMs) have demonstrated excellent therapeutic performance in various disease models following i.v. administration [20-23].

Besides physicochemical properties, the route of administration also plays an important role in the PK and biodistribution patterns of nanomedicines [24]. The most commonly used i.v. route provides direct access to the general circulation. However, i.v. administration requires hospitalisation and the presence of qualified healthcare staff and equipment. Therefore, the use of an alternative route of administration avoiding such healthcare costs is certainly of great interest. Subcutaneous (s.c.) injection represents a convenient route of administration which could allow patients to self-administer nanomedicines at home. S.c. injection administers the therapeutics to the interstitial area underlying the dermis of the skin. Although s.c. administered nanomedicines do not have direct access to the bloodstream, evidence is available

demonstrating that they can be absorbed by the regional lymphatics draining the s.c. injection site [25, 26]. To reach the general circulation, s.c. administered nanomedicines should first traverse the interstitium to enter the lymphatic capillaries and pass through a system of lymphatic ducts to finally enter the bloodstream, while a fraction of the administered dose can be captured by regional lymph nodes [24, 26]. Possibly, drug molecules are released during the lymphatic transport process and then directly enter regional blood capillaries. The efficiency of s.c. administration of nanomedicines for systemic delivery largely depends upon key physicochemical properties such as particle size [24, 27–31] and surface characteristics [32, 33].

The present study aims to examine the s.c. absorption of drug-containing CCL-PMs in healthy mice. Previously, dexamethasone (DMS) covalently attached to the core of CCL-PMs demonstrated excellent efficacy for the treatment of rheumatoid arthritis and solid tumours following i.v. administration [20, 21]. By employing linkers that contain thioethers with different degrees of oxidation, the release kinetics of DMS from the CCL-PMs was successfully tailored *in vitro* [20]. In the present study, we studied the same DMS-containing CCL-PMs exhibiting different drug release kinetics prepared with the same linker chemistry and investigated their PK profiles and systemic availability in healthy mice following s.c. and i.v. administration. In addition to DMS, we also covalently attached the drug molecule paclitaxel (PTX) to the core of CCL-PMs. The PK profile of the obtained PTX-containing CCL-PMs was also assessed in healthy mice after s.c. and i.v. administration.

2. Materials and Methods

2.1. Materials

Dexamethasone (DMS), 1-hydroxy-7-azabenzotriazole (HOAt), 4-dimethylaminopyridine (DMAP), ammonium acetate, formic acid, *N,N'*-diisopropylcarbodiimide (DIC), *N,N,N',N'*-tetramethylethylenediamine (TEMED), potassium persulfate (KPS), sodium sulfate (Na_2SO_4) and trifluoroacetic acid (TFA) were obtained from Sigma Aldrich (Zwijndrecht, The Netherlands). Paclitaxel (PTX) was obtained from LC laboratories (MA, USA). Acetonitrile (ACN) and dichloromethane (DCM) were purchased from Biosolve (Valkenswaard, The Netherlands) and absolute ethanol was obtained from Merck (Darmstadt, Germany). 2-(2-(Methacryloyloxy)ethylthio)acetic acid (referred as “linker 1”, L1), 2-(2-(methacryloyloxy)ethylsulfinyl)acetic acid (referred as “linker 2”, L2) and 2-(2-(methacryloyloxy)ethylsulfonyl)acetic acid (referred as “linker 3”, L3) were synthesised as described previously [20]. The other chemicals were used as received.

2.2. Synthesis of (methacrylated) block copolymer

A block copolymer containing a hydrophilic methoxy poly(ethylene glycol) (mPEG, $M_n = 5000$) block and a thermosensitive block composed of a random copolymer of N-(2-hydroxypropyl) methacrylamide monolactate (HPMAmLac₁, 47 mol%) and N-(2-hydroxypropyl) methacrylamide dilactate (HPMAmLac₂, 53 mol%) was synthesised as previously described [22]. Next, 11 mol% of the lactate side chains was derivatised with methacrylic acid according to a previously reported protocol [22] (80% yield). The critical micelle temperature (CMT) of the methacrylated block copolymer was ca. 10 °C. The weight average molecular weight (M_w) and polydispersity of the methacrylated block copolymer (as determined by gel permeation chromatography (GPC) [16]) were 27 kDa and 2.2, respectively. The number average molecular weight (M_n) of the thermosensitive pHPMAmLac_n block (as determined by nuclear magnetic resonance (NMR) [22]) was 15 kDa.

2.3. Synthesis and analysis of DMS derivatives

DMS was covalently coupled to L1, L2 and L3 to obtain DMS derivative DMSL1, DMSL2 and DMSL3, respectively, as described in detail by Crielaard *et al.* [20] (Figure 1).

Figure 1. Chemical structures of dexamethasone derivatives containing thioethers with different degrees of oxidation. (A) DMSL1, (B) DMSL2 and (C) DMSL3.

2.4. Synthesis and analysis of PTX derivative

L1 was conjugated to the hydroxyl group at the C-2' position of PTX to obtain PTX derivative PTXL1 (**Figure 2**). In brief, L1 (2.34 mmol) was dissolved in DCM (100 mL). Next, DMAP (5.62 mmol), PTX (2.34 mmol), DIC (2.81 mmol) and HOAt (2.81 mmol) were added and the mixture was stirred at room temperature for 2 d. Thereafter, the mixture was washed with water (150 mL), yielding a two phase system. The organic layer was separated and the aqueous layer was extracted with DCM (100 mL). Next, the combined organic layers were dried over Na_2SO_4 and evaporated in vacuo to obtain a yellow oil. The resulting oil was purified by column chromatography using heptane/ethyl acetate (4/1 to 1/1) and the pooled fractions were evaporated in vacuo to obtain PTXL1 as a white solid (75% yield).

NMR characterisation of PTXL1 was recorded using a Gemini 400 MHz spectrometer (Varian Associates Inc. NMR Instruments, Palo Alto, CA) and DMSO-d_6 was used as solvent.

The molecular mass of PTXL1 was determined using electrospray ionisation mass spectrometry (ESI-MS) on a Shimadzu liquid chromatography–mass spectrometry (LC-MS) QP8000 in positive ion mode. A XBridge® 3.5 μm C18 column (50 $\times$ 2.1 mm) (Waters) was used with a gradient from 5% eluent A (0.1% formic acid in ACN)/95% eluent B (0.1% formic acid in water) to 98% eluent A/2% eluent B in 3.5 min at a flow of 0.8 mL/min. The diode array detector was set between 220 and 320 nm. The mass used to identify PTXL1 was 1040.13 Da.

Figure 2. Synthesis scheme of paclitaxel derivative PTXL1

2.5. Preparation of drug-containing CCL-PMs

A series of DMS-containing core-cross-linked polymeric micelles (DMSL_x-CCL-PMs, $x=1, 2$ or 3) and PTX-containing core-cross-linked polymeric micelles (PTXL1-CCL-PMs) were prepared using the fast heating method [16, 34]. In brief, an ice-cold aqueous solution of methacrylated mPEG-*b*-pHPMAmLac_n block copolymer (830 μ L, 24 mg/mL) was mixed with TEMED (25 μ L, 120 mg/mL), both dissolved in ammonium acetate buffer (150 mM, pH 5). Subsequently, the drug derivatives namely DMSL1, DMSL2, DMSL3 or PTXL1 (100 μ L, 20 mg/mL drug equiv., dissolved in ethanol) were added, followed by rapid heating to 60 °C while stirring vigorously for 1 min to form PMs. The micellar dispersions were then transferred into a vial containing KPS (45 μ L, 30 mg/mL) dissolved in ammonium acetate buffer (150 mM, pH 5) at room temperature. The PMs were covalently stabilised by polymerisation of the methacrylate moieties on the block polymer under a N₂ atmosphere at room temperature for 1 h to yield drug-containing CCL-PMs. The final feed concentrations of block copolymer and drug equivalents were 20 and 2 mg/mL, respectively. Next, the micellar dispersions were filtered through 0.2 μ m cellulose membrane filters to remove potentially formed aggregates. Prior to *in vivo* studies, drug-containing CCL-PMs were purified and concentrated using Vivaspin™ sample concentrators (MWCO 1000 kDa) at 3000 rpm (4 °C), using ammonium acetate buffer (150 mM, pH 5) as the washing solution.

2.6. Characterisation of drug-containing CCL-PMs

2.6.1. Particle size

The size of drug-containing CCL-PMs was measured by dynamic light scattering (DLS) using a Malvern ALV/CGS-3 Goniometer. DLS results are given as a *z*-average hydrodynamic diameter (Z_{ave}) and a polydispersity index (PDI).

2.6.2. Zeta potential

The zeta potential of drug-containing CCL-PMs dispersions was measured using a Malvern Zetasizer Nano-Z (Malvern Instruments, Malvern, UK) with universal ZEN 1002 dip cells and DTS (Nano) software (version 4.20) at 25 °C. Zeta potential measurements were performed in ammonium acetate buffer (150 mM, pH 5) at a polymer concentration of 10 mg/mL.

2.6.3. Determination of non-entrapped and total DMS contents in DMSLx-CCL-PMs by UPLC

In DMSLx-CCL-PMs (x=1, 2 or 3), the DMS that is not covalently attached to the core of the CCL-PMs is defined as non-entrapped DMS. The concentrations of non-entrapped DMS in DMSLx-CCL-PMs were determined by ultra-performance liquid chromatography (UPLC) (Waters) equipped with an Acquity HSS T3 1.8 μ m column (2.1 $\times$ 50 mm) (Waters) and an Ultraviolet/Visible (UV/Vis) detector (TUV, Waters) set at 235 nm. To this end, DMSLx-CCL-PMs were diluted 10-fold in a mixture of ACN/H₂O (20%/80% (v/v)) and 7 μ L of the resulting solution was injected. A gradient from 100% eluent A (27% ACN/73% H₂O/0.1% formic acid (v/v/v)) to 100% eluent B (90% ACN/10% H₂O/0.1% formic acid (v/v/v)) was used at a flow of 1 mL/min. DMS standards dissolved in ACN/H₂O (20%/80% (v/v)) mixture were used to prepare a calibration curve (linear between 0.2 and 50 μ g/mL).

The concentrations of total (entrapped plus non-entrapped) DMS in DMSLx-CCL-PMs were determined by measurement of released DMS (i.e. DMS released from the CCL-PMs due to the hydrolysis of ester linkage) in borate buffer solution (100 mM) adjusted to pH 11 at 37 °C. The release was considered complete when a plateau in DMS concentration was reached (after 12, 7 and 3 h for DMSL1-CCL-PMs, DMSL2-CCL-PMs and DMSL3-CCL-PMs, respectively). The detailed analytical method is described in section 2.7. The entrapment efficiency (EE) of DMS was calculated using the UPLC data as follows:

$$\%EE = \frac{\text{Amount of entrapped DMS}}{\text{Amount of DMS equiv. added}} \times 100\%$$

2.6.4. Determination of non-entrapped and total PTX contents in PTXL1-CCL-PMs by UPLC

In PTXL1-CCL-PMs, the PTX that is not covalently attached to the core of the CCL-PMs is defined as non-entrapped PTX. The concentration of non-entrapped PTX in PTXL1-CCL-PMs was determined by UPLC (Waters) using an Acquity HSS T3 1.8 μ m column (2.1 $\times$ 50 mm) (Waters) and a UV/Vis detector (TUV, Waters) set at 227 nm. The mobile phase consisted of 50% ACN/50% H₂O/0.1% formic acid (v/v/v) and the flow rate was 1 mL/min. Before injection, PTXL1-CCL-PMs were diluted 10-fold in a mixture of ACN/H₂O (70%/30% (v/v)) and 7 μ L of the resulting solution was injected. PTX standards dissolved in ACN/H₂O (70%/30% (v/v)) mixture were used to prepare a calibration curve (linear between 0.2 and 40 μ g/mL).

The concentration of total (entrapped plus non-entrapped) PTX in PTXL1-CCL-PMs was determined by measurement of released PTX upon hydrolysis of the sulfide ester bonds in phosphate buffer (100 mM, pH 7.4) containing 1% (v/v) polysorbate 80 (as to solubilise the released PTX) at 60 °C. The release was considered complete when a plateau in PTX concentration was reached (after 92 h). The detailed analytical method is described in section 2.7. The EE was calculated using the UPLC data as follows:

$$\%EE = \frac{\text{Amount of entrapped PTX}}{\text{Amount of PTX equiv.added}} \times 100\%$$

2.7. *In vitro* drug release from drug-containing CCL-PMs

DMSL_x-CCL-PMs (x=1, 2 or 3) were diluted in phosphate buffer (100 mM, pH 7.4, supplemented with 15 mM NaCl) to obtain a DMS concentration of max. 40 µg/mL. PTXL1-CCL-PMs were diluted in phosphate buffer (100 mM, pH 7.4, supplemented with 15 mM NaCl) that additionally contained 1% (v/v) polysorbate 80 (as to solubilise the released PTX) to obtain a PTX concentration of max. 40 µg/mL. The release of drug (DMS or PTX) was monitored at 37 °C by injecting 7 µL of the whole mixture in a UPLC system (Waters) equipped with an Acquity HSS T3 1.8 µm column (2.1 × 50 mm) (Waters) and a UV/Vis detector (TUV, Waters).

For DMSL_x-CCL-PMs, the mobile phase consisted of 27% ACN/73% H₂O/0.1% formic acid (v/v/v), the flow rate was 1 mL/min and the UV detector was set at 235 nm. For PTXL1-CCL-PMs, the mobile phase consisted of 50% ACN/50% H₂O/0.1% formic acid (v/v/v), the flow rate was 1 mL/min and the UV was set at 227 nm. The percentage of drug release was calculated as follows:

$$\%Release\ of\ drug = \frac{\text{Amount of released drug}}{\text{Amount of total drug}} \times 100\%$$

2.8. Pharmacokinetic studies

Animal experiments were conducted in compliance with the national regulations and approved by the local ethical committee for animal experimentation. All animals were housed in a temperature-controlled room (22 ± 3°C) with 55 ± 15% relative humidity, a photoperiod of 12/12 h and free access to water and pelleted rodent chow.

First, the PK profiles of a series of DMSL_x-CCL-PMs (x=1, 2 or 3) were assessed *in vivo*. In this study, C57Bl/6J male mice (8–12 weeks age, Charles River) weighing ~25

g were randomly divided into six groups of three animals. A single dose of DMSL1-CCL-PMs (20 mg DMS equiv. per kg), DMSL2-CCL-PMs (15 mg DMS equiv. per kg) or DMSL3-CCL-PMs (15 mg DMS equiv. per kg) was injected into the flank (s.c. administration) or the tail vein (i.v. administration) of the animals.

Similarly, to assess the PK profile of PTXL1-CCL-PMs, C57Bl/6J male mice (8–12 weeks age, Charles River) weighing ~25 g were randomly divided into two groups of three animals. A single dose of PTXL1-CCL-PMs (25 mg PTX equiv. per kg) was injected into the flank (s.c. administration) or the tail vein (i.v. administration) of the animals.

Blood samples were serially collected in tubes containing EDTA via cheek puncture from three mice per sampling time. Within 30 min after sampling, the blood samples were centrifuged for 10 min at 10,000 rpm (4 °C) and the supernatant was stored at -20 °C until analysis. The quantification of plasma levels of total DMS and total PTX is described in section 2.9 and 2.10.

PK data are presented as the mean $\pm$ SEM of 3 animals per group. PK parameters were calculated using Multifit pharmacokinetic software (University of Groningen, The Netherlands) by a two compartment nonlinear model as described previously [35]. To mention, in the present study, the the area under the plasma concentration-time curve (AUC) of total drug levels obtained following s.c. administration relative to the AUC attained via the i.v. route is denoted as the **systematic availability** (%) of drug-containing CCL-PMs.

2.9. Determination of total DMS contents in mouse plasma

Plasma levels of total (entrapped plus released) DMS were determined by UPLC. In brief, ACN (40 μ L) was added to mouse plasma (20 μ L). The mixture was vortexed for 10 s and centrifuged at 10,000 rpm (4 °C) for 2 min. The supernatant was added to 100 mM Na₂HPO₄ solution adjusted to pH 12 (40 μ L) and incubated at 37 °C for 2 h to allow for complete drug release. Next, 7 μ L of the mixture was injected in a UPLC system (Waters) equipped with an Acquity HSS T3 C18 1.8 μ m column (50 $\times$ 2.1 mm) (Waters) and a UV/Vis detector (TUV, Waters) set at 240 nm. A gradient from 100% eluent A (20% ACN/80% H₂O/0.1% formic acid (v/v/v)) to 100% eluent B (90% ACN/10% H₂O/0.1% formic acid (v/v/v)) was used at a flow of 1 mL/min.

2.10. Determination of total PTX contents in mouse plasma

Plasma levels of total (entrapped plus released) PTX were determined by UPLC. In brief, phosphate buffer (500 mM, pH 7.4) (20 μ L) was added to mouse plasma (20 μ L). The mixture was vortexed for 10 sec and then incubated at 60 °C for 2 d. Next, ACN (80 μ L) was added and the mixture was vortexed for 10 sec, followed by centrifugation at 12,000 rpm (4 °C) for 5 min. Next, 7 μ L of the supernatant was injected into a UPLC system (Waters) equipped with an Acquity HSS T3 C18 1.8 μ m column (2.1 $\times$ 50 mm) (Waters) and a UV/Vis detector (TUV, Waters) set at 227 nm. A gradient from 100% eluent A (45% ACN/55% H₂O/0.1% formic acid (v/v/v)) to 100% eluent B (90% ACN/10% H₂O/0.1% formic acid (v/v/v)) was used at a flow of 1 mL/min.

2.11. Statistical analysis

Statistical significance was analysed using two-tailed unpaired Student's t-test. A p-value < 0.05 was considered statistically significant. The calculated logP (cLogP) values of the synthesised drug derivatives were obtained using ChemBioDraw Ultra 14.

3. Results and Discussion

3.1. A series of DMSL_x-CCL-PMs with varying drug release kinetics

As reported previously [20], dexamethasone (DMS) was covalently attached within core-cross-linked polymeric micelles (CCL-PMs) via a sulfide (DMSL1), sulfoxide (DMSL2) or sulfone (DMSL3) ester bond, yielding a series of DMS-containing CCL-PMs (DMSL_x-CCL-PMs, x=1, 2 or 3). These DMSL_x-CCL-PMs were neutrally charged and possessed mean hydrodynamic sizes of 68 $\pm$ 7 nm with low polydispersity index (PDI, < 0.2), which are typical for such CCL-PM systems [21, 22, 36, 37]. Moreover, their comparable mean hydrodynamic size (distribution) and drug entrapment efficiency (90 $\pm$ 5%) indicate that the key physicochemical properties of these DMSL_x-CCL-PMs are not affected by the type of linker used.

The different linkers used served to modulate the release kinetics of DMS, which was evaluated under physiological conditions (pH 7.4, 37 °C) (**Figure 3**). Compared to DMSL1-CCL-PMs (containing the sulfide ester linkage, < 6% release in 5 days), drug release from the CCL-PMs was accelerated when sulfoxide (DMSL2, $t_{1/2}$ = 15.3 d) or sulfone (DMSL3, $t_{1/2}$ = 8.2 d) ester linkages were employed. The varying drug

release profiles are attributed to the different hydrolysis rates of the thioether ester bonds involved, with faster drug release when the sulfur is in a higher oxidation state, which thereby exhibits a stronger electron-withdrawing effect resulting in an ester bond that is more susceptible to hydrolysis. *In vitro*, the DMS release rate increased in the order of DMSL1 < DMSL2 < DMSL3, which is in good agreement with the previous findings of Crielaard *et al.* [20]. Using this CCL-PMs system, tailorable drug release kinetics was achieved not only with a small molecular weight drug DMS, but recently also with the nonapeptide leuprolide by employing L1 and L2 [36].

Figure 3. Representative *in vitro* DMS release curves from DMSL1-CCL-PMs, DMSL2-CCL-PMs and DMSL3-CCL-PMs, in phosphate buffer (100 mM, pH 7.4, supplemented with 15 mM NaCl) at 37 °C

3.2. High systemic availability of s.c. administered DMSLx-CCL-PMs

To investigate the impact of a) route of administration and b) drug release kinetics on the PK profile of the polymeric nanomedicines, DMSLx-CCL-PMs were injected into healthy mice via the s.c. and i.v. route of administration. The plasma concentrations of total (entrapped plus released) DMS were monitored over time (**Figure 4**) and the calculated PK parameters are presented in **Table 1**.

Figure 4. Plasma levels of total (entrapped plus released) DMS after a single s.c. or i.v. injection of (A) DMSL1-CCL-PMs, (B) DMSL2-CCL-PMs and (C) DMSL3-CCL-PMs into healthy mice. Data are expressed as the mean $\pm$ SEM (n=3)

Table 1. Pharmacokinetic parameters related to total (entrapped plus released) DMS in plasma following a single s.c. or i.v. injection of DMSL1-CCL-PMs (20 mg/kg), DMSL2-CCL-PMs (15 mg/kg) and DMSL3-CCL-PMs (15 mg/kg) into healthy mice (n=3)

	Intravenous				Subcutaneous		
	DMSL1- CCL-PMs	DMSL2- CCL-PMs	DMSL3- CCL-PMs		DMSL1- CCL-PMs	DMSL2- CCL-PMs	DMSL3- CCL-PMs
$t_{1/2}$ (h)	21.5	16.3	17.8	$t_{1/2}$ (h)	18.3	17.9	15.2
AUC $_{\infty}$ (h* μ g/ mL)	5847	3637	2075	AUC $_{\infty}$ (h* μ g/ mL)	2139	978.8	548.5
% AUC $_{\infty}$ attained (vs. i.v. route)	100	100	100	% AUC $_{\infty}$ attained (vs. i.v. route)	37	27	26
CL (mL/kg/h)	3.42	4.12	7.23	CL/F (mL/kg/h)	9.35	15.3	27.4
V $_{ss}$ (mL/ kg)	106	97.5	185	V $_{ss}$ /F (mL/ kg)	248	396	601
MRT (h)	31.1	23.6	25.6	MRT (h)	26.5	25.8	22.0

$t_{1/2}$ = Half-life of elimination; AUC $_{\infty}$ = Area under the plasma concentration-time curve; CL = Clearance; V $_{ss}$ = Steady-state volume of distribution; MRT = Mean residence time; CL/F = Systemic clearance relative to bioavailability; V $_{ss}$ /F = Steady-state volume of distribution relative to bioavailability

Of note, in the present study the AUC of total drug levels obtained after s.c. administration divided by that attained after i.v. administration (the latter being by definition 100%) is denoted as the systematic availability (%) of DMSLx-CCL-PMs. Similar to other drug-containing CCL-PMs [36, 37], DMSLx-CCL-PMs demonstrated prolonged circulation properties after i.v. administration, as evidenced by a significantly extended half-life of elimination ($t_{1/2} = 18.5 \pm 2.7$ h) compared to DMS administered as free drug ($t_{1/2} = 3-4$ h) [38]. Following s.c. administration, in case of all three types of DMSLx-CCL-PMs, plasma levels of total DMS gradually increased and peaked at 24.5 ± 2.2 h after injection (Figure 4). Thereafter, the disappearance of total DMS from the blood followed the same pattern as that attained after i.v. injection (Figure 4). Moreover, plasma levels of total DMS were detectable for at least 4 days and possessed comparable elimination half-lives (Table 1) after s.c. and i.v. administration. These results demonstrate that, once entering the bloodstream, s.c. administered DMSLx-CCL-PMs behave as if they were i.v. administered. Since the release of DMS from CCL-PMs is intrinsically slow under physiological conditions (Figure 3), premature release of DMS before entering the bloodstream (e.g. at the s.c. injection site) is not expected to be extensive. Thus the plasma concentration versus time curve of total DMS is mostly to be attributed to DMS stably entrapped within the CCL-PMs. This is also evidenced by the prolonged circulation properties of the s.c. administered DMSLx-CCL-PMs ($t_{1/2} = 17.1 \pm 1.7$ h) and their similar plasma disappearance patterns observed after T_{max} in comparison

with the i.v. injected counterparts.

The s.c. absorption of DMSLx-CCL-PMs was rather rapid, given the fact that total DMS was already detected in blood at the first sampling time point (0.5-1 h) post s.c. administration (Figure 4). Remarkably, a s.c. injection of DMSLx-CCL-PMs resulted in high systemic availability of 26-37% (Table 1), indicating that s.c. absorption is substantial.

Importantly, for all three s.c. administered PMs types, the plasma concentration versus time curves of total DMS exhibited the same pattern and peaked at the same time ($T_{\max} = 24.5 \pm 2.2$ h) (Figure 4). These results suggest that the s.c. absorption of these nanomedicines is not dictated by the type of linker used. Indeed, the interstitial diffusion, lymphatic absorption and blood elimination of nanocarriers are primarily dependent on their particle size and surface characteristics [25, 26]. Thus the similar PK results obtained are likely the consequence of the comparable small size and neutral zeta potential of the three PMs types evaluated.

As can be concluded from Table 1, the different drug release kinetics had only a minor impact on the PK profiles of s.c. administered DMSLx-CCL-PMs. Presumably, because of the intrinsically slow and controlled drug release properties, the absorption and blood elimination of the intact DMSLx-CCL-PMs were already completed before considerable DMS release took place. Obviously, release of DMS is aimed to occur at pathological target sites but certainly not wanted in the lymphatics and the systemic circulation. In line with our objective, while minimally affecting the PK profiles, the use of various linkers potentially allows for highly different drug release profiles only when these s.c. administered nanomedicines have accumulated at the target sites and, as a consequence, potentially different efficacy outcomes might be obtained. This important aspect regarding the ability to tailor efficacy outcomes utilising our tuneable linker technology will be addressed in future studies.

3.3. Paclitaxel-containing core-cross-linked polymeric micelles

We additionally entrapped the well-known drug paclitaxel (PTX) in the CCL-PMs to address the potential of the latter for targeted delivery of a large variety of agents. Similar to DMS, the release and therapeutic effect of PTX are not aimed to occur in the general circulation. Hence we covalently attached PTX to the core of CCL-PMs through a sulfide ester bond, i.e. the least hydrolytically sensitive ester linkage in this study, yielding PTX-containing CCL-PMs (PTXL1-CCL-PMs) with slow drug release kinetics. To obtain PTXL1-CCL-PMs, first, PTX was esterified at its C-2' hydroxyl group with L1 to obtain PTX derivative PTXL1 (Figure 2). The

synthesised PTXL1 was purified by column chromatography and obtained as a white solid with high purity (> 95%). The identity of the compound was confirmed by ^1H NMR and LC-MS (**Figure 5**). PTXL1 was covalently attached to CCL-PMs upon polymerisation of the methacrylate moieties in PTXL1 as well as in the polymer lactate side chains to obtain PTXL1-CCL-PMs as an opalescent dispersion.

Figure 5. Characterisation of PTXL1. (A) ^1H NMR spectrum and (B) LC-MS result.

Similar to DMSL1-CCL-PMs, the obtained PTXL1-CCL-PMs had an almost neutral zeta potential and a hydrodynamic size of about 70 nm with relatively low PDI (< 0.2). However, in the present study the entrapment of PTXL1 within the CCL-PMs was not optimised, resulting in moderate drug entrapment efficiency (i.e. 45%). Since in the same CCL-PMs system high %EE above 75% can be attained with a similar taxane drug docetaxel [37], the entrapment of PTXL1 within the CCL-PMs is expected to be substantially more efficient upon formulation optimisation. Compared to DMSL1-CCL-PMs ($\sim 3\%$ release in 3 days), drug release from PTXL1-CCL-PMs was significantly faster ($\sim 15\%$ release in 3 days) (**Figure 6**). Considering that in both cases the release of native drug from the CCL-PMs is driven by chemical hydrolysis of the same sulfide ester bond, the different drug release rates are attributed to the divergent chemical structures of these compounds. PTX renders a stronger electron withdrawing effect on the sulfide ester bond than does DMS, leading to more rapid hydrolysis of the ester linkage and thereby faster drug release from the CCL-PMs. In a recent study, the release kinetics of another taxane docetaxel covalently attached to the CCL-PMs via the same ester linkage was evaluated under the same condition (pH 7.4, 37°C) (data not shown). Compared to docetaxel ($\sim 5\%$ release in 3 days), the release kinetics of PTX from the CCL-PMs was also significantly faster. The dissimilar release kinetics of these taxane drugs can be explained by the stronger electron withdrawing ability of the latter provided by the acetate ester at the C-10 position.

Figure 6. Representative *in vitro* PTX release curve in case of PTXL1-CCL-PMs incubated in phosphate buffer (100 mM, pH 7.4, supplemented with 15 mM NaCl) containing 1% (v/v) polysorbate 80 at 37°C

3.4. High systemic availability of PTXL1-CCL-PMs

Similarly, PTXL1-CCL-PMs were injected into healthy mice via the s.c. and i.v. route of administration. The plasma levels of total (entrapped plus released) PTX were monitored (**Figure 7**) and the relevant PK parameters were calculated (**Table 2**). After s.c. injection of PTXL1-CCL-PMs, total PTX was already detectable in plasma at the first sampling time point (0.5-1 h), which continued to increase till reaching the maximum concentration at 20.4 h after administration (Figure 7), corroborating the gradual s.c. absorption of PTXL1-CC-PMs from the s.c. injection site. As also seen with DMSLx-CCL-PMs, after T_{max} , the disappearance of total PTX from the blood followed the pattern as if administered i.v. (Figure 7). In fact, at 48 h post administration and beyond, the plasma disappearance curve of s.c. administered PTXL1-CCL-PMs was parallel to and almost overlapped with that attained via the i.v. route. These results indicate that at 48 h post administration, s.c. absorption of PTXL1-CCL-PMs was close to complete. Remarkably, high systemic availability of 42% was obtained following a s.c. injection of PTXL1-CCL-PMs.

Moreover, local irritation was not observed in the animals receiving a s.c. injection of PTXL1-CCL-PMs. The good tolerability profile indicates that PTX was stably entrapped at the s.c. site of injection, which in turn enables the utilisation of s.c. administered CCL-PMs for anticancer therapies. This again points to the importance of controllable (anticancer) drug release kinetics, which can be achieved using the tuneable CCL-PMs system described in the present study.

Figure 7. Plasma levels of total (entrapped plus released) PTX after a single s.c. or i.v. injection of PTXL1-CCL-PMs into healthy mice. Data are expressed as the mean $\pm$ SEM ($n=3$).

Table 2. Pharmacokinetic parameters related to total (entrapped plus released) PTX in plasma following a single i.v. or s.c. injection of PTXL1-CCL-PMs (25 mg/kg) in mice (n=3)

Intravenous		Subcutaneous	
$t_{1/2}$ (h)	16.7	$t_{1/2}$ (h)	14.3
AUC $_{\infty}$ (h* μ g/ mL)	11996	AUC $_{\infty}$ (h* μ g/ mL)	4980
% AUC $_{\infty}$ attained (vs. i.v. route)	100	% AUC $_{\infty}$ attained (vs. i.v. route)	42
CL (mL/kg/h)	2.08	CL/F (mL/kg/h)	5.02
V $_{ss}$ (mL/ kg)	50.5	V $_{ss}$ /F (mL/ kg)	103
MRT (h)	24.2	MRT (h)	20.6

$t_{1/2}$ = Half-life of elimination; AUC $_{\infty}$ = Area under the plasma concentration-time curve; CL = Clearance; V $_{ss}$ = Steady-state volume of distribution; MRT = Mean residence time; CL/F = Systemic clearance relative to bioavailability; V $_{ss}$ /F = Steady-state volume of distribution relative to bioavailability

In rodents, the anatomical site of s.c. injection plays a pivotal role in the s.c. absorption of e.g. biological drugs [39] and nanomedicines [26]. Previously, Oussoren *et al.* found that < 60% injected dose (ID) of liposomes remained at the site of injection after s.c. administration to the foot (dorsal side or footpad) of rats and the corresponding systemic availability was 30-40%. In contrast, when s.c. administered at the flank of the animals, ca. 95% ID of liposomes were retained at the s.c. injection site while the systemic availability of the liposomes was merely 3% [40]. A similarly low systemic availability of 3% was also reported by Gautier *et al.* after s.c. administration of growth hormone releasing factor-containing nanoparticles to the same anatomical sites [41]. Remarkably, in the present study we demonstrate that even when administered at the flank of the animals, systemic availability of up to 42% was achieved following a single s.c. dose of drug-containing CCL-PMs, which accordingly can be considered high. Moreover, in this study, peak levels representing 28.6% ID of PTX-containing CCL-PMs were found in the blood following s.c. administration. Such values can be considered very high, as substantially lower levels of nanomedicines (e.g. peak levels as low as 0.2% ID) were reported to enter the circulation after s.c. administration of other nanoparticulate formulations such as oligonucleotide-containing lipid nanoparticles [42] and etoposide-loaded tripalmitin nanoparticles into mice [43]. Our promising results strongly point to the opportunity of utilising the s.c. route for systemic delivery of CCL-PMs to treat various diseases.

To further increase the systemic availability of CCL-PMs, alternative anatomical sites of s.c. injection should be considered. Moreover, since the physical, chemical and physiological attributes of s.c. tissues can strongly affect the s.c. absorption outcome [44], additional measures such as massage of the s.c. injection site may be applied

to stimulate the lymphatic absorption of interstitial fluid and thereby enhance the systemic availability of s.c. administered nanomedicines [45].

4. Conclusions

In this study, we covalently attached DMS and PTX to the core of CCL-PMs via varying ester linkages and evaluated their PK profiles in healthy mice after a single s.c. and i.v. dose. Remarkably, the CCL-PMs had up to 42% systemic availability after s.c. injection into the flank of mice. Moreover, the s.c. absorption of DMS-containing CCL-PMs is not strongly affected by their intrinsically different drug release properties provided by the use of different linkers. In conclusion, as an alternative to the i.v. route, s.c. administration of CCL-PMs is a realistic option to attain high systemic availability and, from a practical point of view, should be potentially considered for self-administration which is more convenient and reduces healthcare costs.

Acknowledgements

This work was supported by Cristal Therapeutics (Maastricht, the Netherlands).

Abbreviations

ACN	Acetonitrile
AUC	Area under the plasma concentration-time curve
CCL-PMs	Core-cross-linked polymeric micelles
CL	Clearance
CL/F	Systemic clearance relative to bioavailability
CMT	Critical micelle temperature
DCM	Dichloromethane
DIC	<i>N,N'</i> -Diisopropylcarbodiimide
DLS	Dynamic light scattering
DMAP	4-Dimethylaminopyridine
DMS	Dexamethasone
DMSLx-CCL-PMs	Dexamethasone-containing core-cross-linked polymeric micelles
EE	Entrapment efficiency
EPR	Enhanced permeability and retention
ESI-MS	Electrospray ionisation mass spectrometry
GPC	Gel permeation chromatography
HPMAmLac ₁	<i>N</i> -(2-hydroxypropyl) methacrylamide monolactate
HPMAmLac ₂	<i>N</i> -(2-hydroxypropyl) methacrylamide dilactate
HOAt	1-Hydroxy-7-azabenzotriazole
ID	Injected dose
i.v.	Intravenous
KPS	Potassium persulfate
L1	2-(2-(Methacryloyloxy)ethylthio)acetic acid
L2	2-(2-(methacryloyloxy)ethylsulfinyl)acetic acid
L3	2-(2-(Methacryloyloxy)ethylsulfonyl)acetic acid
LC-MS	Liquid chromatography-mass spectrometry
M _n	Number average molecular weight
MRT	Mean residence time
M _w	Weight average molecular weight
Na ₂ SO ₄	Sodium sulfate
NMR	Nuclear magnetic resonance
PDI	Polydispersity index
PK	Pharmacokinetics
PMs	Polymeric micelles
PTX	Paclitaxel
PTXL1-CCL-PMs	Paclitaxel-containing core-cross-linked polymeric micelles
s.c.	Subcutaneous
t _½	Half-life of elimination
TEMED	<i>N,N,N',N'</i> -Tetramethylethylenediamine
TFA	Trifluoroacetic acid
UPLC	Ultra-performance liquid chromatography
UV/Vis	Ultraviolet/visible
V _{ss}	Steady-state volume of distribution
V _{ss} /F	Steady-state volume of distribution relative to bioavailability
Z _{ave}	Z-average hydrodynamic diameter

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Chapter 6

Summary and perspectives

1. Summary

Polymeric micelles are nanosized assemblies of amphiphilic block copolymers that are characterised by a core-shell structure. The hydrophobic core of polymeric micelles can accommodate (hydrophobic) drugs, leading to enhanced drug 'solubility' and stability. The hydrophilic shell and the small hydrodynamic sizes (generally < 100 nm) allow polymeric micelles to have prolonged circulation kinetics *in vivo* and thereby great potential for passive targeting via the enhanced permeability and retention (EPR) effect [1, 2]. The application of polymeric micelles for drug delivery purposes was first pioneered by Bader *et al.* in 1984 [3], and since then polymeric micelles of varying compositions have been developed [4-6]. In particular, extensive research has been conducted to reinforce the *in vivo* structural stability, drug retention and targeting ability of polymeric micelles for broader biomedical applications [7-9]. Already, a variety of nanomedicines based on polymeric micelles have entered clinical trials [10]. In this thesis, we describe the expansion of a polymeric micellar system based on thermosensitive and biodegradable methoxy poly(ethylene glycol)-*b*-poly[N-(2-hydroxypropyl)methacrylamide-lactate] (mPEG-*b*-pHPMAmLac_n) block copolymers for broad pharmaceutical applications.

Chapter 1 provides a general introduction to nanomedicines, with a particular focus on polymeric micelles composed of thermosensitive mPEG-*b*-pHPMAmLac_n block copolymers. Particularly, the necessity to stabilise the micellar structure by means of core-cross-linking and the need for covalent drug attachment are described. The aim of the thesis and the research topics are outlined at the end.

As a biocompatible polymer, pHPMAm has been widely investigated as a building block for polymeric micelles [11]. In this thesis, mPEG-*b*-pHPMAmLac_n, a block copolymer containing a hydrophilic mPEG ($M_n=5000$) block and a thermosensitive pHPMAmLac_n ($n=1$ or 2) block has been utilised to form polymeric micelles. This block copolymer is characterised by a so-called critical micelle temperature (CMT), below which the thermosensitive pHPMAmLac_n block and thus the block copolymer including the PEG segment as such is water-soluble. At temperatures above the CMT, the thermosensitive block becomes insoluble and the amphiphilic block copolymer self-assembles into polymeric micelles with a core-shell structure. Importantly, *in vivo*, the integrity of the micellar structure and the stable entrapment of payloads in polymeric micelles should be ensured prior to reaching the target sites. To achieve the former, the block copolymers can be cross-linked in the micellar core, yielding core-cross-linked polymeric micelles (CCL-PMs) [9, 12]. To stably entrap drugs in the micelles during systemic circulation, drug molecules can be covalently attached to the micellar core [13].

Previously, small molecule drugs covalently entrapped in CCL-PMs have demonstrated improved therapeutic efficacy in animal models [11, 14, 15]. The promising results of these proof-of-concept studies corroborate the use of this nanomedicine platform for the treatment of solid tumours and inflammatory diseases. To expand this nanomedicine platform for even broader applications, a higher tuneability of the pharmaceutical properties of the micellar system is essential. In **Chapter 2**, the modulation of CCL-PMs regarding particle size, drug release kinetics and carrier degradation characteristics is described. The particle size of (drug-entrapped) CCL-PMs was tailored by varying the molecular weight of the constituting block copolymer, which was easily achieved by altering the feed ratio between monomer and initiator. As a result, a series of (drug-entrapped) CCL-PMs with varying hydrodynamic sizes between 30 and 100 nm were attained. To tailor the drug release kinetics, the model drug docetaxel (DTX) was first derivatised with a linker containing a thioether group, which was then covalently attached to the core of the CCL-PMs through a hydrolysable ester linkage. By using linkers that contain thioether groups of different oxidation degrees and thereby generating ester linkages of divergent hydrolytic sensitivity, the release kinetics of DTX from the CCL-PMs could be significantly varied *in vitro*. Furthermore, the degradation kinetics of CCL-PMs was tuned through the modulation of cross-linking. Previously, methacrylate was used as the crosslink which was attached to the block copolymer via an ester bond. In an aqueous medium, the CCL-PMs can only fully disintegrate after all the crosslinks are cleaved as a result of hydrolysis. Accordingly, under physiological conditions the degradation of the CCL-PMs is anticipated to be relatively slow. To attain CCL-PMs with potentially faster degradation kinetics, different types and densities of crosslinks were applied. *In vitro*, the use of a linker (i.e. L2) as the crosslink as well as a lower crosslink density yielded CCL-PMs with significantly faster degradation kinetics. Taken together, the results presented in Chapter 2 demonstrate the high tuneability of this nanomedicine platform based on CCL-PMs.

Polymeric micelles have been recognised as a favourable drug carrier for tumour targeting [16]. To date, various micelle-entrapped cytotoxic drugs have been developed for systemic administration, such as paclitaxel [17], cisplatin [18] and camptothecin [19]. Next to oncology, in an earlier study, Crielaard *et al.* reported the remarkable therapeutic efficacy of dexamethasone (DMS) covalently entrapped in CCL-PMs in rat models of rheumatoid arthritis [14]. To demonstrate that this CCL-PMs system also holds significant potential for the treatment of solid tumours, in **Chapter 3**, we covalently attached an anticancer drug DTX to the core of CCL-PMs via a thioether ester linkage, yielding DTX-entrapped CCL-PMs (DTX-CCL-PMs). The covalent conjugation allowed for sustained release of DTX under physiological conditions *in vitro*. *In vivo*, DTX-CCL-PMs demonstrated superior therapeutic efficacy in mice

bearing MDA-MB-231 tumour xenografts as compared to the marketed formulation of DTX (Taxotere®). Strikingly, even a single intravenous (i.v.) injection of DTX-CCL-PMs enabled complete regression of both small (150 mm³) and established (550 mm³) tumours, leading to 100% survival of the animals. These remarkable antitumour effects of DTX-CCL-PMs are attributed to their enhanced tumour accumulation and anti-stromal activity. Besides excellent therapeutic efficacy, DTX-CCL-PMs also exhibited superior tolerability in healthy rats as compared to Taxotere®. These preclinical data strongly support clinical translation of this nanomedicinal product for the treatment of solid tumours.

Admittedly, over the last two decades, polymeric micelles have been intensively employed for the delivery and targeting of small molecule drugs [10]. So far, the utilisation of the CCL-PMs platform significantly improved the therapeutic index of anticancer drug doxorubicin [11], dexamethasone [14, 15] and DTX (**Chapter 3**) in various animal models. Besides small molecule drugs, peptides of various sizes and derivation have also been increasingly used as therapeutic drugs in many disease areas [20]. However, the use of peptides as drugs is often challenged by their poor pharmacokinetics due to rapid renal clearance and enzymatic degradation [21]. To overcome these obstacles, a variety of drug carriers have been employed for the delivery of therapeutic peptides. Among all nanocarriers, micelles have not been recognised as a suitable candidate for this purpose due to the charge and hydrophilic nature of many peptide drugs [22]. To investigate the feasibility of peptide delivery using this nanomedicine platform, in **Chapter 4**, a model therapeutic peptide leuprolide was covalently conjugated to the core of CCL-PMs via a hydrolysable thioether ester linkage. *In vitro*, leuprolide was released from the CCL-PMs in a sustained manner. *In vivo*, the pharmacokinetic profile of the leuprolide nanomedicine was evaluated in healthy rats following a single i.v. injection. Compared to the soluble peptide, leuprolide entrapped in CCL-PMs showed a prolonged circulatory half-life (14.4 h) in healthy rats and the released leuprolide was detected in blood for at least 72 hours. Importantly, the released peptide remained biologically active as demonstrated by an initial increase and then decrease in plasma testosterone levels. This study shows that covalent attachment of peptides to CCL-PMs via hydrolytically sensitive (ester) bonds is a promising approach for achieving sustained systemic levels of peptides after i.v. administration. To date, only a handful of studies have demonstrated the entrapment of therapeutic peptides in polymeric micelles for drug delivery purposes. However, in all these cases the peptide-containing nanomedicines have been administered following non-i.v. (e.g. intraocular [23]) routes. To the best of our knowledge, this is the first study describing the feasibility of i.v. delivery of peptides covalently entrapped in nanoparticles.

Although i.v. delivery is the most common route of administration for nanomedicines, subcutaneous (s.c.) administration may serve as a good alternative for patients as it offers the possibility of self-administration and thereby may reduce healthcare costs. In **Chapter 5**, the feasibility of s.c. administration of CCL-PMs-based nanomedicines is investigated. Intuitively, once entering the bloodstream, the s.c. administered nanomedicines should have the same biological fate as those administered i.v.. Therefore, the pharmacokinetic profile and in particular the systemic availability of the nanomedicines are evaluated after i.v. and s.c. administration into healthy rats. To understand the effect of drug release kinetics on the pharmacokinetic profile of the nanomedicines, the small molecular weight drug DMS was derivatised with three thioether-containing linkers, respectively, which allowed DMS to be covalently attached to the core of CCL-PMs through different thioether ester linkages. The use of different linkers resulted in divergent release kinetics of DMS *in vitro*, but did not significantly alter the pharmacokinetic profile of these nanomedicines after s.c. administration. Remarkably, a single s.c. dose of the DMS-containing CCL-PMs resulted in high systemic availability of these nanomedicines, as evidenced by an AUC between 26-37% relative to the AUC obtained after i.v. injection. Next to DMS, the drug paclitaxel (PTX) was also covalently attached to the CCL-PMs via a sulfide ester bond. Similarly, high systemic availability of PTX-containing CCL-PMs was achieved after s.c. administration into healthy mice (42%, AUC relative to that obtained after i.v. administration). These results demonstrate that s.c. administration is a promising route to attain high systemic availability of CCL-PMs, enabling a potentially more patient-friendly and cost-effective treatment approach than the i.v. route.

Finally, this **Chapter 6** summarises the results described in this thesis and provides perspectives on the use of this nanomedicine platform based on CCL-PMs.

2. Perspectives

In recent years, CCL-PMs based on mPEG-*b*-pHPMAmLac_n block copolymers have been employed as drug carriers to promote the targeting of small molecular weight drugs to tumour or inflammatory tissues in animal models following i.v. administration. Preclinical studies of nanomedicines based on CCL-PMs have demonstrated promising results, which in turn advocate a) the development of a nanomedicinal product based on CCL-PMs towards clinical use and b) the expansion of this nanomedicine platform for broad therapeutic applications. These two aspects are addressed in this thesis:

- Product development

A nanomedicinal product containing chemotherapeutic DTX was developed. The ease of manufacturing and the excellent preclinical data of this nanomedicinal product strongly support its clinical translation.

- Platform expansion

- The pharmaceutical properties of (drug-entrapped) CCL-PMs with respect to particle size, drug release kinetics and carrier degradation characteristics were independently tailored.
- Besides small molecular drugs, the applicability of the CCL-PMs platform for therapeutic peptides was established in an animal model following i.v. administration.
- Besides the most commonly employed i.v. alternative, the s.c. route of administration has proven to be an attractive option for systemic delivery of nanomedicines based on CCL-PMs.

To best utilise and further expand this versatile CCL-PMs platform, perspectives are provided with respect to the following aspects.

2.1. Pharmaceutical development

The *in vivo* disposition of nanomedicines is strongly influenced by their pharmaceutical properties, in particular, particle size [24, 25]. In Chapter 3, the potent anticancer agent DTX was covalently entrapped in CCL-PMs. This anticancer nanomedicine had a hydrodynamic size of 66 nm and showed remarkable antitumour efficacy in tumour xenografts. Recent studies on anticancer nanomedicines demonstrate that a hydrodynamic size below 50 nm is optimal for tumour penetration [26, 27]. It is therefore tempting to speculate that the antitumour efficacy of this DTX nanomedicine may be enhanced by further downsizing. In **Chapter 2**, we successfully varied the hydrodynamic size of DTX-entrapped CCL-PMs between 30 and 100 nm. Next, the feasibility of further downsizing CCL-PMs, e.g. in the range of 15-30 nm, should be investigated.

Covalent drug attachment to nanocarriers via hydrolysable linkages represents an attractive approach to modulate the drug release kinetics. As described in Chapter 2, by employing linkers that contain thioethers of different oxidation degrees (i.e. L1, L2 and L3), the native drug can be released at divergent rates *in vitro*. Further, the latter can be fine-tuned by conjugating multiple linkers to multiple reactive moieties

of the drug molecule, as demonstrated by the drug derivative DTX(L2)₂ in Chapter 2. Thus it may be hypothesised that the release kinetics of a given drug from the CCL-PMs can be fully tailor-made through the utilisation of a combination of linkers. This hypothesis should be examined using the same model drug DTX or other drug candidates.

Similarly, by using L2 as a crosslinker, the degradation rate of the CCL-PMs was successfully modulated *in vitro*, with a degradation time of ca. one month under physiological conditions (pH 7.4, 37 °C) (Chapter 2). To enable even more rapid degradation of the CCL-PMs, polymerisable linkers containing a more hydrolytically sensitive (ester) bond, e.g. L3, can be used as the crosslinker. Importantly, by derivatising the drug and block copolymer with proper linkers, the release of drug and degradation of the CCL-PMs can occur at similar rates. Thus upon complete drug release the drug carrier is also degraded and eliminated from the body, precluding potential toxicities induced by long-term particle accumulation, if any.

In Chapter 2, three pharmaceutical parameters of (drug-entrapped) CCL-PMs, namely particle size, drug release kinetics and carrier degradation characteristics, were individually tailored. Next, the combination of these properties into one CCL-PMs system should be realised, which, if successful, will facilitate the generation of a library of nanomedicines for broad applications.

2.2. Scale-up manufacturing

As the field of drug delivery advances, nanomedicines of diverse structural and production complexity have been developed over the past decade. For non-clinical studies, nanomedicine production can be performed at a laboratory scale. However, to attain a nanomedicinal product for patients, the feasibility of GMP-production should be taken into account. Previously, Hrkach *et al.* reported the clinical-scale manufacturing of prostate-specific membrane antigen (PSMA)-targeted poly(lactico-glycolic acid) (PLGA) nanoparticles containing DTX, also known as BIND-014 [28]. Prior to purification and ligand conjugation, the DTX-containing nanoparticles were first manufactured via a complicated nanoemulsion process, including steps of solution preparation, high-energy emulsification and particle quench. In comparison, DTX-entrapped CCL-PMs can be prepared in a more straightforward process, which only consists of solution preparation and a single-step production. Importantly, during the manufacturing of DTX-entrapped CCL-PMs, only a small fraction (10% v) of organic solvent is introduced, which is subsequently removed through the use of Tangential flow filtration (TFF). Another advantage of CCL-PMs is the easy sterilisation process for their pharmaceutical production. Since polymeric micelles are free from micro-sized particles, they can be sterilised by filtration through filters

with e.g. 0.22 μm pore size [29]. Together, these easy steps corroborate the simple production scale-up of nanomedicines based on CCL-PMs. To ensure a long shelf-life as well as easy transportation and storage, freeze-dried nanomedicinal products based on CCL-PMs should be developed and their corresponding stability should be assessed.

2.3. Pharmacokinetics and biodistribution profile

The primary objective of using polymeric micelles as drug carriers is to improve drug disposition in the body directed towards a better therapeutic outcome. Due to the small hydrodynamic size and stealth properties, polymeric micelles can have a long residence time in the systemic circulation after i.v. administration. The prolonged circulation kinetics in turn allows the delivery and targeting of the entrapped payloads to tumour and inflammatory tissues owing to the EPR effect. These advantageous features of polymeric micelles have attracted considerable academic and commercial attention. Likewise, PEGylated liposomes also have a prolonged circulation profile following systemic administration. However, in case of tumour targeting, PEG-modified liposomes do not penetrate deeply into the tumour tissue likely due to their relatively large size (≥ 100 nm). In contrast, polymeric micelles are reported to have higher tumour penetration abilities, which in turn may translate into superior antitumour responses [6]. In this thesis, it has been well established that covalent entrapment of small molecular weight drug DTX in CCL-PMs based on mPEG-*b*-PHPMAmLac_n block copolymer (66 nm) significantly improves the antitumour efficacy of the chemotherapeutic agent (Chapter 3). It is considered that with an even smaller size, the tumour extravasation, penetration and thereby antitumour response of this and other anticancer nanomedicines can be further elevated. To validate this hypothesis, DTX nanomedicines based on CCL-PMs of varying sizes between 15 and 65 nm should be evaluated *in vivo* with respect to pharmacokinetics, tumour localisation, antitumour efficacy and safety profile. Next to particle size, the influence of drug release kinetics and carrier degradation characteristics on the circulation, biodistribution profile and tolerability of this anticancer nanomedicine can also be examined in animal models.

2.4. Subcutaneous administration

S.c. injection is widely employed as a delivery route for compounds with low oral bioavailability or as a means to prolong their systemic exposure. Compared to the most commonly used i.v. route, s.c. treatment represents a more patient-friendly and less costly option. Owing to the lymphatic pathway, nanoparticles administered s.c. may also have access to the systemic circulation [30]. To

investigate the systemic availability of s.c. administered nanomedicines based on CCL-PMs, blood levels of total (entrapped plus released) drug were monitored following s.c. and i.v. administration to the animals (Chapter 5). Remarkably, high systemic availability (26-42%) of nanomedicines based on CCL-PMs was attained following s.c. administration. Nevertheless, it has to be remarked that since the main aim of the study was to determine the systemic availability of s.c. administered nanomedicines, only total drug levels in blood was monitored. To better understand the pharmacokinetics and bioavailability of the native drug, blood levels of drug released from the s.c. administered nanomedicines should also be determined *in vivo*. Ideally, the polymer and the drug should be radiolabeled with different isotopes to determine their localisation in not only blood but also other tissues such as the lymph nodes. This is because the metastasis of most tumours initially spreads through the surrounding lymphatic tissue and ultimately forms tumours therein. To provide an effective anticancer chemotherapy for the treatment of lymphatic metastatic tumours, lymphatic targeted nanomedicines have been explored in the past decades to target anticancer drugs to regional lymph nodes via s.c. administration [31]. The small size of CCL-PMs presented in the thesis renders it a perfect nanocarrier candidate to enter the lymphatic vessels [32] and to penetrate the microvasculature of lymphatic metastatic tumours. Therefore, the localisation of s.c. administered CCL-PMs in lymph nodes should be investigated, which, if efficient, will support the utilisation of this nanomedicine platform for lymphatic targeted therapy following s.c. administration.

2.5. Active targeting

EPR-mediated drug targeting is considered as a passive approach. Besides the utilisation of the EPR effect, drug targeting can be further enhanced by means of active targeting. Active targeting is afforded by the use of targeting ligands on the surface of nanocarriers, including small molecules [33], peptides [34], antibodies [35], affibodies [36] and aptamers [37]. In earlier studies, an anti-epidermal growth factor receptor (anti-EGFR) nanobody EGa1 was conjugated to the surface of pyridylthio propionate (PDP)-modified CCL-PMs, which demonstrated greater binding and uptake by EGFR over-expressing cancer cells than the non-targeted counterpart [38]. Moreover, the attachment of the nanobody also enhanced the *in vivo* antitumour efficacy of doxorubicin-entrapped CCL-PMs in tumour xenografts [39]. These promising results corroborate the use of the CCL-PMs platform for active drug targeting. Besides the PDP moiety, the hydrophilic PEG block can also be modified with other functional groups, such as an azide group, making the coupling of a variety of targeting molecules derivatised with alkyne groups via 'click' chemistry possible [40-42].

It is noteworthy that the active targeting strategy is also faced with substantial challenges and the clinical benefit of the additional targeting ligand still has to be proven. Taking BIND014 (i.e. PSMA-targeted PLGA nanoparticles containing DTX) as an example, although in a preclinical study BIND014 outperformed the non-targeted nanomedicine in prostate cancer xenografts, the efficacy benefit may be deemed modest. In a review article, Goldberg *et al.* summarises the challenges for the clinical translation of actively targeted anticancer nanomedicines, such as identifying the validated receptors and leaping the biological hurdles [43]. After travelling from the systemic circulation to the tumour vasculature, anticancer nanomedicines must penetrate deeply into the tumour parenchyma and then diffuse into the hypoxic core. This process may be impeded by the heterogeneity of tumours and barriers such as heterogeneous circulation and increased interstitial pressure within tumours [43]. In addition, the authors also pointed out the need to 1) control the physicochemical properties (e.g. particle size and surface characteristics) of actively targeted nanomedicines and 2) scale-up for clinical production with assured quality control. These criteria can be fulfilled using the CCL-PMs platform as presented in this thesis. Therefore, CCL-PMs with active targeting ability and a small particle size property (< 50 nm) should be manufactured and evaluated in tumour xenografts. In addition, the optimal number of targeting ligands per particle should also be assessed *in vitro* and *in vivo*.

2.6. Various active pharmaceutical ingredients

So far, a handful of active pharmaceutical ingredients (API) have been covalently entrapped in CCL-PMs based on mPEG-*b*-pHPMAmLac_n block copolymers and assessed for their pharmacokinetics and/or therapeutic performance in animal models (Table 1).

Based on the promising results presented in the thesis, we consider this nanomedicine platform based on CCL-PMs to be highly valuable for substantially improving the therapeutic performance of currently marketed drugs. In addition, drugs that previously failed in clinical trials may also be reconsidered by utilising CCL-PMs, as these drugs may not only benefit from enhanced efficacy but also from reduced side effects (as shown in this thesis).

Owing to the hydrophobicity of the micellar core, poor entrapment of hydrophilic payloads in polymeric micelles is generally assumed. However, covalent attachment of hydrophilic compounds such as leuprolide to the core of CCL-PMs resulted in moderate drug entrapment efficiency (35-40%) and loading content (3-4%) (Chapter 4). Accordingly, this finding advocates the use of this CCL-PMs system for covalent entrapment of APIs with diverse hydrophobicity. Besides small molecule drugs

(**Chapter 2, 3 and 5**) and peptides of small (**Chapter 4**) or large (data not shown) molecular weights, covalent entrapment of biologics of larger molecular size in the CCL-PMs should also be considered.

Table 1. Preclinical *in vivo* evaluation of drug-entrapped CCL-PMs composed of mPEG-*b*-pHPMAmLac_n block copolymers

API	API type	Linker	Route of administration	<i>In vivo</i> outcome	Ref
Doxorubicin	Small molecule	Hyd-MA (hydrazone)	i.v.	Improved antitumour efficacy	[11]
Dexamethasone		L3	i.v.	Improved efficacy in rheumatoid arthritis	[14, 44]
		L2	i.v.	Improved antitumour efficacy	[15]
		L1, L2, L3	s.c. and i.v.	High systemic availability of s.c. administered nanomedicines	Chap 5
Paclitaxel		L1	s.c. and i.v.	High systemic availability of the s.c. administered nanomedicine	Chap 5
Docetaxel		L3	i.v.	Improved pharmacokinetic profile, antitumour efficacy, tumour accumulation, anti-stromal activity and tolerability	Chap 3
Leuprolide	Peptide*	L1	i.v.	Improved pharmacokinetic profile; Release of bioactive peptide	Chap 4

*although not evaluated *in vivo*, therapeutic peptides of larger molecular sizes (26- and 39-mer peptides) have also been successfully entrapped in the CCL-PMs (data not shown).

In the future, CCL-PMs as dual carriers of different APIs may also be developed. Indeed, combined therapy with drugs of different therapeutic effects has shown promise for the treatment of various diseases, yet the control over the release behavior of each drug has been considered as a great challenge. In recent years, combination therapy platforms that allow for simultaneous incorporation and independent release of drugs have arisen [45, 46]. Besides these novel carriers, CCL-PMs may also serve as a good candidate for the co-delivery of drugs and dual modality therapy. As demonstrated in **Chapter 2**, the release kinetics of a drug from the CCL-PMs is dictated by its intrinsic physiochemical properties and the type and/or number of polymerisable linkers used. Thus it may be speculated that in case of dual entrapment of APIs, the release kinetics of each API may be controlled independently by attaching each to the core of CCL-PMs through a certain linker.

In conclusion, this thesis demonstrates the high versatility and broad applicability of the CCL-PMs platform composed of mPEG-*b*-pHPMAmLac_n block copolymers. As a pioneer, an anticancer nanomedicinal product based on this platform was developed, which demonstrated excellent therapeutic performance in animal models and great potential for clinical use.

Abbreviations

Anti-EGFR	Anti-epidermal growth factor receptor
API	Active pharmaceutical ingredients
CCL-PMs	Core-cross-linked polymeric micelles
CMT	Critical micelle temperature
DMS	Dexamethasone
DTX	Docetaxel
DTX-CCL-PMs	Docetaxel-entrapped core-cross-linked polymeric micelles
EPR	Enhanced permeability and retention
i.v.	Intravenous
mPEG- <i>b</i> -pHPMAmLac _n	Methoxy poly(ethylene glycol)- <i>b</i> -poly[N-(2-hydroxypropyl) methacrylamide-lactate]
PDP	Pyridyldithio propionate
PLGA	Poly(lactic-co-glycolic acid)
PSMA	Prostate-specific membrane antigen
s.c.	Subcutaneous
TFF	Tangential flow filtration

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Appendices

Nederlandse Samenvatting

Acknowledgments

Curriculum Vitae

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Nederlandse samenvatting

Polymeren micellen zijn deeltjes met nanometer afmetingen die opgebouwd zijn uit zogenaamde blok co-polymeren. Deze polymeren vormen in water een micelle structuur die gekarakteriseerd wordt door een kern en een mantel. De kern van een polymeren micelle is uitermate geschikt voor het insluiten van water-onoplosbare geneesmiddelen waardoor de uiteindelijke oplosbaarheid van deze geneesmiddelen vele malen beter wordt. De water-oplosbare mantel en de geringe grootte (meestal kleiner dan 100 nm) helpen de micellen langer in de bloedbaan te blijven waardoor het geneesmiddel langer zijn werk kan doen. Bij kanker therapie kunnen de nanodeeltjes zo in verhoogde mate tumorweefsel binnen dringen, en op langere termijn voor betere behandeling van de ziekte zorgen.

Sinds 1984 zijn diverse types polymeren micellen ontwikkeld als nano-medicijn voor biomedisch gebruik, waarvan er een aantal inmiddels getest zijn in de mens. Belangrijke aandachtspunten in de ontwikkeling van geneesmiddelbevattende polymeren micellen zijn de stabiliteit in het lichaam na toediening en de mate waarin ze de verdeling van het geneesmiddel over het lichaam veel gunstiger laten worden. In dit proefschrift is de brede toepasbaarheid van polymeren micellen om het therapeutisch profiel van diverse geneesmiddelen te verbeteren onderzocht en beschreven.

Hoofdstuk 1 bevat een algemene introductie over nano-medicijnen gebaseerd op polymeren micellen die gemaakt worden uit blok co-polymeren. Vooral de noodzaak om de micellen te stabiliseren door de polymeren aan elkaar te koppelen met behulp van zogenaamde cross-linkers en het verankeren van geneesmiddelen in de micellaire kern met specifieke linkers staat in dit hoofdstuk centraal. De gebruikte blok co-polymeren zijn temperatuurgevoelig en aldus vormen deze in water boven een bepaalde temperatuur spontaan polymeren micellen met de eerder beschreven kern-mantel structuur. Middels de cross-link stap worden zowel de polymeren als de geneesmiddel-linker combinatie stabiel aan elkaar gebonden in de kern van de micellen. De verhoogde therapeutische effectiviteit van deze gecrosslinkte geneesmiddelbevattende micellen is inmiddels aangetoond in tumordragende muizen en in diermodellen voor chronische ontstekingen. Door het verder optimaliseren van de farmaceutische samenstelling van de polymeren micellen kan naar verwachting het aantal medische toepassingen verder worden uitgebreid. In **Hoofdstuk 2** is beschreven hoe de deeltjesgrootte van de polymeren micellen, de manier en snelheid waarop zij ingesloten geneesmiddelen afgeven en de uiteindelijke afbraak kunnen worden geoptimaliseerd. De deeltjesgrootte kan eenvoudig worden gestuurd door

langere of kortere polymeren te gebruiken als bouwsteen waardoor de micellen zo klein als 30 of zo groot als 100 nm kunnen worden gemaakt. Om de controle over de afgifte van een geneesmiddel uit de nanodeeltjes te kunnen bestuderen is het anti-kanker geneesmiddel docetaxel als modelstof gebruikt. Door docetaxel met behulp van verschillende types linkers te binden in de kern, kan de afgiftesnelheid van het geneesmiddel heel precies worden gecontroleerd. De afbraak van de nanodeeltjes zelf kan worden gestuurd door gebruik te maken van verschillende soorten cross-linkers waarmee de polymeren met elkaar worden verbonden. De experimenten zoals uitgevoerd in Hoofdstuk 2 tonen aan dat de polymeren micellen kunnen worden aangepast al naar gelang het gewenste gedrag in het lichaam (rational design). Polymeren micellen worden vooral gezien als middel om anti-kanker geneesmiddelen een gunstigere verdeling over het lichaam te geven en effectiever in tumorweefsel te laten ophopen.

In **Hoofdstuk 3** staan de experimenten beschreven waarin de gestabiliseerde polymeren micellen met docetaxel getest zijn in diersmodellen. Deze polymeren micellen bleken, in vergelijking met docetaxel zelf, een superieure effectiviteit te hebben in een muizenmodel voor borstkanker. Zelfs een eenmalige injectie van de docetaxel bevattende polymeren micellen resulteerde in een volledig verdwijnen van de tumoren en 100 % overleving van de dieren. Bovendien bleek in gezonde ratten dat de docetaxel bevattende polymeren micellen minder bijwerkingen hadden in vergelijking met docetaxel zelf. Ook dierexperimenten met andere geneesmiddelen zoals doxorubicine en dexamethasone hebben inmiddels aangetoond dat een betere effectiviteit met minder bijwerkingen wordt gevonden, wanneer deze geneesmiddelen ingesloten worden in polymeren micellen.

Een nieuwere klasse van geneesmiddelen, zogenaamde peptides, worden vaak te snel in het lichaam afgebroken danwel afgevoerd. In **Hoofdstuk 4** is onderzocht of deze peptides ook ingesloten zouden kunnen worden in de polymeren micellen. Als modelsysteem is hiervoor het therapeutisch peptide leuprolide gebruikt. Na intraveneuze toediening blijken leuprolide bevattende polymeren micellen veel langer in de bloed circulatie van ratten aanwezig te zijn in vergelijking met leuprolide zelf. Bovendien blijkt het uit de polymeren micellen vrijkomende leuprolide nog steeds biologisch actief te zijn. Deze studies hebben voor het eerst laten zien dat de polymeren micellen ook grote potentie hebben voor de intraveneuze toediening van peptides. Naast intraveneuze toediening van nanomedicijnen kan ook onderhuidse toediening interessant zijn omdat deze toedieningsvorm ook door de patient zelf kan worden toegepast. In **Hoofdstuk 5** is daarom gekeken naar de mogelijkheid om polymeren micellen te maken die gebruikt kunnen worden voor onderhuidse toediening. Hiervoor werden polymeren micellen gemaakt die de geneesmiddelen

dexamethason of paclitaxel bevatten. Vervolgens werd de beschikbaarheid van deze geneesmiddelen in het bloed van ratten vergeleken na onderhuidse toediening of rechtstreekse injectie in de bloedbaan. Uit deze studies blijkt dat ook na onderhuidse toediening van polymeren micellen het geneesmiddel in hoge mate beschikbaar komt in het bloed. Deze studie toont dus aan dat ook onderhuidse toediening van polymeren micellen mogelijk is, oftewel een patiëntvriendelijkere en meer kostenbesparende manier van toediening behoort tot de mogelijkheden. Tenslotte worden in **Hoofdstuk 6** de in dit proefschrift beschreven resultaten samengevat en een toekomstperspectief geschetst voor het gebruik van op deze polymeren micellen gebaseerde nanomedicijnen.

Acknowledgements

In 2009, I came to the Netherlands to pursue my graduate study. And six good years later, my thesis is accomplished and my Ph.D. journal is coming to an end. Along the way, so many people have helped me, getting closer and closer to the finishing line. I could not have done it without their support.

My Ph.D. story would not have an opening chapter if it wasn't for my promoter Prof. G. Strom. Gert, thanks for creating this opportunity at Twente and all the critical suggestions given. As you said, the setup of my Ph.D. appointment is not the most common kind, however, with your effort I am about to become your very first Ph.D. graduating from the UT. Thanks for that!

My sincere gratitude is extended to my other promoter, Prof. W.E. Hennink. Wim, it was because of your involvement, this thesis can be put into place today. I respect your professionalism and attitude towards science and I thank you for always responding and commenting on my writing in no time. Your valuable insight makes the finalisation of my thesis much smoother than I could expect.

This thesis could not have been accomplished without the guidance of my co-promoter, Dr. J. Prakash. Jai, I feel lucky you stepped in as my co-supervisor. Because of your patience and generous guidance, I slowly picked up scientific writing and many other useful techniques. In fact, I finished my very first publication together with you, and I would never forget typing numbers into your "PK" laptop:) Your encouragement meant a great deal, thank you!

Cristianne, I hope this paragraph wouldn't turn out to be a novel as I have so many things to thank you for. I first met you in November 2009, when you "interviewed" me for a research internship focusing on "core-cross-linked polymeric micelles". During that nine-month internship, I learned a lot from you and was deeply motivated by your encouragement in pursuit of a Ph.D.. I am glad I contacted you at the end of my Minor research internship, which then led to the writing of a Master thesis under your supervision. Immediately after that, I got a job offer from you at Cristal Therapeutics and happily accepted it. As I mentioned to you, it was my very first job and I was (and still am) looking forward to working with you. Shortly after this, you helped me establish the connection with Gert. With your enthusiastic help, I was given a Ph.D. position at Twente, in collaboration with CT. As Gert said, it is a luxurious position, for I have had SO MUCH support from CT and the colleagues. And I thank you for consistently standing on my side and having my best interest at heart. You were the most closely involved in my Ph.D. project and have spent a great deal of time helping

me coming this far. I could not have done it without your support! Thanks for your dedication, trust and encouragement, which made moving to Maastricht a not so hard decision. It is amazing how you (and others) turned CT from a start-up into what it is today. I look forward to even greater achievements.

To my colleagues at **Cristal Therapeutics**, I enjoyed working with you in Utrecht and in Maastricht and I thank you all for your contributions.

Ethlinn, you simply “set the path straight” for me and a “thank you” is not enough. I wish you all the best in life and in learning mandarin:) Please let me know if you ever plan to visit China again.

Paul, your help is essential for my Ph.D. thesis and it was great having you sitting next to me for the entire years.

Anne, Martin, Gerard, you all helped and contributed a great deal, thank you!

Joost, thanks for granting me the opportunities.

Jan, you always have an answer/solution, it is a pleasure working with you.

Rob, thanks for the Dutch summary and many more.

Jimmy, many thanks for all the “small” favors.

Annelies, thanks to you I easily found an apartment in Maastricht; and it is great having you as my “neighbour” in the office.

Michiel, it is wonderful to have a humours analytic expert around.

Nancy, thanks for all the help and arrangements in everyday life.

Jeroen, Ilva, Inge and other colleagues, I thank you for your great support.

My sincere “thanks” also goes to the **Department of Pharmaceutics, Utrecht University**. During my stay at the Department, I experienced many happy moments and met great people there. Mies, you always came to the rescue. I really appreciate your generous help throughout the whole time, as well as your cheerful spirit and Friday afternoon singing. Joep, you are always there to lend a helping hand, thanks for all the cool TEM pictures, I wish you all the best! Barbara, thanks for arranging all the hospitality agreements and many more! Burcin, Isil, Dandan, Yinan, James, Jia, I enjoyed all our chats and I wish you all the best in everything you do. Amr, we are both appointed at UT yet spending most of the hours working in Utrecht. I wish you the best of luck in the big apple and I hope to see you soon! Marina (my daily supervisor of the Major research internship), Kristel, Mehrnoosh, Ebel, Georgi, Neda,

Negar, Anna and other colleagues...it was great having you around.

Of course I could never forget the friends from the lunch group, with whom I shared great moments and memories.

My dear paranymph Mereltje, I am glad you sitted in front of me for the entire two and a half years. With you being around I was never bored. Thanks for your visits to Maastricht and for showing that bread, pindakaas and cucumber do come together.

Weiluan (Louann), my other dear paranymph, you have always been so considerate. I enjoyed all our chats and I'm certain you will do great at work and in life!

Filis, I missed having lunch next to you. It was a great honor being your paranymph, and I wish you, Agon and your unbelievably adorable girls a great life in Denmark.

Sima, your accompany is wonderful. I like your artistic sides and attitude toward life! Please don't ever change.

Yang & Qingxue, it was a true pleasure knowing you two, I wish you all the best in Guangzhou and I look forward to having dim sum together.

Andhyk, thanks for always being there to help and for arranging the trip to Iceland, should do it again some time. Good luck with the last phase of your Ph.D.!

Luis, you convinced me that PT is forever invincible. You have always been patient and helpful (and sarcastic in a good way). I wish you and Marta all the best in Warsaw.

Edu, you have two of the most beautiful girls in the world! You and your family are terribly missed. I wish you all the best in Dublin.

Bo, Kohei, Orn (Good luck with the last last phase of Ph.D.), Oil and Anastasia, hope to see you again soon.

I also want to thank the **BST** group at the **University of Twente**. Although my work is not based in Enschede, every time I visited the group I was warmly received. Karin, you are always there to help and that's why I never hesitated to shoot you an email whenever I had administrative puzzles to figure out. Thanks for making the last phase of my Ph.D. a lot lot easier. Ruchi, thanks for your contribution into my thesis chapter. Karin P, Iris, Praneeth, Dwi, Guoying and other colleagues, thanks for your friendliness and I wish you success in your Ph.D. and future career.

I also would like to take this opportunity to thank the friends I met in the Netherlands, Ying, Lingjing, Jinbao, Yang, Yu Z, Yu C, Jia, Yunpeng.....I learned something from each one of you. Xiaomei, Za, Yu...thanks for your visits and accompany in the Netherlands. Mengling, your friendship is precious to me. I am happy even we are geographically apart we could still find a way to chat, just like the good old days.

亲爱的蜜蜂，感谢你无条件的支持与信任，使我顺利度过为博士论文奋战的岁月。你教会了我乐观，以及凡事都要勇于尝试。为此我感到无比幸运，且无所畏惧。

亲爱的豆爸，是你的激励使我决心迎接这份挑战,并最终完成了博士论文。感谢你对我的教导，信任与关爱，我愿为此成为更好的人。

Qizhi (Rachel)

2015 August, Maastricht

Curriculum Vitae

Qizhi Hu was born on 04 April 1987 in Beijing, China. In 2005, she graduated from the Affiliated High School of Peking University, China. In 2009, she obtained her Bachelor of Science degree at the School of Pharmaceutical Sciences, Peking University. In the same year, she participated in the Master program “Drug Innovation” at Utrecht University, with the Utrecht Excellence Scholarship. During this time, she performed scientific research at the Department of Pharmaceutics in Utrecht University and in University of Washington, USA. She obtained her Master’s degree in 2011. From October 2011 to June 2012, she worked at Cristal Therapeutics as a formulation scientist. Thereafter, she started her PhD project at the Department of Biomaterials Science and Technology, University of Twente, under the supervision of Prof. dr. G. Storm and Prof. dr. ir. W.E. Hennink. The results of this project are described in this thesis.

List of publications

1. **Q. Hu**, E.V.B. van Gaal, P. Brundel, H. Ippel, T. Hackeng, C.J.F. Rijcken, G. Storm, W.E. Hennink, J. Prakash, A novel approach for the intravenous delivery of leuprolide using core-cross-linked polymeric micelles, *Journal of Controlled Release*, 205 (2015) 98-108.
2. **Q. Hu**, C.J.F. Rijcken, R. Bansal, W.E. Hennink, G. Storm, J. Prakash, Complete regression of breast tumour with a single dose of docetaxel-entrapped core-cross-linked polymeric micelles, *Biomaterials*, 53 (2015) 370-378.

List of abstracts

1. **Q. Hu**, G. Storm, A. Vroon, E.J. van Hoogdalem, C.J.F. Rijcken, E.V.B. van Gaal. CriPec® Docetaxel: Nanomedicine with improved therapeutic index in oncology. 3rd Conference on Innovation in Drug Delivery – APGI, Sep 2013, Pisa, Italy.
2. **Q. Hu**, C.J.F. Rijcken, G. Storm, J. Prakash. Complete regression of breast tumour with a single dose of docetaxel-entrapped core-cross-linked polymeric micelles. FIGON Dutch Medicine Days, Oct 2014, Ede, The Netherlands.
3. **Q. Hu**, C.J.F. Rijcken. CriPec® as a versatile nanomedicinal platform with broad applicability for an enhanced therapeutic performance. FIGON Dutch Medicine Days, Oct 2015, Ede, The Netherlands.