Polyelectrolyte Multilayer-Stabilized Paclitaxel Nanocrystals

A thesis submitted to attain the degree of

DOCTOR OF SCIENCES of ETH ZÜRICH
(Dr. sc. ETH Zürich)
presented by

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2016
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ABSTRACT

Side effects related to exposure of healthy tissues to a cytotoxic drug represent one of the major limitations of conventional systemic chemotherapy. Another important obstacle emerges from the poor aqueous solubility of many potent chemotherapeutics, which often imposes the use of large doses of potentially harmful solubilizing excipients. Nanotechnology could help solve these problems, due to the possibility to formulate hydrophobic drugs in well tolerated nanocarriers, and because of the ability of a drug delivery nanosystem to accumulate locally in tumor tissue via the enhanced permeability and retention (EPR) effect.

Especially, drug nanocrystals (NCs), which are colloidal dispersions composed almost entirely of a hydrophobic drug with minimum content of non-drug excipients, can locally deliver high doses of a therapeutic. However, achieving tumor accumulation with intravenously injected NCs is challenging because of their uncontrolled dissolution characteristics or rapid uptake by the mononuclear phagocyte system (MPS). Chapter 1 shows how the limitations of the conventional chemotherapy can be overcome with nanomedicines and specifically, provides an insight into characteristics and challenges related to drug NCs.

In this work, paclitaxel (PTX) was used as a model hydrophobic drug to produce NCs of ~200 nm. To address the issues related to rapid dissolution and the MPS uptake of the NCs, a layer-by-layer (LbL) approach was adopted to coat PTX NCs with alternating layers of oppositely-charged polyelectrolytes, using a PEGylated copolymer as the top layer. LbL coating is a simple and versatile technique based on the sequential deposition of molecular species on planar surfaces or colloidal templates. Its relevance in drug delivery primarily emerges from its versatility to control the release rate of the cargo encapsulated within the colloidal core. Chapter 2 presents the insight into the LbL technique, specifically in relation to solid drug cores, including drug NCs.

Chapter 3 reports the fabrication and physicochemical characteristics of the LbL-coated PTX NCs. It was shown that the LbL coating composed of six layers of poly(L-arginine) and poly(styrene sulfonate) with poly(L-lysine)-graft-PEG as topmost layer successfully slowed down dissolution in comparison to the non-coated NCs and to Abraxane® (an approved PTX nanoformulation) and provided colloidal stability in physiologically-relevant media. In Chapter 4, the formulation’s performance in the biological environment (cells and tumor-bearing mice) was assessed. The presence of coating had no intrinsic effect on cell viability at the concentrations tested. Nevertheless, the pharmacokinetic and biodistribution profiles indicated that the NCs were rapidly cleared from the bloodstream due to their deposition in MPS organs (i.e. liver and spleen). This might be a consequence of the shedding of the top layer
(PEGylated polyelectrolyte) from the NCs’ surface. Overall, this work questions whether approaches that rely solely on electrostatic interactions for retaining coatings on the surfaces of NCs are appropriate for *in vivo* use. **Chapter 5** provides a discussion on the major achievements of this thesis and an outlook, including the potential solutions for increasing the retention of the PEGylated polyelectrolytes on the NCs’ surface.
ZUSAMMENFASSUNG


Kapitel 3 beschreibt die Herstellung und physikochemischen Eigenschaften der LbL-beschichteten PTX-NK. Es zeigte sich, dass die Umhüllung von PTX-NK mit sechs Schichten von Poly(l-arginin) und Poly(styrolsulfonat) sowie von Poly(l-lysin)-graft-PEG als äusserste Schicht die Auflösung von PTX-NK im Vergleich sowohl zu nicht beschichteten PTX-NK als auch zu Abraxane® (eine zugelassene PTX-Nanoformulierung) erfolgreich verlangsamt.
Chapter 1

Background and Purpose
1.1. Chemotherapy of cancer

Cancer is one of the leading death causes in both developed and developing countries. According to the most recent report of the American Cancer Society, 14.1 million new cancer cases and 8.2 million cancer-associated deaths occurred in the world’s population in 2012 alone, and it is estimated that these numbers will raise to 21.7 and 13 million by 2030, respectively, due to general growth of the population, ageing of the society and adoption of lifestyles that are known to increase cancer risk (e.g. tobacco and alcohol consumption, stress, obesity, improper diet, etc.). Therefore, the treatment and cure of cancer remains one of the greatest challenges of medicine. The conventional strategies for cancer treatment include surgery, radiation and chemotherapy. In addition, immunotherapies, which employ the patient’s own immune system to defeat the cancer with the use of e.g. antibodies, cytokines or cell-based therapies are on the market.

Among these strategies, chemotherapy based on systemic administration of a single or a combination of cytotoxic drugs remains the major therapeutic approach for cancer treatment. Chemotherapy is used either alone, or as (neo)adjuvant therapy together with surgery and/or radiation. The development of chemotherapeutics for cancer treatment was initiated in 1940s when the effects of vesicant warfare gases had been investigated by the United States Department of Defense. The observation that bone marrow and lymph nodes were markedly depleted in men exposed to mustard gas led two pharmacologists, Alfred Gilman and Louis Goodman from Yale School of Medicine, to the assumption that this chemical might as well destroy the lymphoid tumor. Accordingly, when evaluating the therapeutic potential of the mustard compounds in tumor-bearing mice, they discovered that particularly nitrogen mustard caused tumor depletion. Encouraged by this finding, the physician Gustav Lindskog administered nitrogen mustard in a non-Hodgkin’s lymphoma patients and noticed marked remission of the tumor. Although the effect lasted only a few weeks and was followed by further disease progression, this study established a proof-of-principle that systemically administered chemical compounds may be used to induce tumor regression. Since the discovery of the anticancer activity of nitrogen mustard, many cytotoxic compounds based on small molecules have been identified and approved for their use in the clinics. These compounds can be classified according to their mechanism of action. For example, some drugs directly damage DNA such as alkylating (e.g. cyclophosphamide, nitroureas, nitrogen mustard), alkylating-like (platinum-based drugs) or intercalating agents (anthracyclines). Others, such as nucleoside analogues (e.g. gemcitabine, 5-fluorouracil, cytarabine), are incorporated in the DNA and RNA and interfere with cell metabolism and nucleic acid synthesis. Taxanes and vinca alkaloids (e.g.
paclitaxel (PTX), vincristine) interact with microtubules during mitosis thus disrupting the cell division process. Finally, topoisomerase inhibitors (e.g. irinotecan or SN-38) increase the steady state of topoisomerase complexes that cleave the DNA, which leads to cell death.⁴

Despite its widespread use, conventional chemotherapy with cytotoxic drugs faces several limitations. Their lack of selectivity towards cancerous tissues results in damage of rapidly proliferating healthy cells, generating serious adverse effects. Multidrug resistance (MDR), which can involve enhanced drug efflux outside the cell by various energy-dependent membrane transport proteins (e.g. P-glycoprotein), prevents the drug from reaching the therapeutic concentrations inside the cell and limits the therapeutic effect. Moreover, the poor aqueous solubility of many potent anticancer drugs requires the use of solvents, which further promotes systemic toxicity. Nanotechnology offers tools that help to overcome these limitations and improve the safety and efficacy of the existing cytotoxic anticancer drugs, which will be discussed in the next sections of this chapter.⁶

### 1.2 Improving chemotherapy with nanomedicines.

#### 1.2.1 Reducing systemic toxicity and increasing selectivity with nanomedicines.

In general, nanomedicines range in size from 1 – 1000 nm and embrace a wide variety of structures and materials for therapeutic and diagnostic purposes. To form an anticancer nanomedicine, a cytotoxic drug can be (i) conjugated to a protein or a polymer, (ii) embedded within a nanocarrier or a nanoparticle (e.g. liposome, polymeric nanoparticle, micelle, nanoshells) or (iii) constitute the solid core of the nanoparticle (drug nanosuspension).⁶ Herein, the concept of nanomedicines will be discussed in the context of nanocarrier and nanosuspension-based systems.

The extensive research devoted in the past decades to the development of nanomedicines for cancer treatment resulted in few nanoformulations of anticancer drugs being approved by the Food and Drug Administration (FDA) or the European Medicinal Agency (EMA) (Table 1.1). These formulations have the advantage of inducing significantly less dose-limiting side effects compared to the administration of drugs in solution. For example, cardiotoxic effect of free anthracyclines (doxorubicin and daunorubicin) is markedly reduced when they are administered as liposomal formulations (Doxil®, Myocet®, DaunoXome®) since they lead to negligible amounts of free drug in the bloodstream and consequently minimal
Chapter 1: Background and Purpose

distribution to the heart. Moreover, Abraxane®, the albumin-bound PTX nanoparticles of pure drug, markedly reduce the risk of hypersensitivity in comparison to its first formulation Taxol®, as the effect was related to the presence of solubilizing excipients (Cremophor EL/ethanol).

Furthermore, the intrinsic physicochemical properties of nanomedicines enable their use for targeting solid tumors and increase the specificity and efficacy of the treatment. In particular, their size can be tuned to facilitate their passive accumulation in malignant tissues via the enhanced permeability and retention (EPR) effect, and their large surface-to-volume ratio enables their decoration with tunable densities of high affinity ligands actively targeting the particles to the tumor cells/environment.

Passive tumor targeting

The concept of passive targeting by the EPR effect was introduced in 1986 by Matsumura and Maeda, upon observation of preferential distribution and prolonged retention of dye-labeled proteins of molecular weight higher than 30 kDa in the tumor tissue. The effect relies on intrinsic properties of solid tumors, such as rapid angiogenesis, which in order to supply nutrients to the fast growing tumor tissue promotes the formation of discontinuous and irregular blood vessels with fenestrations ranging in size from 200 – 2000 nm (depending on the tumor type and localization). The defective blood vessels facilitate the extravasation of macromolecular drugs and nanomedicines of sizes typically less than 200 nm into the tumor interstitium and constitutes the “enhanced permeability” part of the EPR effect. Moreover, depending on the type and stage of the tumor, the impaired lymphatic system in the malignant tissue can prevent the uptake of the interstitial fluids, which in healthy tissue is drained back into the blood circulation. Thus, once the nanomedicine or macromolecular drug extravasates into the tumor tissue, it is not cleared efficiently and instead accumulates in the tumor interstitium, which denotes the “enhanced retention” part of the EPR effect (Figure 1.1).

Importantly, long blood circulation time of nanomedicines (> 6 h) is a pre-requisite for their efficient passive targeting. However, colloidal particles with hydrophobic surfaces are often prone to adsorption of specific blood components, such as opsonin proteins, which is followed by the recognition and phagocytosis by the macrophages of the mononuclear phagocyte system (MPS). This process can occur within minutes after their intravenous (i.v.) administration and is the main clearing route of the colloids from the bloodstream, constituting a major obstacle for achieving the long circulation times necessary for passive targeting. Thus, to reduce opsonization and subsequent clearance by the MPS, the surface of the nanomedicines are often decorated with flexible hydrophilic polymers (e.g. poly(ethylene glycol) (PEG),
polysaccharides, poly(vinyl pyrrolidone), poly(vinyl alcohol)) that provide a steric barrier for the adsorption of the opsonins and confers stealth properties to the nanomedicines.\textsuperscript{12, 13} Due to its low toxicity, PEG is the most frequently used hydrophilic polymer for this purpose. Moreover, recent strategies for increasing circulation time include decorating the surface of the nanocarriers with the membrane constituents (e.g. CD-47 self-peptides or whole membrane isolates) of naturally long-circulating blood components like e.g. erythrocytes or leukocytes.\textsuperscript{14, 15}

![Figure 1.1. Graphical representation of passive and active tumor targeting with drug nanocarriers.](image)

The marketed PEGylated liposomal doxorubicin nanoformulation (Doxil\textsuperscript{®}/Caelyx\textsuperscript{®}) is a classic example of passively targeted nanomedicine, which achieve mean circulation half-life time of ~70 h and thus exploit the EPR effect. Its non-PEGylated version (Myocet\textsuperscript{®}) is also able to accumulate in the tumor tissue efficiently despite having a markedly shorter circulation in the blood stream and similar observations apply as well as to the non-PEGylated liposomal formulation of daunorubicin (DaunoXome\textsuperscript{®}).\textsuperscript{7, 9, 16} However, although these nanomedicines increase the local drug concentration in the vicinity of the tumor and decrease its systemic toxicity, the benefits in overall survival rates in patients compared to traditional chemotherapy are in fact only marginal.\textsuperscript{17} This is likely due to the inherent complexity and heterogeneity of the EPR effect among different tumor types and individual patients, as well as the lack of
appropriate preclinical animal models that accurately predict the passive targeting in humans. Thus, much of the current research is devoted to enhancing the EPR effect, understanding the interpatient variability and tuning the physicochemical properties of the formulations and the tumor microenvironment to maximize the therapeutic outcomes of passively targeted nanomedicines.\textsuperscript{12, 18}

Table 1.1. FDA and EMA-approved anticancer nanomedicines for \textit{i.v.} administration.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Nanoformulation type</th>
<th>Commercial name (Company)</th>
<th>Indication</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin</td>
<td>PEGylated liposomes</td>
<td>Doxil®/Caelyx® (Janssen)</td>
<td>Ovarian cancer, AIDS-related Kaposi’s sarcoma, multiple myeloma</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>Liposomes</td>
<td>Myocet® (Teva)</td>
<td>Metastatic breast cancer</td>
</tr>
<tr>
<td>Daunorubicin</td>
<td>Liposomes</td>
<td>DaunoXome® (Galen)</td>
<td>Advanced HIV-associated Kaposi’s sarcoma</td>
</tr>
<tr>
<td>PTX</td>
<td>Albumin-bound drug nanoparticles</td>
<td>Abraxane® (Celgene)</td>
<td>Advanced non-small cell lung cancer, advanced pancreatic and breast cancer</td>
</tr>
<tr>
<td>Vincristine</td>
<td>Liposomes</td>
<td>Marquibo® (Spectrum Pharmaceuticals)</td>
<td>Philadelphia chromosome negative acute lymphoblastic leukemia</td>
</tr>
<tr>
<td>Irrinotecan</td>
<td>Liposomes</td>
<td>Onivyde® (Merrimack)</td>
<td>Metastatic pancreatic adenocarcinoma</td>
</tr>
<tr>
<td>Mitamurtide</td>
<td>Liposomes</td>
<td>Mepact® (Takeda)</td>
<td>High grade non-metastatic osteosarcoma</td>
</tr>
<tr>
<td>Cytarabine</td>
<td>Liposomes</td>
<td>Depoeye® (Mundipharma)</td>
<td>Lymphomatous menignitis*</td>
</tr>
<tr>
<td>Cis-platin</td>
<td>Liposomes</td>
<td>Lipoplatin® (Regulon Inc.)</td>
<td>Pancreatic cancer**</td>
</tr>
</tbody>
</table>

*lymphoma complication; **phase 3 clinical trials ongoing in EU

\textit{Active tumor targeting} Active targeting of the nanomedicine to a tumor (also denoted as ligand-targeting or receptor-mediated targeting) involves the decoration of the nanocarrier’s surface with a ligand binding generally to a receptor overexpressed on the tumor cells or on the tumoral endothelium (Figure 1.1). In the former case, binding of the nanocarrier to a specific receptor on the tumor cell can trigger endocytosis, which increases the intracellular concentration of the drug.\textsuperscript{6} Drug nanocarriers with ligands targeting several classes of internalization–prone receptors have been investigated, such as the transferrin receptor\textsuperscript{19}, the folate receptor\textsuperscript{20}, membrane glycoproteins (\textit{e.g.} epithelial cell adhesion molecule, (EpCAM))\textsuperscript{21, 22} and the epidermal growth factor receptors (\textit{e.g.} human epidermal receptor-2 (HER2)).\textsuperscript{23} The ability of a targeted nanocarrier to reach the tumor interstitium is dictated by the EPR effect.\textsuperscript{24} In many cases, the attachment of a targeting ligand does not affect the pharmacokinetics and biodistribution of the nanocarrier and thus its overall accumulation in the tumor tissue but it can enhance its intracellular uptake, which in turn translates into increased antitumor efficacy.\textsuperscript{25–28}
For example, when tested in a pre-clinical animal model bearing human HER2-positive breast cancer tissue, MM-302 (doxorubicin PEGylated liposomes decorated with an antibody fragment targeting HER-2, currently in phase 2 clinical trial) were deposited to the same extent in the tumor as the non-targeted version. Notwithstanding, in contrast to the non-targeted liposomes which primarily distributed in the extracellular stroma of the tissue, MM-302 was internalized in tumor cells. However, in certain cases, the overall pharmacokinetics and biodistribution of the nanocarrier may be unfavourably altered by the presence of targeting agents. For instance, increasing the surface concentration of the targeting ligand conjugated via PEG chains can be counterproductive as it may shield the stealth properties conferred to the nanocarrier and increase its opsonization and MPS accumulation, which in turn decrease circulation times and tumor accumulation. In contrast, the direct conjugation of a targeting moiety on the surface of the nanocarrier may mask its charged or hydrophobic surface, reducing its non-specific interactions with the plasma proteins and its recognition by macrophages, ultimately leading to prolonged circulation times. Furthermore, the use of full antibodies entails a risk Fc fragment-mediated immune reaction and increased clearance of the nanocarrier, which is mainly dependent on the orientation of the attached antibody. Overall, the way in which targeting ligands influence the nanocarriers’ tumor accumulation is highly complex and more studies will be required for its full understanding.

Targeting the tumoral endothelium to destroy its vascular system and deprive the cancer cells from nutrients and oxygen is an indirect strategy to target solid tumors. Notably, it does not rely on the EPR effect and instead allows the nanosystem to bind at the targeted site and get internalized without the need of extravasation into the tumor tissue. Moreover, a drug nanocarrier bound to the tumoral endothelium can potentially serve as a drug depot to release the cytotoxic drug in the vicinity of malignant cells. However, in comparison to direct targeting of tumor cells, this approach has been more rarely investigated in combination with cytotoxic drug nanocarriers. Among the proteins on the tumoral endothelium that can be targeted the vascular endothelial growth factor (VEGF) receptor, αβ integrin receptor and matrix metalloproteinases (e.g. aminopeptidase N) are some of the most prominent examples. Targeting the αβ integrin receptor is especially beneficial due to its expression not only on the tumor endothelium but also on the surface of many tumor cells (e.g. melanoma, prostate cancer or breast cancer), which allows for synergistic action of the delivered cytotoxic drug. For example, liposomes decorated with a peptide containing the arginine-glycine-aspartic acid (RGD) sequence that targets the integrin receptor, exhibited binding to both tumor vascular endothelium and mouse melanoma (B16F0) tumor xenograft cells in an animal model. The
systemic administration of these targeted doxorubicin-loaded liposomes significantly reduced tumor growth and increased survival time compared to the non-targeted versions.\textsuperscript{37}

\textbf{1.2.2. Addressing MDR with nanomedicines}

Among the many drug resistance mechanisms that exist, the resistance mediated by the cells’ drug efflux pumps of the ATP-binding cassette transporter family represents one of the most prominent hurdles for conventional chemotherapy. Direct approaches developed to overcome this obstacle by increasing the drug influx/efflux ratio include the use of compounds binding to the efflux pump or to components that affect its function, and the silencing of resistance-related mRNAs. Although neither of these approaches have so far been approved for clinical use either due to toxicity issues or to obstacles related to the intracellular delivery of nucleic acids\textsuperscript{38} nanotechnology could enable the simultaneous and synergistic delivery of a cytotoxic drug and an anti-MDR compound in a single delivery vehicle. For example, lapatinib, a drug inhibiting the functions of the P-gp efflux pump protein, was co-delivered in a polyelectrolyte shell coating of PTX nanocrystal to a drug-resistant cell line, resulting in increased cytotoxic activity compared to the PTX formulation without lapatinib.\textsuperscript{39} In another study, mesoporous silica nanoparticles co-delivering doxorubicin and siRNA against P-gp efflux pump proteins enhanced the therapeutic response in a drug-resistant tumor in mice.\textsuperscript{40} Moreover, the passive and active targeting of nanocarriers can also indirectly contribute to overcoming MDR by acting as a depot in the vicinity of the tumor or by increasing the amount of the drug delivered to the cell, respectively.\textsuperscript{38}
1.2.3. Overcoming solubility issues with nanomedicines

Many of the potent cytotoxic drugs used for chemotherapy exhibit poor aqueous solubility (as it is the case of taxanes and camptothecins for instance), which often hampers their clinical development. Conventional strategies for improving their solubility involve chemical modifications (such as salts or prodrug strategies) and the use of surfactants and co-solvents; however, the former approach can alter the activity of the drug (e.g. prodrug approach) whereas the latter often implies additional toxicity issues.

Nanotechnology offers means for delivering unmodified drugs with the use of non-toxic and/or biodegradable materials. Hydrophobic drugs can be formulated in lipid-based nanocarriers (e.g. embedded within the phospholipid bilayer of liposomes or in the core of solid lipid nanoparticles or nanoemulsions), or incorporated in the hydrophobic part of polymer-based nanosystems (e.g. polymeric nanoparticles and polymeric micelles) (Figure 1.2). However, in these nanoformulations, particle components (i.e. polymers, surfactants, lipids) often significantly exceed the amount of the drug itself, yielding low drug loadings, which constitutes their major disadvantage. Drug nanosuspensions (i.e. drug nanocrystals (NCs) and amorphous drug nanoparticles) represent another distinct group of nanomedicines, where the solid core consists entirely of the hydrophobic drug and the stabilizing excipients comprise a small fraction of the formulation. By minimizing the presence of non-drug compounds in the formulation, drug nanosuspensions enable the delivery of large amounts of the active compound. Anticancer drug nanosuspensions constitute the focus of this thesis, and their physicochemical properties and issues concerning their use as drug delivery systems for tumor targeting will be discussed in the following sections.
Figure 1.2. Graphical representation of different types of drug nanocarriers for the delivery of hydrophobic drugs. a - d) Polymer-based systems. a) In polymeric nanoparticles a hydrophobic drug is dispersed within the polymer matrix. b) Polymeric micelles are formed by self-assembly of amphiphilic polymers, and the hydrophobic core serves as a drug reservoir. c) Polymeric nanoshells are vesicles with a liquid oil core containing the drug surrounded by a polymeric membrane. d) In polymersomes the membrane of the vesicle is composed of self-assembled amphiphilic block copolymer and the poorly water soluble drug is associated with the hydrophobic blocks. e - g) Lipid-based systems. e) In liposomes a hydrophobic drug is associated in a bilayer membrane. f,g) Solid lipid nanoparticles and nanoemulsions contain a solid or liquid oil core, respectively, encapsulating the drug, and are stabilized by phospholipids or surfactants. h, i) Drug nanosuspensions. h) Albumin-stabilized nanoparticles are composed of pure solid drug (usually amorphous). i) Nanocrystals are composed of pure solid drug in a crystalline state stabilized with surfactants or amphiphilic polymers.

1.3. Drug nanosuspensions – physicochemical considerations

To appreciate the advantages and limitations of drug nanosuspensions in chemotherapy, it is crucial to understand their physicochemical properties. Drug nanosuspensions can be fabricated through top-down processes where the coarse powder size is reduced to a nanometer scale (e.g. wet milling, high pressure homogenization, sonication-assisted disintegration), or through bottom-up processes, where a solubilized drug is precipitated into particles (e.g. nanoprecipitation, emulsification-evaporation) (both described in more detail in Chapter 2). Regardless of the fabrication process, the newly formed surface area (ΔA) generates an excess of Gibbs free energy (ΔG) as described by Equation 1 (γ is the interfacial tension), which tends to be minimized by suspension agglomeration. Thus, the addition of a surface-active stabilizer...
during the miniaturization process reduces the interfacial tension and the overall free energy of the system, contributing to the NCs’s stabilization.\textsuperscript{45}

\[ \Delta G = \gamma \cdot \Delta A \]  \hspace{1cm} \textbf{Equation 1}

Ionic and non-ionic surface-active compounds can be used for particle stabilization, which aside from lowering the interfacial tension, stabilize particles by electrostatic and steric repulsion forces, respectively. The electrostatic repulsion is based on the overlapping of the electrical double layers surrounding the particles in aqueous media, which is described by Derjaguin–Landau–Verwey–Overbeek (DLVO) theory.\textsuperscript{46} This stabilization mechanism is mainly sensitive to ionic strength and pH variations due to its influence on the surface charge of the particle. The steric effect emerges from the solvation of the hydrophilic part of the non-ionic surfactant extending into the aqueous medium. When the particles approach each other, their close contact involving interpenetration of the surfactant’s hydrophilic chains accompanied by the release of water molecules, is thermodynamically disfavored. As the solvation effect decreases with the increase of temperature, steric stabilization becomes susceptible to temperature variations. Clearly, due to their complementary nature, a combination of both electrostatic and steric stabilization principles can be particularly beneficial for obtaining stable suspensions.\textsuperscript{47}

Moreover, size reduction to the nanometer scale confers specific physicochemical properties to the particles. Essentially, it leads to an increase in the saturation solubility \((C_s)\) of the drug dissolved from the nanoparticle, a phenomenon described by Ostwald-Freundlich equation. This emerges from the fact that the equilibrium between dissolution and recrystallization for particles with high curvature (\textit{i.e.} below \(\sim\) 1 µm) is shifted in favor of dissolution.\textsuperscript{48}

Increased saturation solubility also further enhances dissolution velocity \((dC/dt)\) of the nanosuspensions, because, as described by Noyes-Whitney equation (\textbf{Equation 2}), it increases the concentration gradient \((C_s-C_x)\) over the diffusional distance \(h\) \((C_x\) is the concentration of the drug in bulk solution). Furthermore, the smaller the particles are, the shorter the diffusional distance \(h\) and the higher the specific surface area \((A)\) they possess, which further contributes to their accelerated dissolution.\textsuperscript{49}

\[
\frac{dC}{dt} = \frac{D \cdot A \cdot (C_s-C_x)}{h} \]  \hspace{1cm} \textbf{Equation 2}

Another important aspect that affects both the particle’s dissolution velocity and saturation solubility is their crystalline state. For amorphous particles, the high lattice energy
of the disordered molecular structure is thermodynamically unstable, thus their dissolution velocity is substantially accelerated compared to crystalline. Moreover, since the saturation solubility is a function of lattice energy (besides the particle size), the amorphous particles exhibit increased saturation solubility in comparison to crystalline ones.\textsuperscript{50}

1.4. Nanosuspensions in pharmaceutics

The availability of drug nanosuspensions in the market is so far mainly limited to orally administered drugs. Due to the increased dissolution velocity and saturation solubility explained above, nanosuspensions substantially increase the bioavailability of orally administered poorly soluble drugs. The absorption of the active compound is likely promoted by the large drug concentration gradient achieved between the gut and the blood vessels and possibly, by the improved adhesiveness of the nanoparticles to the gut mucosa given their increased contact surface area. Examples of orally administered drug NCs on the market are Rapamune\textsuperscript{®} (Pfizer, sirolimus, immunosuppressive drug), Emend\textsuperscript{®} (Merck, aprepitant, antiemetic drug) or Tricor\textsuperscript{®} (Abbot Laboratories, fenofibrate, treatment of primary hypercholesterolemia or mixed dyslipidemia). Concerning parenteral formulations, only the Abraxane\textsuperscript{®} drug nanoparticles (described previously) have reached the market.\textsuperscript{51,52}

1.5. Towards passive tumor targeting with drug NCs

Ideally, after i.v. injection, drug particle dissolution and the release of the cytotoxic compound should follow the extravasation into the tumor tissue or the uptake by cancer cells. However, rapid dissolution of small drug nanoparticles (as described by the Noyes-Whitney equation) in the bloodstream constitutes a major obstacle for achieving long circulation times, a pre-requisite for efficient passive tumor targeting. Thus, pharmacokinetic and biodistribution profiles of i.v.-injected drug nanosuspensions are often similar to those of drug solutions, but with a more favorable toxicity profile due to the absence of solubilizing excipients.\textsuperscript{53,54} For example, although higher tumor accumulation of Abraxane\textsuperscript{®} (compared to Taxol\textsuperscript{®}) is generally attributed to the EPR effect, the immediate dissolution of this albumin-stabilized amorphous PTX nanoparticle formulation rather excludes this targeting mechanism.\textsuperscript{55} Several studies demonstrated solution-like pharmacokinetics of i.v.-injected nanosuspensions of anticancer drugs or drug candidates in animal models.\textsuperscript{56-58} For example, similar plasma concentration profiles were observed in rats for NCs of the antitumor compound SNX-2112 of \textasciitilde200 nm stabilized by poloxamer188 \textit{versus} drug solutions, and only \textasciitilde20\% higher liver and spleen
content at early time points post-injection. In fact, *in vitro* drug release studies revealed the same release profiles of the drug from a solution and NCs under sink conditions, which is indicative of the rapid dissolution of the latter.\(^{56}\)

In line with the Noyes-Whitney equation, increase of the nanoparticles’ size results in a decrease of their dissolution rate, which alters their pharmacokinetics and biodistribution profiles. Slowly dissolving particles are cleared from the bloodstream by macrophages and distributed in the MPS organs (*i.e.* liver and spleen) consequently preventing their deposition at the tumor site. Gao and co-workers have demonstrated the differences in the pharmacokinetics and biodistribution of 100 and 900 nm nanocrystals of poloxamer 188/lecitin-stabilized oridonin in rats. While small NCs behaved *in vivo* similarly to drug solutions, the large NCs accumulated in the liver, spleen and lungs. Notably, at later time points increased drug serum concentrations were observed for the large NCs due to their dissolution in the phagocytic cells which released the drug back into the systemic circulation.\(^{59}\)

Moreover, some small particles may also display similar biodistribution profiles to those of large particles.\(^{53, 60}\) For example, Hollis *et al.* fabricated PTX NCs of \(~200\) nm without the use of any stabilizer. After injection to tumor-bearing mice, NCs accumulated extensively in the MPS organs (*i.e.* liver, spleen, lungs), which is presumably an indicative of their *in vivo* agglomeration. Consequently only a little fraction of the drug was found in tumor.\(^{60}\)

Taking into account these examples, it is clear that achieving long circulation times and passive tumor targeting requires a certain balance between the nanoparticle’s size, dissolution rate and surface properties. In addition, resolving the obstacles for passive tumor accumulation to enable the contact between the intact particles and the targeted cells is a pre-requisite for active targeting.

Aiming at simultaneously slowing down the dissolution rate of the particles and reducing their uptake by macrophages, our group previously investigated the suitability of an amphiphilic block copolymer of methoxy-PEG with a cross-linkable hydrophobic part for stabilization of PTX NCs fabricated by in a wet milling process. However, although crosslinking of the polymer’s hydrophobic blocks directly on the NC surface *via* copper-catalyzed 1,3-cycloaddition helped to retain the integrity of the coating and prevented its shedding, it did not alter the dissolution rate of the NCs.\(^{61}\) In another investigation from our laboratory, the block copolymer of methoxy-PEG and \(\alpha\)-propargyl-\(\delta\)-valerolactone-\(co\)-\(\varepsilon\)-caprolactone was modified with different alkanes of varying hydrophobicity using thiol-yne chemistry. The underlying hypothesis was that the dissolution rate of the PTX NCs could be
tuned by varying the strength of the hydrophobic interactions of the particle surface with the aforementioned stabilizers. However, this approach also appeared to be invalid since no correlation of hydrophobicity of the stabilizer with dissolution rate was observed. The layer-by-layer (LbL) coating of drug nanoparticles with a multilayer shell of oppositely charged polyelectrolytes constitutes a promising approach to tune the particles’ dissolution kinetics. Indeed, several examples exist in literature where this technique was applied to reduce the dissolution of anticancer drug nanoparticles. The LbL coating technique has been employed in this doctoral work and is thoroughly discussed in Chapter 2.

1.6. The approach of this thesis

In this doctoral thesis, PTX (Figure 1.3) was chosen as a model hydrophobic anticancer drug. The pharmacological mode of action of PTX relies on its binding to the cell’s microtubules, specifically to β-tubulin in a αβ-tubulin heterodimer. In the mitosis process, the dynamics of microtubule polymerization/depolymerisation controls the separation of chromosomes. Binding of PTX stabilizes the polymerized structures and prevents their depolymerisation, thus blocking the cells in the G2/M phase of the cell cycle and ultimately leading to apoptosis. The extremely low aqueous solubility of PTX (less than 0.1 µg mL⁻¹) makes the formulation of this drug a challenging task. In the formulation Taxol®, PTX is solubilized with Cremophor EL and ethanol, but the presence of these agents causes severe side effects and also imposes non-linear pharmacokinetics because of the drug’s entrapment in Cremophor EL micelles. These issues were addressed in the succeeding formulation Abraxane®, which eliminated the excipient-related toxicity and enabled linear dose-dependent pharmacokinetics of the drug. However, the toxicity due to the systemic exposure of solubilized PTX to the whole body (e.g. alopecia, fatigue, neuroptenia, sensory neuropathy), is still a pertinent problem.

Therefore, the aim of this doctoral work was to produce PTX NCs able to passively deposit in the tumor tissue via the EPR effect, and to evaluate their in vitro and in vivo performance. To this end, NCs were coated LbL with a PEGylated shell of polyelectrolytes in order to simultaneously tune their dissolution rate and reduce their uptake by macrophages (Figure 1.4). LbL coating is a simple and versatile technique based on the sequential deposition of molecular species on planar surfaces or colloidal templates.
Chapter 1: Background and Purpose

Figure 1.3. Chemical structure of PTX.

Chapter 2 introduces this technique and presents the advances in this field with a focus to solid drug nano- and microparticles. Chapter 3 describes the development and physico-chemical characterization of the formulation. Firstly, the fabrication of electrostatically stabilized drug NCs using wet milling process is described, followed by the screening of a series of polyelectrolytes for the LbL particle stabilization and of the PEGylated polyelectrolyte for the top coating layer. Secondly, the study of their influence on colloidal stability and dissolution rate of the NCs is presented. In Chapter 4, the formulation’s characterization in biological systems (cells and mice) is shown in comparison to Abraxane®. The formulations’ impact on the viability of the human colon adenocarcinoma (HT-29) cell line, as well as their pharmacokinetics and biodistribution profiles in HT-29 tumor-bearing mice are presented. Finally, Chapter 5 provides general conclusions on the studies carried out and gives an outlook towards passively targeted LbL-coated drug NCs for anticancer treatment.

Figure 1.4. Graphical representation of the system designed in this thesis.
Chapter 2

Layer-by-layer coating of solid drug cores: a versatile method to improve stability, control release and tune surface properties

This chapter is published:

Chapter 2: LbL coating of solid drug cores

2.1. Introduction

The effectiveness of pharmacologically active compounds is strongly subordinated to their accessibility to target tissues and organs. Accordingly, modern pharmaceutical formulation and drug delivery aim at improving a compound’s efficacy by achieving therapeutic concentrations at a specific site for a desired period of time. Various factors, such as route of administration, pharmacological mode of action and physicochemical properties of a drug, influence the design of an optimal dosage form.68, 69 Among the many physicochemical parameters that must be considered in the formulation process, the molecule’s solubility is of critical importance. Around 70% of the drugs emerging nowadays from high throughput screenings are poorly water soluble, which often greatly complicates their clinical development.41 Chemical approaches for improving the solubility involve formation of salts of ionizable compounds or syntheses of soluble prodrugs. However, those modifications can only be applied to a limited number of compounds and moreover bear the risk of reducing the drug’s activity. In addition, other existing strategies, relying on the use of surfactants, co-solvents and polymeric/lipidic particulate systems often imply high excipients-to-drug ratio and may entail toxicity issues.42, 70 Besides the aforementioned approaches, the reduction of drug powders to the micro or nanoscale is a valuable formulation strategy enabling high drug loading with marginal risk of excipient-related toxicity. In the resulting micro and nanosized drug particles - also referred to as micro/nanosuspensions, drug micro/nanoparticles or drug micro/nanocrystals - the cores of the dense, solid state particles consist purely of the drug, while stabilizers ideally comprise a merely small fraction of the formulation.45, 71, 72 In addition, the miniaturization process increases the specific surface area and accelerates the dissolution velocities, which ultimately has a beneficial impact on the drug release and associated bioavailability.73 In order to precisely control the release kinetics and protect the drug from degradation, strategies such as the layer-by-layer (LbL) assembly method can be coupled to the production of micro/nanocores. The LbL technique based on sequential layering of chemical species was first introduced by the group of Decher in early 90’s, who showed the feasibility of alternating the adsorption of oppositely charged polyelectrolytes on a planar surface, and demonstrated the linear relationship between the number of deposited layers and the overall thickness of the shell.74 The concept was translated to solid drug cores by Caruso et al. in 2000, when catalase microsized “biocrystals” were coated with positively charged poly(allylamine hydrochloride) (PAH) and negatively charged poly(styrene sulfonate) (PSS) to successfully protect the protein from degradation by proteases.75 The same coating was then described for microcrystalline pyrene and fluorescein solid cores and interestingly, the core dissolution rate was observed to
depend on the number of coating layers, suggesting the method’s promising application in drug delivery.  
Subsequently, a genuine drug (ibuprofen) was coated with polyelectrolyte multilayers of chitosan/dextran sulfate, which resulted in the prolongation of the core dissolution time and decisively established the utility of LbL coatings in suspension-based drug delivery systems.  

The LbL coating was also applied on a sacrificial template (e.g. mesoporous SiO₂, CaCO₃ or hydrogels) that is dissolved after the layering process is terminated. In this case, the drug is loaded into the hollow spheres obtained after removal of the core by temporarily manipulating the permeability of the shell, or included in a porous template prior to the LbL assembly.  

The latter method, used for example with CaCO₃ as sacrificial core that can be dissolved under mild conditions, is suitable for the encapsulation of sensitive macromolecular compounds, such as proteins or nucleic acids. However, those approaches result in lower drug content compared to solid drug cores and are out of the scope of this review. Moreover, the LbL concept has found broad applications in many other biomedical (drug-eluting stents, surgical sutures) and non-biomedical fields (electrodes, batteries or solar cells) and the reader is invited to consult other review articles for more information on these topics.

In general, through the sequential deposition of chemical or colloidal species (polyelectrolytes, nanoparticles, lipids or dyes) on colloidal templates, the LbL method allows to tune the release of therapeutic compound from inside the core by modulating the permeability of the coating. The interplay between coating species can be based on hydrophobic interactions, hydrogen bonds, covalent bonding or complementary base pairing, although most commonly the multilayer build-up is governed by electrostatic interactions between oppositely charged entities. Moreover, the incorporation of specific responsive components in the multilayers may allow the drug to be released at specific sites under certain environmental conditions (e.g. presence of enzymes, change in pH or ionic strength or external stimuli).

Although almost 15 years have elapsed since the concept of LbL on drug micro and nanocores was introduced, the advancement in this field has been fairly slow. In this article, we review the developments in LbL formulations of micro- and nanosuspensions of poorly soluble drugs and proteins, and identify the major obstacles that are impairing their translation to the (pre)clinical stages.
2.2. Preparation of template drug particles for LbL assembly

The production of well-dispersed template micro or nanocores constitutes the initial and crucial step for the generation of LbL-coated drug suspensions. Steric and electrostatic repulsions are the two main mechanisms through which colloidal suspensions can be stabilized. For LbL the electrostatic stabilization principle is largely preferred as it enables further electrostatic interactions between the charged coating species and the particle surface. The intrinsic charge that particles of some poorly water soluble drugs bear in water is generally sufficient for the adsorption of the first polyelectrolyte coating layer, which can be performed during or immediately after the size reduction process. When the intrinsic charge is insufficient for this, an ionic stabilizer that strongly interacts with the surface of the colloidal particle via non-electrostatic interactions must be used. Additionally, in order to avoid the formation of separate drug-free polyelectrolyte complexes during the LbL process, the particles need to be purified from the excess of non-adsorbed polyelectrolyte prior to LbL assembly or alternatively the minimum amount of polyelectrolyte needed to reverse the surface charge must be determined by titration.

The top-down and bottom-up approaches used to manufacture suspensions of poorly water-soluble drugs (Figure 2.1) as well as protein core fabrication are described in this section.

2.2.1. Hydrophobic drugs

- Microcores

The classical concept of drug micronization has been employed since many decades to improve the oral bioavailability of class II drugs which exhibit good permeability but low solubility. Top-down micronization methods include those based on powder comminution by mechanical force such as pressure, friction, attrition or milling (i.e. grinding in mortar, jet milling, pearl-ball milling or high pressure homogenization), while bottom-up involve controlled production processes such as spray drying or controlled crystallization. On average, micronization produces particles of sizes ranging from two to a few tens of micrometers. Table 2.1 lists the methods and stabilizers employed for fabrication of drugs microcores for LbL.

Top-down approaches. Although drug grinding with mortar and pestle does not provide precise control over the size of the particles, it is a commonly used method to prepare drug microcores in academic laboratories. It produces crystalline drug microparticles of sizes
from ca. 2 µm to 60 µm and in some cases, the size distribution may be further narrowed by fractionation of the suspension by sedimentation. Depending on the initial net charge of the drug particles, positively or negatively charged polyelectrolytes may be applied as first coating layer.

**Figure 2.1.** Graphical representation of the most frequently used top-down and bottom-up approaches for production of drug micro- and nanocores for LbL assembly. a) Mortar and pestle disintegration (microcores only); b) sonication-assisted disintegration; c) wet milling; d) precipitation or emulsification followed by solvent evaporation; e) sonication-assisted precipitation.

**Bottom-up approaches.** Compared to top-down methods, bottom-up approaches have been seldomly used for the preparation of template microcrystals for LbL assembly. For example, positively charged hydrocortisone microcrystals of around 10 µm were successfully obtained by bottom-up precipitation from acetone without the use of any stabilizer. The size could be tuned by varying the acetone/water ratio and the evaporation temperature. Another strategy to obtain solid cores of drugs that exhibit pH-dependent solubility is altering the pH of the medium. For example, microparticles of naproxen, which is soluble in alkaline pH, could
be obtained by lowering the pH of the solution in presence of vitamin E tocopheryl poly(ethylene glycol succinate) (TPGS) as stabilizer.111

Table 2.1. Top-down and bottom-up approaches employed for fabrication of drug microcores for LbL assembly.

<table>
<thead>
<tr>
<th>Method</th>
<th>Active compound</th>
<th>First coating layer</th>
<th>Diameter [µm]</th>
<th>Zeta potential</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Top-down</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mortar and pestle disintegration</td>
<td>Ibuprofen</td>
<td>Chitosan&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.5-31</td>
<td>+</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>Ibuprofen</td>
<td>HSA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15-60</td>
<td>+</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>Furosemide</td>
<td>PSS&lt;sup&gt;b&lt;/sup&gt;</td>
<td>~ 5</td>
<td>-</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>Vitamin K&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Alginate&lt;sup&gt;a&lt;/sup&gt;</td>
<td>~10</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Indomethacin</td>
<td>PSS&lt;sup&gt;a&lt;/sup&gt;</td>
<td>~ 2</td>
<td>-</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>Indomethacin</td>
<td>Alginate&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5-10</td>
<td>-</td>
<td>102-</td>
</tr>
<tr>
<td></td>
<td>Indomethacin</td>
<td>PDDA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>n/a</td>
<td>+</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>Ketoprofen</td>
<td>PSS&lt;sup&gt;b&lt;/sup&gt;</td>
<td>~ 6</td>
<td>-</td>
<td>106</td>
</tr>
<tr>
<td></td>
<td>Sinomenine</td>
<td>Chitosan&lt;sup&gt;b&lt;/sup&gt;</td>
<td>~ 2</td>
<td>+</td>
<td>108</td>
</tr>
<tr>
<td>Bottom-up</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Precipitation by pH change</td>
<td>Naproxen</td>
<td>TPGS&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11-20</td>
<td>-</td>
<td>111</td>
</tr>
<tr>
<td>10-Hydroxycamptothecin</td>
<td>Dipyridamol</td>
<td>Tween 80&lt;sup&gt;a&lt;/sup&gt;</td>
<td>n/a</td>
<td>-</td>
<td>112</td>
</tr>
<tr>
<td></td>
<td>Hydrocortisone</td>
<td>HPMC&lt;sup&gt;c&lt;/sup&gt;</td>
<td>~5-10</td>
<td>-</td>
<td>110</td>
</tr>
<tr>
<td>Precipitation from organic solvent</td>
<td>Hydrocortisone</td>
<td>Alginate&lt;sup&gt;b&lt;/sup&gt;</td>
<td>~ 10</td>
<td>-</td>
<td>109</td>
</tr>
</tbody>
</table>

<sup>a</sup>polymer/stabilizer added during core fabrication process; <sup>b</sup>drug particles mixed with the solution of the first coating layer after their fabrication; <sup>c</sup>hydroxypropylmethylcellulose; n/a: not available.

- Nanocores

For many poorly soluble compounds, the specific surface area created by micronization is not sufficiently large to adequately enhance the dissolution velocity and increase the bioavailability, thus requiring further decrease of the drug particles’ size to the nanometer scale. Nanosuspensions exhibit enhanced saturation solubility of the drug dissolved from nanoparticles, a phenomenon explained by the Ostwald-Freundlich equation which links the reduced radius and increased surface energy/interfacial tension with the improved saturation solubility.113 Moreover, given their reduced size nanoparticles can also be injected i.v. as aqueous suspensions, broadening their potential applications compared to drug microparticles.71, 114, 115 Nevertheless, drug powder nanosizing presents additional challenges to micronization and requires more energy input and more efficient stabilizers. Table 2.2 lists the methods and stabilizers employed for the fabrication of drug nanocores for LbL application.

Top-down approaches. The most often utilized top-down approach for the fabrication of nanocores for LbL assembly is the sonication-assisted powder disintegration. In this method, the drug powder is typically dispersed in water and sonicated for 20-60 min using a powerful
ultrasonic probe processor. The addition of polyelectrolyte before or just after termination of sonication prevents the aggregation of the obtained nanoparticles.\textsuperscript{53, 64, 95, 116-121} In general, the size of the formed nanoparticles is decreased by increasing the time and the power of sonication. Moreover, the presence of gas releasing agents (\textit{i.e.} NH\textsubscript{4}HCO\textsubscript{3}) in the dispersing medium has been shown to increase the effective intensity of ultrasounds and facilitate powder breakdown, ultimately leading to smaller particles.\textsuperscript{118, 119}

Wet milling (also called nanocomminution or nanogrinding) is another top-down approach that was employed to produce template NCs for LbL. In this method, the drug powder is placed in a milling chamber together with beads and a stabilizer solution, and the size reduction process occurs by mechanical attrition of the drug powder by the beads, while the stabilizer is simultaneously adsorbed (usually amphiphilic block copolymers or surfactants). In our recent work, PTX was milled with several positively charged polyelectrolytes but in spite of the intrinsic negative charge reported for bare PTX nanocores, none of the cationic polyelectrolytes tested generated a stable suspension. Only milling in presence of negatively charged PSS produced NCs with size around 170 nm,\textsuperscript{122, 123} suggesting that particle stabilization during the wet milling process might not be governed by electrostatic interactions but instead by specific interactions between the aromatic groups of PTX and PSS repeating units or by hydrophobic interactions.\textsuperscript{124} Despite its numerous advantages, wet milling has not been broadly employed to produce template suspensions for LbL, probably due to the difficulty of applying this technique when very small amounts of drug are available. An interesting top-down approach which is especially suited for small volumes and could be potentially applied to fabricate drug NCs for LbL is femtosecond laser ablation.\textsuperscript{125, 126}

\textbf{Bottom-up approaches.} Nanoprecipitation has been used to prepare template particles for LbL coating. In this technique, the dissolved drug is mixed with an anti-solvent, resulting in supersaturation followed by nucleation and crystal growth. The presence of stabilizers in an aqueous phase and/or use of sonication can prevent agglomeration and slow down the crystal growth resulting in smaller particles.\textsuperscript{62, 63, 127-129} The presence of small concentrations of non-ionic poly(vinylpyrrolidone) (PVP) or poly(vinyl alcohol) (PVA) in the water phase was shown to be beneficial in combination with an ionic component (\textit{e.g.} alginate or sodium docusate (AOT)), as it introduced additional steric stabilization and increased the viscosity and density of the medium, which ultimately enhanced the colloidal stability during core formation.\textsuperscript{62, 129, 130} As for microcores, precipitation may also be based on the pH-dependent drug solubility. This principle was applied to obtain semi-crystalline meloxicam nanoparticles (\textit{ca.} 100 nm) for LbL by precipitation in a microfluidic continuous flow rotating tube processor.\textsuperscript{120}
Solvent evaporation/emulsification is a widely used approach to produce polymeric nanoparticles encapsulating hydrophobic drugs,\textsuperscript{44,131} however, it is rarely adopted to fabricate drug nanocores. In this method, a drug dissolved in an organic solvent is emulsified into a non-miscible continuous phase to form discrete emulsion droplets, followed by evaporation of the solvent and formation of solid particles. When water is employed as the continuous phase, the stabilizer can be directly dissolved in it.\textsuperscript{132} Alternatively, if the emulsification is performed in another immiscible organic solvent (\textit{e.g.} eucalyptol or \textit{n}-heptan), the obtained particles can be collected by centrifugation and then suspended in the aqueous phase.\textsuperscript{133-135} For example, nanocores of hydrophobic drugs (\textit{i.e.} PTX, nifedipine, furosemide or isoxyl) of 65-170 nm in diameter were obtained for LbL assembly with the latter method and without the use of any stabilizers during core formation process.\textsuperscript{135}

Despite the high probability of obtaining amorphous particles by bottom-up approaches, the majority of publications report the formation of crystalline particles. However, it should be noted that in most of the cases the crystalline state was not confirmed by X-ray diffraction, but was instead concluded by visual examination of the particles’ morphological structure by electron microscopy. Besides the risk of forming amorphous particles (when not desired), the use of organic solvents constitutes a disadvantage common to all bottom-up approaches as they have to be removed to avoid toxicity issues. Moreover, due to hands-on time and many steps required for the following LbL coating, the complexity and length of the core preparation process should be rather avoided. Therefore, simple top-down disintegration methods involving powder processing in an aqueous phase (such as sonication or wet milling) would be deemed the most suitable for this purpose.
Table 2.2. Top-down and bottom-up approaches employed for fabrication of drug nanocores for LbL assembly.

<table>
<thead>
<tr>
<th>Method</th>
<th>Active compound</th>
<th>First coating layer</th>
<th>Diameter (nm)</th>
<th>Zeta potential</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Top-down</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sonication-assisted</td>
<td>Tamoxifen</td>
<td>PAH(^a)</td>
<td></td>
<td>+</td>
<td>64, 118</td>
</tr>
<tr>
<td>disintegration</td>
<td>Tamoxifen</td>
<td>PDDA(^a)</td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Curcumin</td>
<td>PAH(^a)</td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Curcumin</td>
<td>Protamine sulfate(^a)</td>
<td>100-220</td>
<td>+</td>
<td>118</td>
</tr>
<tr>
<td></td>
<td>Resveratol</td>
<td>Chitosan(^a)</td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PTX</td>
<td>PAH(^a)</td>
<td></td>
<td>+</td>
<td>63, 118</td>
</tr>
<tr>
<td></td>
<td>PTX</td>
<td>Chitosan(^a)</td>
<td>150</td>
<td>+</td>
<td>39, 118</td>
</tr>
<tr>
<td></td>
<td>PTX</td>
<td>PDDA(^a)</td>
<td>100</td>
<td>+</td>
<td>64</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>Chitosan(^b)</td>
<td>n/a</td>
<td></td>
<td>+</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td>Ibuprofen</td>
<td>PAH(^a)</td>
<td>120</td>
<td>+</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>Docetaxel</td>
<td>Chitosan(^a)</td>
<td>200</td>
<td>+</td>
<td>116</td>
</tr>
<tr>
<td>Wet milling</td>
<td>PTX</td>
<td>PSS(^a)</td>
<td>170</td>
<td>-</td>
<td>122</td>
</tr>
<tr>
<td><strong>Bottom-up</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Precipitation from</td>
<td>PTX</td>
<td>PAH(^a)</td>
<td>100</td>
<td>+</td>
<td>63</td>
</tr>
<tr>
<td>organic solvent</td>
<td>PTX</td>
<td>PVP, AOT, Polysorbate 80(^a)</td>
<td>170-180</td>
<td>-</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>Camptothecin</td>
<td>BSA, PVP(^a)</td>
<td>&lt;150</td>
<td>+</td>
<td>62, 129</td>
</tr>
<tr>
<td></td>
<td>Curcumin</td>
<td>PAH(^a)</td>
<td>80</td>
<td>+</td>
<td>128</td>
</tr>
<tr>
<td></td>
<td>PTX</td>
<td>Protamine sulfate(^a)</td>
<td>80</td>
<td>+</td>
<td>130</td>
</tr>
<tr>
<td></td>
<td>PTX</td>
<td>Alginate, PVA(^a)</td>
<td>100</td>
<td>-</td>
<td>120</td>
</tr>
<tr>
<td>Precipitation by pH change</td>
<td>Meloxicam</td>
<td>Poloxamer 188(^a)</td>
<td>&lt;100</td>
<td>-</td>
<td>120</td>
</tr>
<tr>
<td>Solvent evaporation/</td>
<td>Dexamethasone</td>
<td>PAH(^b)</td>
<td>48 / 150(^b)</td>
<td>+</td>
<td>134</td>
</tr>
<tr>
<td>emulsification</td>
<td>Dexamethasone</td>
<td>PDDA(^b)</td>
<td>200</td>
<td>+</td>
<td>133</td>
</tr>
<tr>
<td></td>
<td>PTX</td>
<td>Alginate, PVA(^a)</td>
<td>64-147</td>
<td>-</td>
<td>132</td>
</tr>
<tr>
<td></td>
<td>PTX</td>
<td>PDDA(^b)</td>
<td>156</td>
<td>+</td>
<td>135</td>
</tr>
<tr>
<td></td>
<td>Furosemide</td>
<td>PSS(^b)</td>
<td>174</td>
<td>-</td>
<td>135</td>
</tr>
<tr>
<td></td>
<td>Nifedipine</td>
<td>PDDA(^b)</td>
<td>110</td>
<td>+</td>
<td>135</td>
</tr>
<tr>
<td></td>
<td>Isoxyl</td>
<td>PSS(^b)</td>
<td>65</td>
<td>-</td>
<td>135</td>
</tr>
<tr>
<td></td>
<td>Spray drying</td>
<td>Artemisinin</td>
<td>766</td>
<td>-</td>
<td>136</td>
</tr>
</tbody>
</table>

\(^a\)polymer/stabilizer added during core fabrication process; \(^b\)drug particles mixed with the solution of the first coating layer after their fabrication; \(^c\)particles obtained after passing the crude suspension through 0.1 or 0.2 µm filter.

2.2.2. **Protein particles**

Since protein particles can also be considered as solid cores of therapeutically active compounds, they have also been included in the present review. Most of the advancements for this type of particles have emerged from the research on insulin, given the invaluable benefits that its controlled release would bring for diabetic insulin-dependent patients. With aims such as prolonged hypoglycemic effect and protection from degradation in the gastric fluid after oral administration, LbL encapsulation of insulin has been investigated.
Chapter 2: LbL coating of solid drug cores

Solid insulin crystals can be directly coated with the first polymer layer by suspending the protein powder in a solution of oppositely charged polyelectrolyte.\textsuperscript{100, 137} Alternatively, salting out is a general method for the preparation of protein aggregates that has been successfully applied to produce charged insulin aggregates for LbL assembly.\textsuperscript{138-141} The technique relies on the disruption of the hydration shell on the protein surface, as a result of the screening of electrostatic charges by salt ions and consequent aggregation and precipitation. Salt concentration and pH have been thus identified as important parameters that must be optimized to obtain small and well-dispersed aggregates. Indeed, insufficient salt concentration may entail low precipitation yields resulting from the high solubility of insulin, while larger aggregates may be formed above a certain ionic strength as a result of excessive screening of electrostatic charges. Moreover, salting out in medium of lower pH may lead to smaller aggregates due to higher ionization of the insulin particles.\textsuperscript{139} Insulin aggregates can be also formed by complexation with a polyanion (e.g. dextran sulfate) at pH below pI, or with polycation (e.g. chitosan) at pH above the pI.\textsuperscript{140-142} Although in this case the encapsulated particles do not consist purely of the active compound, the weight fraction of polymer incorporated in the insulin core was found to be rather low (~10%), therefore this type of particles is also included in the present review.\textsuperscript{140, 142} In the complexation method, precipitation yield and insulin content in the particles can be improved by performing the complexation in PEG solution, which lowers the solubility of the protein via the excluded volume effect.\textsuperscript{142} Insulin can be co-precipitated with another protein in the aggregate formation step, granting the possibility to add an additional function to the produced particles. For example, the co-encapsulation of insulin with small amounts of protease inhibitors has been described as a strategy to hamper its enzymatic hydrolysis upon release in the gut.\textsuperscript{141, 143}

Importantly, insulin’s solubility is low around pI and increases when shifting from this point. Moreover, the protein-polyelectrolyte interactions are distorted as a consequence of changes of their ionization degrees triggered by pH alteration. Therefore, compared to weak polyelectrolytes (\textit{i.e.} chitosan or poly(α,β-L-malic acid)),\textsuperscript{139, 142} using strong polyions as first coating layer/complexing agent (\textit{e.g.} dextran sulfate, PDDA)\textsuperscript{140, 141, 143} increases the stability and reduces the pH-dependent release of insulin from the solid cores. This principle is especially relevant for achieving core stability at acidic pH and avoiding premature protein release in the stomach after oral administration.

Although insulin has been the most explored protein for subsequent LbL encapsulation, the concepts developed can be extrapolated to other proteins (\textit{i.e.} aggregates of chymotrypsin were employed as model for multilayer encapsulation).\textsuperscript{144, 145}
2.3. Coating materials and LbL methodology

Among the numerous charged materials available for LbL assembly, polyelectrolytes have been the most commonly used. In this section, coating materials employed for LbL assembly on drug solid cores are reviewed.

2.3.1 Polyelectrolytes

Employed polycation-polyanion combinations (including synthetic polyelectrolytes (non- and biodegradable) and naturally derived ones, such as polysaccharides) for LbL encapsulation of drug cores are summarized in Table 2.3. The primary function of polyelectrolyte coatings on drug cores is to control their dissolution and/or to confer them colloidal stability as well as preventing the degradation of the encapsulated active compound. Those aspects are discussed in more details in Sections 2.4 and 2.5.

Table 2.3. Combinations of coating materials used for electrostatic LbL assembly on drug micro and nanocores.

<table>
<thead>
<tr>
<th>Polycation</th>
<th>Polyanion</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAH (^{a})\</td>
<td>PSS (^{a})\</td>
<td>63, 64, 95, 100, 118, 120, 128, 130, 133-135, 144, 145, 64, 99, 101, 105, 106, 137,</td>
</tr>
<tr>
<td>PDDA (^{a})\</td>
<td>PSS (^{a})\</td>
<td>146</td>
</tr>
<tr>
<td>Gelatin (^{b),c})\</td>
<td>PSS (^{a})\</td>
<td>99, 106, 117, 125, 146</td>
</tr>
<tr>
<td></td>
<td>Dextran sulfate (^{b),c})\</td>
<td>111</td>
</tr>
<tr>
<td></td>
<td>Alginate (^{b),c})\</td>
<td>108, 111, 136</td>
</tr>
<tr>
<td></td>
<td>Dextran sulfate (^{b),c})\</td>
<td>98, 117, 132, 142, 143</td>
</tr>
<tr>
<td></td>
<td>Alginate (^{b),c})\</td>
<td>63, 102-104, 108, 116, 118, 136</td>
</tr>
<tr>
<td>Chitosan (^{b),c})\</td>
<td>Heparin (^{b),c})\</td>
<td>142</td>
</tr>
<tr>
<td></td>
<td>Chondroitin sulfate (^{b),c})\</td>
<td>135</td>
</tr>
<tr>
<td></td>
<td>Polyacrylic acid (^{d})\</td>
<td>111</td>
</tr>
<tr>
<td></td>
<td>Citrus pectin (^{b),c})\</td>
<td>120</td>
</tr>
<tr>
<td>PLL (^{a),c})\</td>
<td>Alginate (^{b},c})\</td>
<td>100</td>
</tr>
<tr>
<td>PLL-b-PEG (^{a})\</td>
<td>Heparin (^{b},c})\</td>
<td>62, 129</td>
</tr>
<tr>
<td>Poly(L-arginine) (^{a})\</td>
<td>PSS (^{a})\</td>
<td>123</td>
</tr>
<tr>
<td>BSA (^{b),c})\</td>
<td>Protamine sulfate (^{b),c})\</td>
<td>63, 129</td>
</tr>
<tr>
<td>Protamine (^{b),c})\</td>
<td>Dextran sulfate (^{b),c})\</td>
<td>140</td>
</tr>
</tbody>
</table>

\(^{a})\) synthetic; \(^{b})\) naturally derived; \(^{c})\) biodegradable
Chapter 2: LbL coating of solid drug cores

2.3.2 Other coating materials

The use of systems/compounds such as nanoparticles, lipids or proteins as shell components yielding functional coatings has been under broad investigation in LbL-based systems.$^{85, 147}$ Herein, the advances in preparation of functional coatings on solid drug cores will be discussed.

An interesting approach was applied for covering ibuprofen microcrystals with charged compounds that abound in the body: human serum albumin and l-α-dimyristoylphosphatidic acid, a constituent lipid of biological membranes. Coating the drug core with such lipid/protein complexes would enable the incorporation of membrane-specific components (e.g. receptor channels) for molecular recognition or camouflaging the drug particles with complexes of naturally long-circulating vehicles (e.g. blood cells) to evade the immune system.$^{14, 98}$

Additional beneficial properties of the coating may be gained by including nanoparticles in the multilayer shell.$^{112, 148}$ For example, inclusion of paramagnetic particles could potentially enable magnetic-driven delivery of the drug cores to the target disease area. For example, preliminary experiments demonstrated that applying a magnet to a vessel containing dipyridamole particles coated with shell incorporating superparamagnetic iron (II, III) oxide nanoparticles resulted in their clustering, which suggest their potential for magnetic guidance in vivo.$^{112}$

In addition, drugs can be embedded in the coating in order to enhance the efficacy or reduce the side effects of the core drug. For example, negatively charged lapatinib, an epidermal growth factor inhibitor which can reverse multidrug resistance (by inhibiting the function of P-gp pump) was delivered within the polyelectrolyte multilayer encapsulating PTX NCs and this concept was further successfully validated in vitro (see section 2.6.1). (Figure 2.2a).$^{119}$ A similar design was applied to hydrocortisone crystals encapsulated within multilayers containing insulin, which could potentially alleviate the increased glucose levels induced by this steroid drug, however, the lack of experiments in an animal model keeps this study genuinely conceptual.$^{109}$ Moreover, shells exhibiting exceptional stability can be obtained by assembling Fe$^{3+}$ ions and polysaccharides (e.g. dextran sulfate) that interact via both electrostatic and chemical complexation mechanisms. These multilayers remain intact over wide pH range, rendering them especially useful for encapsulation of orally administered compounds. The performance of this coating on solid drug core has been demonstrated in an animal model (see Section 2.6.2).$^{110, 138}$

Overall, limited attention has been so far devoted to advanced functional coatings on drug cores and the majority of the studies involve only the physico-chemical characterization
of the system. Thus, in many cases the advantage of applying those multilayer coatings on drug cores compared to other LbL-based vehicles remains unknown requiring additional studies in biologically relevant environments.

Figure 2.2. Graphical representation of systems containing alternative materials as LbL coatings. a) Lapatinib co-delivered in the shell encapsulating PTX nanoparticles. b) Viability of OVCAR-3 cells upon exposure to free PTX, LbL-encapsulated PTX nanoparticles without and with lapatinib in the shell (LbL-PTX and LbL-PTX/lapatinib, respectively). (P<0.05 *, P<0.01**, P<0.001***). Reprinted with permission from Vergara et al. \(^3\) Copyright (2011) Elsevier c) TiO\(_2\) nanoparticles used as coating to protect nifedipine against photodegradation. Percentage of nifedipine remaining intact after exposure of the drug cores encapsulated with shells containing one and three TiO\(_2\) layers to a light dose of 60 000 lux for 500 h and its shelf life (time required for the drug to decompose to 90% of its initial content) after exposure to the same light dose vs. crude non-encapsulated drug.\(^{14\text{a}}\)
2.3.3 Last layer modification

The relevance of the last coating layer in governing the interactions with the tissue/cell of interest with LbL systems has been under broad investigation. The following section is dedicated to the use of specific coating materials for the last coating layer in relation to LbL-coated solid drug cores.

- Introducing stealth properties

LbL-coated, slowly dissolving nanoparticles can be opsonized and rapidly cleared from the bloodstream by the MPS after their \( i.v. \) administration. In general, opsonisation can be reduced by functionalizing the surface of the drug nanoparticle with hydrophilic polymers such as PEG. PEGylation of LbL-coated drug nanoparticles has been achieved after termination of LbL assembly by covalent attachment to specific reactive groups of the polymer of the last coating layer (\( e.g. \) via amine or carboxylic groups). Alternatively, high density of PEG can be achieved directly via electrostatic interactions using copolymers with polyelectrolytes. For example, our group introduced a PEG layer on the surface of LbL encapsulated PTX NCs by employing a copolymer composed of PEG grafted to PLL (PLL-g-PEG). Unfortunately, this polymer was found to desorb from the surface of the particles in serum, resulting in negative outcomes of the \( in vivo \) study in mice (see section 6.2). Our study implied that the electrostatic interactions were not sufficient to retain this PEGylated layer in the presence of serum proteins. Therefore, the stability of the PEGylated layers on the drug nanoparticles and their consequent ability to reduce opsonisation should be thoroughly evaluated \( in vitro \). The use of naturally derived, high molecular weight polysaccharides (\( i.e. \) dextran sulfate, hyaluronic acid or alginate) may be an alternative for preventing opsonisation as these polyelectrolytes have been shown to confer some stealth properties and prolong the \( in vivo \) circulation times of other LbL-coated quantum dots and polymeric nanoparticles.

- Improving adhesiveness

As stated before, drug nanoparticles are characterized by increased adhesiveness due to their large surface area. This is especially relevant for particles for oral and pulmonary administration, as mucoadhesion can promote the drug’s bioavailability. This effect can be further enhanced using specific mucoadhesive polymers such as chitosan as the last coating layers.
2.3.4 Coating techniques

In LbL assembly, the coating is performed by sequential exposure of the drug particle to oppositely charged coating entities (Figure 2.3a). To avoid drug loss during coating, the deposition of the multilayers should be performed in the conditions at which the active compound presents the lowest solubility and highest stability. For example, camptothecin crystals must be coated at low pH to minimize the hydrolysis that otherwise occurs at neutral or slightly alkaline conditions.62, 129

The conventional method for multilayer assembly involves incubating the suspension with a solution containing a large excess of oppositely charged polyelectrolyte, after which the excess of non-adsorbed polyion needs to be removed by centrifugation-redispersion cycles. However, this procedure requires considerable hands-on time and is especially impractical when working with very small colloids that are difficult to pelletize.154, 155 In an alternative method called “washless” or “titration” protocol, which was first studied in details by Bantchev et al. for TiO2 colloidal sub/microcores, the coating compound is added in the final amount that will be fully adsorbed on the colloidal template. This eliminates the intermediate centrifugation steps required to remove the excess of polyelectrolyte before deposition of the next layer and thus minimizes the risk of particle loss.154 The minimum amount of polyelectrolyte needed for reversing the surface charge of a particle is determined from the plateau onset of zeta potential which is measured after addition of each fraction. Once determined, the amounts of polyelectrolytes needed to achieve charge reversal can be added at once. This method, however, requires careful titration for each polyelectrolyte pair at each deposition condition (e.g. ionic strength, pH). On the other hand the centrifugation-dispersion requires less optimization and allows the screening of a large number of polymer pairs in a relatively short period of time. Moreover, titration entails the risk of particle aggregation when the surface charge is low or almost completely screened and electrostatic stabilization is absent. This can be minimized by applying sonication during the coating process95 and/or employing block copolymers of poly(L-lysine) and PEG (PLL-b-PEG), which in the absence of electrostatic repulsions reduce particle aggregation at low surface charge due to the steric stabilization by PEG chains.62, 129

LbL coating of colloid templates can be also performed in other processes, for example using fluidized bed, microfluidics devices or electrophoresis155-158 and it is envisaged that those can also be applied for encapsulation of solid drug cores.
Figure 2.3. Graphical representation of LbL assembly and release process. a) General protocol of electrostatic LbL assembly on drug cores based on sequential exposure to oppositely charged coating species. The polyelectrolyte can be added in excess and the unbound polymer removed by centrifugation, or added in the amount that is fully adsorbed on the particle (determined by titration). b) Release mechanism from LbL encapsulated drug particles based on diffusion of solvent molecules through the multilayers to dissolve the core followed by diffusion of free drug through the shell. c) Cumulative release of free drug from bare dexamethasone nanoparticles and those encapsulated with 4.5 and 8.5 bilayers of PDDA/PSS. Reprinted with permission from Zhou et al.\textsuperscript{133} Copyright (2014) American Chemical Society.

2.4. LbL to enhance stability

Important benefits that may be gained by applying LbL coatings include the increase of chemical stability of the encapsulated active compound as well as the enhancement of colloidal stability of the whole suspension.

2.4.1. Chemical stability

The presence of a multilayer shell can help preserve the stability of the core drug, for instance by providing protection against light-triggered degradation.\textsuperscript{108, 148} Accordingly, microcrystals of the light-sensitive drug nifedipine were coated with negatively charged titanium dioxide nanoparticles (in combination with PDDA), which acted as a filter against light and conferred photoprotective properties (Figure 2.2c).\textsuperscript{148} Moreover, LbL helps to keep the drug in the solid form, which makes it less prone to chemical or enzymatic degradation. For instance, while in neutral and slightly alkaline aqueous solutions the active lactone form of camptothecin is quickly hydrolyzed, LbL encapsulation of solid drug particles prevented its degradation.\textsuperscript{62, 129}
LbL entrapment of insulin aggregates has also been applied to hamper the inactivation of the protein in gastric environment after oral administration.\textsuperscript{142, 143}

### 2.4.2. Colloidal stability

LbL coating can also prevent particle growth in suspensions through electrostatic stabilization and by providing a barrier for dissolution, which counteract aggregation and Ostwald ripening, respectively.\textsuperscript{105, 129, 135} For example, while LbL encapsulated nanoparticles of PTX, isoxyl, furosemide and nifedipine remained stable for over 120 days, their non-encapsulated versions increased in size up to several micrometers.\textsuperscript{135} Moreover, introducing PEG as the outermost layer or within inner multilayer shells (e.g. PLL-\textsuperscript{b}-PEG) improves the colloidal stability via steric repulsion, which, in contrast to electrostatic stabilization is much less sensitive to ionic strength. This property is important to achieve colloidal stability in physiologically relevant media.\textsuperscript{62, 122, 129}

### 2.5. LbL to control drug release

The ultimate purpose of LbL assembly of polyelectrolytes on solid drug particles is to modulate the release of the active compound under conditions that are dictated by the administration route. For the oral route, the goal is mainly to create a shell that will preserve the integrity, ensure the colloidal stability and substantially reduce the dissolution rate of the drug particle at gastric pH.\textsuperscript{106, 159} These issues are relevant for particles of compounds that are inactivated at low pH and in presence of enzymes (e.g. insulin).\textsuperscript{142, 143} They are also pertinent to those which might pose the risk of gastric irritation as a result of exposure to high drug concentration (e.g. many non-steroidal anti-inflammatory drugs (NSAIDs)).\textsuperscript{78, 103, 106} Importantly, efficient control over the dissolution of particles with LbL coatings in intestinal conditions (and consequently over systemic absorption of the drug) is highly desirable as it enables the drug concentration to be kept at the therapeutic level for prolonged periods of time. Similarly, LbL coatings may also control the dissolution of particles and the absorption of drugs administered via other routes (e.g. subcutaneous, pulmonary), as well as introduce sustained release of locally acting drugs (e.g. for ocular or topical delivery).

Long circulation time of nanoparticles is a prerequisite for passive accumulation in the tumor tissue via the enhanced permeability and retention (EPR) effect.\textsuperscript{6} LbL-coatings can be potentially applied to reduce the dissolution rate and thereby extend the circulation time of anticancer drug nanoparticles (e.g. PTX, tamoxifen or camptothecin) in the bloodstream after intravenous administration.\textsuperscript{53, 62, 129, 130, 132, 134} However, since slowly dissolving nanoparticles
can be opsonized and rapidly cleared from the bloodstream by the MPS, PEGylation needs to be considered as discussed in section 2.3.3.

2.5.1. **Mechanism of release of LbL encapsulated drug cores**

The dissolution of LbL encapsulated drug particles involves firstly the diffusion of the solvent molecules through the shell, followed by the diffusion of the dissolved compound to the outside through the multilayer (Figure 2.3b). Consequently, the shell permeability determines the overall release rate of the encapsulated drug particle. Two mechanisms of permeation occurring simultaneously at the shell should be considered: permeation through water filled pores formed within the multilayers, and permeation through the bulk polyelectrolyte multilayer shell. Thus, the shell’s permeability is determined by the size and concentration of its pores and also by its thickness. It is noteworthy that the drug core dissolution may also lead to a large concentration gradient across the multilayer, resulting in increased osmotic pressure inside the capsule. This may result in polyelectrolytes’ rearrangement and formation of larger pores within the multilayer, therefore accelerating the diffusion of the drug through the shell at the initial stages of dissolution.89, 160

The shell thickness as well as the pores’ size and concentration may be altered for example by changing the number and type of adsorbed layers and the deposition environment (denoted as the “Inherent permeability of the shells”) or by altering the medium after the layering is terminated (denoted as “Induced permeability of the shells”). These factors are universal for all LbL-based planar and colloidal systems and they will be discussed here in the context of encapsulated drug particles.

2.5.2. **Inherent permeability of the shells**

- Number of coating layers

The most straightforward approach to decrease the permeability of the shell is to increase the number of deposited layers. The lower permeability of thicker shells is related to the longer diffusional pathway that the dissolved drug has to overcome, and to the successive closure of the multilayers’ pores by further layer deposition.160 To quantify the permeability of the shells with increasing number of layers (PAH/PSS) to low molecular weight compounds Antipov and co-workers used fluorescein microcrystals as a model. Permeability values were calculated using the linear range of the release curve where the release rate was constant and were subsequently converted to diffusion coefficients by multiplying the permeability by the shell wall thickness. With each added polyelectrolyte coating, the diffusion coefficient values
decreased prominently for layers one to eight (inner layers), while from the eighth layer onwards (outer layers) the calculated diffusion coefficient remained constant. Thus, it was concluded that while the successive closing of the pores may provide additional barrier to diffusion for the inner layers, the decrease in permeability for the outer layers mostly emerges from the longer diffusional pathway.\textsuperscript{77, 160}

Simply increasing the number of layers prolonged the dissolution of many active compound nano\textsuperscript{63, 64, 117, 132, 135, 136, 139} and microparticles\textsuperscript{78, 98, 99, 111, 142, 161}. For example, whereas the bare dexamethasone particles dissolved completely after 2 h, coating them with 4.5 and 8.5 PDDA/PSS bilayers (9 and 17 single polyelectrolyte layers) resulted in only 65 and 30\% release after 8 h, respectively (Figure 2.3c).\textsuperscript{133}

- **Deposition conditions**

In this section, the factors influencing the structure of the polyelectrolytes during their deposition and subsequently the permeability of the resulting shell will be discussed.

**Ionic strength and pH** In general, the conformational structure adopted by polyelectrolytes depends on their ionization state, which can in turn be modified by changing the pH (for weak polyelectrolytes) or by increasing the ionic strength to compensate the charges. Polyelectrolytes at lower ionization states adopt more globular rather than extended conformations due to lesser interchain repulsions, and since the lateral electrostatic repulsions between the polyelectrolytes of the same charge are weaker the resulting shells are generally thicker. For such films, the thickness increases with the number of deposited layers in an exponential manner as a result of interpenetration of polyelectrolytes in the interior of the multilayer.\textsuperscript{88, 162-164} Given that thicker layers are less permeable, the half-time of release of artemisinin encapsulated within chitosan/alginate multilayers was prolonged by 10-fold when the shell was deposited at 0.5 M NaCl instead of in the absence of salts.\textsuperscript{136} However, it was also demonstrated that shells composed purely of strong polyelectrolytes are more permeable when deposited at higher ionic strengths, due to their “spongy” structure containing more water-filled pores that facilitates the diffusion.\textsuperscript{162, 165} For instance, faster drug release from dexamethasone nanoparticles was achieved when PDDA/PSS deposition was carried out at higher ionic strength compared to pure water.\textsuperscript{133} Importantly, when the salt concentration in the deposition medium exceeds a certain threshold, the interactions between the polymer and the particle surface are prevented by the compensation of all their electrostatic charges and the resulting absence of electrostatic repulsion may ultimately lead to the suspension’s aggregation.
Temperature and organic solvents  The temperature at which the multilayers are deposited and the presence of organic solvent in the coating medium may also influence the permeability of the shell. Thicker, exponentially growing polyelectrolyte films were successfully built at elevated temperatures in the presence of ethanol in the coating medium. The mechanism behind this phenomenon was proposed to be similar to the influence of ionic strength, namely emerging from more globular conformation of polyelectrolytes under these conditions, resulting in the formation of interpenetrated multilayers. Accordingly, it was found that the half-time of release of drugs encapsulated with chitosan/alginate layers was increased by two to six-fold when the multilayers were deposited at elevated temperatures and in presence of ethanol in the coating solution.

2.5.3. Induced permeability of the shells

As stated above, the permeability of the polyelectrolyte multilayer shell may be also induced by changing the conditions of the surrounding medium once the built-up is completed. This property enables the design of responsive LbL systems, for which the drug release may be modulated at conditions prevailing in specific parts of the body or upon applying external stimuli. Much attention has been devoted to fabrication of multilayer shells responsive to a variety of factors, such as pH, ionic strength, temperature, glucose, redox potential, ultrasound or light, however limited number of those approaches has been applied to solid drug cores.

- pH

The interpolyelectrolyte complexes in multilayers are formed in a charge stoichiometric ratio. If this stoichiometry is disturbed by any shift from the adsorption conditions, such as ionic strength or pH, the compensation or occurrence of surplus charges may lead to structural changes of the multilayer. Therefore, the permeability of shells that are composed of at least one weak polyelectrolyte (e.g. PAH/PSS, chitosan/dextran sulfate, gelatin/PSS, etc.), should be responsive to pH. In general, the accumulation of charges of the same sign leads to increased interpolyelectrolyte repulsions, resulting in swelling and to the formation of more free volume cavities, which in turn increase the shell’s permeability. This process can be reversed by altering the pH to decrease the repulsion between polyelectrolytes resulting in shrinking of the shell, closing the pores and lowering the permeability. However, this holds within a certain pH range as the large charge imbalance between polyelectrolytes may also lead to shell disassembly.

The pH-dependent release of several orally administered drugs (e.g. ibuprofen, furosemide, ketoprofen) and insulin from particles encapsulated with pH-sensitive shells was investigated. However, it has to be pointed out that in those cases the differences in the release
rates did not emerge from the pH-controlled permeability of the shells but from the intrinsic pH-dependent solubility of the core compounds. In the case of insulin, its release could be halted at pH below pI when a strong polyelectrolyte (*e.g.* dextran sulfate) was complexed with the protein in the core or adsorbed as the first coating layer or reduced in the case of weak polyelectrolyte (*e.g.* chitosan) (see Section 2.2), *(Figure 2.4a,b).*

- Enzymes

In the studies mentioned so far, release experiments were carried out in buffers simulating gastric and intestinal conditions in the absence of enzymes. It is noteworthy that some biocompatible polyelectrolytes undergo enzymatic degradation in the body, which leads to layer erosion and loss of control over the release. Wang *et al.* showed that chitosan/alginate acid coatings erode from the surface of indomethacin microcrystals as a result of enzymatic degradation of chitosan, leading to accelerated release of the drug. The study revealed that the resistance of the films to enzymes at low pH could be enhanced by increasing the number of deposited chitosan/alginate acid films, raising the deposition temperature and by crosslinking of the multilayers *(Figure 2.4c).* 103, 104

- Temperature

Thermally-induced drug release could be an attractive strategy for obtaining localized response. It was envisaged that the rearrangement of multilayers at elevated temperatures leads to the formation of higher concentrations of free volume cavities which enables faster diffusion through the shell. Indeed, the influence of temperature on the drug release rate from dexamethasone particles encapsulated with PDDA/PSS multilayers was studied, however, for this system, the acceleration in release rate after increasing the temperature from 20 ºC to 60 ºC was found to be minor than expected. 133

Overall, the number of studies concerning the stimuli-responsive systems is not extensive; however this approach is especially worth investigating in combination with solid drug cores, as the accelerated release of a high drug payload from the particle specifically at diseased site may substantially enhance the therapeutic outcome.
Figure 2.4. Graphical representation of pH-dependent release of insulin from the LbL-coated salted-out aggregates: a) coated with chitosan/heparin sulfate (chitosan as the first coating layer). Adapted from Song et al.\textsuperscript{142} with permission. Copyright (2014) Dovepress; b) coated with dextran sulfate/protamine (dextran sulfate as the first coating layer). Adapted from Balabushevich et al.\textsuperscript{140} with permission. Copyright (2004) Springer. c) Graphical representation of drug release triggered by enzymatic degradation of the shell, which can be prevented by shell crosslinking, elevating the temperature of deposition and increasing the number of layers. Graph redrawn from Wang et al.\textsuperscript{103} with permission. Copyright (2007) American Chemical Society.
2.6. **In vitro and in vivo studies**

2.6.1. **In vitro cell experiments**

*In vitro* cellular assays are important tools to preliminarily estimate the efficacy and identify the safety profile of newly developed drug delivery systems. Cytotoxicity studies are often performed to assess the efficacy of LbL-encapsulated anticancer drugs and the safety of the coating materials employed. In many cases, the multilayered hollow capsules do not influence the viability of the cells \(^{168-171}\) and the moderate cytotoxicity triggered by certain systems is usually ascribed to the positive charge of the capsules or to their sedimentation onto the cells. \(^{172, 173}\)

Accordingly, LbL-encapsulated solid nanoparticles of non-cytotoxic drugs did not trigger toxicity in a broad range of concentrations and regardless of the thickness of the shell.\(^{95, 120}\) Moreover, in systems where the solid core constituted cytotoxic compounds the shells did not illicit additional cytotoxic response beyond the expected for the drug itself \(^{122, 130, 132}\) excepting when positively charged particles were used.\(^{116}\) For example, we recently observed comparable toxicity on HT-29 (human adenocarcinoma tumor) cells for PEGylated PTX NCs encapsulated with PSS/poly-(L-arginine) multilayers, PEGylated PTX NCs(without the multilayered shell) and Abraxane\(^{	ext{®}}\) (commercially available nanoparticulate formulation of PTX).\(^{122, 123}\)

It should be pointed out however, that although the intact polyelectrolyte complexes may not induce cytotoxicity in *in vitro* assays, the response could be triggered later on by their degradation products and/or after their dissociation into free polymers. In particular, positively charged polyelectrolytes can induce cell death as a result of interactions with the negatively charged surface of the cell membrane.\(^{174, 175}\) Therefore, the safety of LbL-based materials would require further investigation. However, as in the case of the solid drug cores the overall polymer-to-drug mass ratio is low compared to drug-loaded matrices, the possibility to deliver large quantity of an active compound using minute amounts of polyelectrolytes would minimize the risk of polymer-triggered toxicity.

Moreover, although PEGylation of the outermost layer of encapsulated drug nanocores has been applied in a few cases as a strategy to reduce the nanoparticles’ opsonisation, none of the experiments published so far have considered the uptake of the particles by phagocytic cells (the main cell type involved in nanoparticles’ clearance). However, reduced uptake by those cells demonstrated for some PEGylated multilayer-coated polymeric systems could help to
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draw conclusions about the potential outcome of this approach applied on solid drug cores in vivo.\textsuperscript{152,176}

Efficient and cell-specific uptake can be achieved by functionalizing the surface of the particle with an active targeting moiety.\textsuperscript{6} Although few approaches have been described for the functionalization of multilayer capsules with high affinity ligands,\textsuperscript{177-179} only two publications have reported the preparation of targeted LbL-based drug suspensions with enhanced binding to the cell surface or improved cytotoxicity profile compared to the non-targeted versions in preliminary \textit{in vitro} experiments. However, further tests are required to fully validate the added value of the active targeting in these systems.\textsuperscript{64,132}

The concept of dual drug delivery with LbL colloidal systems was tested with PTX nanoparticles encapsulated with shells containing lapanitib (a drug preventing multidrug resistance) on resistant ovary adenocarcinoma OVCAR-3 cells. Exposure of the cells to the particles resulted in increased percentage of cell death compared to the treatment with free PTX and nanoparticles coated without lapanitib (\textbf{Figures 2.2a,b}). Dual drug delivery with LbL core-shell systems could have synergistic effects as it should enable both drugs to achieve the same pharmacokinetics profiles and temporal co-localization in the cell.\textsuperscript{119}

Moreover, the ability to protect the active compound from degradation and prevent loss of activity was evidenced by the treatment of glioblastoma cells with LbL-encapsulated camptothecin versus the free-drug. It was proposed that the prolonged cytotoxic effect of the former emerged from the slow release of non-degraded camptothecin over the course of the incubation time, in contrast to the quick hydrolysis of a large fraction of the free drug to its inactive form.\textsuperscript{129}

\textbf{2.6.2. \textit{In vivo} data}

\textit{In vivo} studies represent a decisive step towards the clinical translation of newly developed drugs and delivery systems. For drug delivery systems based on supramolecular assembly, it is of critical importance to ascertain the integrity of the interactions in \textit{in vivo} conditions before reaching the tissue of interest. Especially after systemic administration the electrostatically assembled coatings might be destabilized in the presence of proteins or salts. Although some researchers have described the stability and performance of colloidal LbL-based systems \textit{in vivo} (\textit{e.g.} coated liposomes, quantum dots, polymeric nanoparticles),\textsuperscript{149,150,172,180} to the best of our knowledge there is only two reports of animal studies concerning multilayer-encapsulated hydrophobic drug cores. This strategy was applied to promote the bioavailability and prolong the half-life of 10-hydroxycamptothecin after oral administration. The pharmacokinetic studies
performed in mice revealed that after administration of microcrystals encapsulated with ten stable Fe\(^{3+}\)/dextran sulfate bilayers, the half-life and the area under the plasma concentration-time curve increased around 3- and 2-fold respectively, in comparison to the free drug.\(^{110}\)

In a recent investigation from our group, the pharmacokinetics and biodistribution of PEGylated PTX NCs after intravenous administration were studied in tumor xenograft-bearing mice. In spite of the expected long blood circulation times and tumor targeting via the EPR effect, a rapid clearance from the bloodstream and accumulation in liver and spleen were observed. These findings suggested a rapid uptake of the particles by the mononuclear phagocyte system, which could be a result of loss of stealth properties of the outermost layer (see 2.3.3).\(^{122,123}\)

In contrast, LbL-encapsulated insulin particles have been more extensively assessed in rodents. In general, those particles elicited a prolonged hypoglycemic effect after subcutaneous or intrapulmonary administration compared to non-encapsulated ones and to insulin solutions, demonstrating the efficacy of the multilayers in providing sustained release of insulin \textit{in vivo}.\(^{137,138}\) Moreover, insulin crystals that were administered intrapulmonary did not cause lung damage in rats after short term or chronic treatment.\(^{137}\) Higher hypoglycemic effect in diabetic mice was triggered after oral administration of LbL-encapsulated complexed insulin-chitosan particles compared to treatment with non-coated ones, which suggests that the presence of multilayers improved the stability of the protein in the gastric environment.\(^{142}\) Balabushevich \textit{et al.} demonstrated that LbL-encapsulated insulin-dextran sulfate complexes induced hypoglycemic effect after oral administration to rats in contrast to the control saline group. In both cases, the use of mucoadhesive chitosan as the outermost layer could have promoted the bioavailability and absorption of the protein. Moreover, although co-complexation of proteolysis inhibitors in the core was expected to enhance the hypoglycemic effect due to efficient protection from degradation of insulin upon mutual release of both proteins \textit{in vitro}, it resulted only in minor improvement at decreasing the blood glucose level \textit{in vivo}.\(^{141,143}\)


2.7. Discussion and conclusions

In this review, various aspects of LbL assembly on solid drug micro and nanoparticles were discussed, including their preparation, the tuning of their shell permeability, their pharmaceutical applications as well the advantages and remaining challenges of this method (Figure 2.5). The large variety of hydrophobic drugs that can be formulated as solid particles and coated with distinct classes of materials has been evidenced in many proof-of-concept studies. The coatings used varied, ranging from synthetic to naturally-derived polyelectrolytes, to colloids and other active compounds. These coatings enable tuning of the core dissolution rate in vitro, provide protection against the degradation of the encapsulated drug and, in theory, may govern the interactions of the systems with the tissue of interest. However, despite the versatility of the available LbL shells designs, still a limited number of studies focused on the functional and responsive multilayer shells on the solid drug cores. Moreover, the niche in which the LbL-encapsulated solid drug cores might have added value compared to previously developed drug delivery systems has not yet been clearly defined and in addition the performances of existing systems in biological environments have been rarely investigated in sufficient depth. Many of the studies published so far on LbL-coated microcores have focused on well-established orally administered compounds (i.e. NSAIDs or steroid drugs) for which the need for complex LbL systems can be questioned. In contrast, LbL-encapsulation of nanocores of cytotoxic drugs could, in theory, offer great benefits over existing formulations, in particular in terms of the development of long-circulating injectable nanoformulations for tumor targeted therapy based on the EPR effect. However, thorough biodistribution and pharmacokinetics studies are required to ascertain the utility of these designs in anticancer drug delivery. In addition to complementing the passive targeting, the functionalization of nanoparticle surfaces with active targeting moieties could potentially yield delivery systems with tumor specificity. This concept has however not been comprehensively explored in combination with LbL-encapsulated solid drug nanoparticles.

Moreover, there are many aspects regarding the behavior of LbL-coated drug cores in biological systems that remain unknown or poorly understood, which hampers the translation of these delivery systems into clinical research stages. The knowledge about their cellular uptake mechanism and intracellular fate is for instance very limited, and to date only studies on hollow multilayer capsules exist showing that following cellular internalization they accumulate in lysosomal compartments. Another crucial aspect lacking comprehensive studies is the integrity of the different polyelectrolyte coatings and their impact on drug release in vivo. Additional and equally important issues that need to be elucidated are the potential
toxicity and elimination routes of the employed polyelectrolytes, especially polycations and the known non-biodegradable ones. Thus, thorough analysis of the influence of the physicochemical properties and structure of LbL-coated drug cores on their biological performances is a pressing prerequisite for the rational design of these classes of particles and their future translation into the clinic.

**Figure 2.5.** Graphical representation of the advantages and the remaining challenges of the LbL-encapsulated solid drug cores.
Chapter 3

PEGylated layer-by-layer coated paclitaxel nanocrystals – preparation and characterization

A part of this chapter published:

Chapter 3: PEGylated LbL-coated PTX NCs – preparation and characterization

3.1. Introduction

As stated in Chapter 2 slow dissolution of i.v. injected drug NCs is one of the prerequisites for their local deposition at the tumor site via the EPR effect. Among the strategies aimed at reducing the dissolution rate of anticancer drug NCs, stabilizing coatings produced by the LbL assembly of polyelectrolytes are one of the few strategies that have demonstrated some success. Indeed, polyelectrolyte multilayers have been shown to slow down the dissolution of PTX, camptothecin and tamoxifen NCs. However, nanoparticles with charged surfaces are generally susceptible to opsonization and clearance by the MPS, leading to short biological half-lives and accumulation in the liver and spleen, which, again, is deleterious to tumor accumulation. Moreover, electrostatically stabilized suspensions are prone to aggregation in biologically-relevant aqueous media containing relatively high concentrations of salts. Thus, it is desirable to mask such charged coatings with flexible hydrophilic polymers such as PEG because of its ability to hinder the adsorption of plasma proteins and introduce steric stabilization, making nanoparticles less susceptible to ionic strength variations.

Fabrication of LbL-coated drug NCs is a multistep process, involving: i) preparation of electrostatically stabilized nanosized particles in a top-down or bottom-up process; ii) coating of the obtained core with a shell of oppositely charged polyelectrolytes; iii) PEGylation of the last coating layer. Among many techniques used for processing of the microsized drug into nanosized particles (Chapter 2), a top-down wet milling process was selected. This simple method, widely used in industry, does not require organic solvents, yields nanoparticles of a narrow size distribution with a low batch-to-batch variability, and can be easily adapted on a laboratory scale. The addition of a stabilizer compensates the high surface energy emerging from the large surface area formed during the size reduction, which generates a barrier to agglomeration. The selection of a proper stabilizer for production of drug particles via wet milling depends on many factors, such as interactions between specific functional groups of a drug and stabilizer as well as the molecular weight, hydrophobicity and the concentration of the stabilizing agent. Thus, although some general trends could be established (e.g. increased stabilizing efficiency with increasing hydrophobicity of the polymer), the choice of the stabilizer is usually drug-specific and is based on empirical trial-and-error studies. Although several amphiphilic compounds were reported to produce sterically stabilized PTX NCs (e.g. poloxamers, vitamin E tocopheryl-PEG or copolymers of PEG and poly(ε-caprolactone)), limited attention has been devoted to electrostatic stabilization of PTX suspensions by polyelectrolytes in a wet milling process. Thus, in this work, a series of
positively and negatively charged polymers was initially screened to obtain electrostatically stabilized PTX NCs.

In a second step, the NCs were coated in a LbL process with a shell of oppositely charged polyelectrolytes. Among available methods (described in Chapter 2), the technique relying on the incubation of the NCs with the solution containing an excess of oppositely charged polyelectrolytes, followed by the removal of unbound polyelectrolytes by centrifugation was chosen. This straightforward method allowed for screening a large number of polyelectrolyte pairs without tedious optimization steps required to determine the minimum amount of polymer to be fully adsorbed on the NC for each layer. Subsequently, the influence of different coatings on the drug release and the colloidal stability of the PTX NCs was monitored.

The third step included PEGylation of the last coating layer. PEG can be attached chemically to functional groups available in the topmost coating layer, for example via amine or carboxylic groups. Alternatively, a more straightforward approach includes the use of PEGylated polyelectrolytes that are deposited on the surface of the NC via electrostatic interactions. In this work, two constructs of PEGylated polyelectrolytes were tested: a block and a graft copolymer. Notably, the latter platform is advantageous because of the higher and tunable PEG grafting densities achieved, which can result in improved protein repellency and colloidal stability of the NCs.

3.2. Experimental

Materials PTX was obtained from Bolon Pharm (Taizhou, China). Docetaxel (> 99% purity) was bought from Apollo Scientific (Cheshire, UK). Abraxane® (Abraxis Biosciences, Celgene, NJ) was purchased from the pharmacy of the University Hospital Zürich, Switzerland. Poly(styrene sulfonate) (M_w 70 kDa, PSS_70), propyl methacrylate (PrMA) and tert-butyl methacrylate (tBMA) were purchased from ABCR-Chemicals (Karlsruhe, Germany). PSS_32 and PSS_17 (M_w 32 kDa and 16.8 kDa), dextran sulfate sodium salt from Leuconostoc species (M_w 9 – 21 kDa), poly(allylamine hydrochloride) (M_w 15 kDa), anhydrous copper (II) sulfate, mPEG_113-OH (M_n 5000 kDa), 2-bromoisobutyryl bromide and all the used solvents were purchased from Sigma-Aldrich (Buchs, Switzerland). PSS_70 (M_w 70 kDa) coupled to FITC (labeling degree 1:270, mol dye: mol monomer) was purchased from Surflay Nanotec GmbH, (Berlin, Germany). Hyaluronic acid sodium salt (M_w 29 kDa) was purchased from Lifecore Biomedicals (Chaska, MN). Polyamino acids: poly(L-arginine) (M_n 20.6 kDa, PLR), poly(L-
lysine) (Mₙ 17.3 and 42.9 kDa, PLL), poly(l-glutamic acid sodium salt) (Mₙ 15.9 and 30 kDa),
poly-(l-homoarginine hydrochloride) (Mₙ 20.4 and 53.3 kDa, PLHR) were purchased from
Alamanda Polymers (Huntsville, AL). mPEG succinimidyl propionate (mPEG-SPA) (Mₘ 5.17
kDa) was from Shanghai Biochemicals (Shanghai, China) and propargylacetamido-PEG
succinimidyl butanoate (alkyne-PEG-NHS) (Mₘ 4.9 kDa) from Iris Biotech GmbH
(Marktredwitz, Germany).

Sulfo-Cy5-azide and tris(3-hydroxypropyltriazolylmethyl)amine (THPTA) were bought from
Jena Bioscience (Jena, Germany). Sodium ascorbate was bought from Axon Lab AG (Baden-
Dättwil, Switzerland). Bovine serum albumin fraction V (BSA) was purchased from Applichem
(Darmstadt, Germany). Phosphate buffer saline (PBS), Dulbecco’s Modified Eagle Medium
(DMEM) with GlutaMAX™ (high glucose), fetal bovine serum (FBS), penicillin/streptomycin
stock solution (10,000 units mL⁻¹ penicillin and 10,000 µg mL⁻¹ streptomycin) and
trypsin/EDTA 0.25% for cell culture were obtained from Life Technologies (Carlsbad, CA).

Equipment The hydrodynamic diameter and zeta potential of NCs were determined by dynamic
light scattering (DLS) and laser Doppler anemometry, respectively, using a DelsaNano C
particle size analyzer (Beckman Coulter, Brea, CA). The cumulant method was used to report
the measured size of the NCs. PTX concentration was determined by high-performance liquid
chromatography (HPLC) using a system comprised of an autosampler and pump system
(Ultimate 3000, Dionex, Thermo Fisher Scientific, Reinach, Switzerland) equipped with a
diode array detector. Analyte separation was achieved using an Accucore C₁₈ column (100 ×
2.1 mm, 2.6-µm) maintained at 30 °C using a gradient elution method (40% acetonitrile in
water, rising to 70% in 15 min). PTX was detected at 230 nm. The HPLC instruments and
columns were purchased from Thermo Fischer Scientific (Reinach, Switzerland). Proton
nuclear magnetic resonance (¹H NMR) spectra were recorded on Bruker Av400 spectrometer
(Bruker BioSpin, Fällanden, Switzerland) operating at 400 MHz for protons. FTIR spectra were
recorded using an attenuated total reflectance geometry on a Spectrum 65 infrared
spectrophotometer (Perkin-Elmer, Schwerzenbach, Switzerland). Electron microscopy images
of NCs were obtained using a Phillips CM12 (FEI, Hillsboro, OR) TEM equipped with tungsten
cathode operating at 100 kV and a Gatan CCD 794 camera (Gatan Inc., Pleasanton, CA). For
imaging, 4 µL of suspension was adsorbed on a glow-discharged carbon-coated copper grid
and after two washings with water, NCs were negatively stained with 2% (w/v) uranyl acetate
for 1 min and air-dried after blotting with filter paper. For fluorescence and absorbance
measurements, a TECAN Infinite M200 microplate reader (TECAN, Männedorf, Switzerland)
was used. Fluorescent images were obtained using a Leica DMI6000B epifluorescent microscope (Leica Microsystems, Heerbrug, Switzerland).

**Synthesis of methoxy-PEG-b-poly(propyl methacrylate-co-methacrylic acid) (mPEG-b-P(PrMA-co-MAA)).** The PEGylated copolymer was synthesized by atom transfer radical polymerization (ATRP) according to the detailed protocol described elsewhere. Briefly, to obtain a macroinitiator, mPEG$_{115}$-OH was acylated using 2-bromoisobutyryl bromide (4 eq.). The polymerization was carried out using PrMA (25 eq.) and $t$BMA (50 eq.) The final polymer was afforded by deprotection of tert-butyl groups in $t$BMA in 95% trifluoroacetic acid in dimethyl sulfoxide for followed by dialysis for 48 h against water and lyophilization. The final composition was calculated from the $^1$H NMR spectrum in D$_2$O (Appendix, Figure S1) considering the CH$_2$ of PrMA at 4.0 ppm and the signal at 0.7 – 2.5 ppm, using the following formulas (Equations 3 and 4).

\[
PrMA = \frac{A_{4.0\text{ ppm}}}{2}
\]  
Equation 3

\[
MAA = \frac{A_{(0.7-2.5\text{ ppm})}-10 \cdot PrMA-6}{5}
\]  
Equation 4

The final polymer contained 24 units of PrMA and 55 units of MAA.

**Synthesis of PLL-g-mPEG** Two different PLL-g-mPEG were synthesized as described in the literature from PLL (M$_n$ 17.3 kDa, approximately 100 lysine repeating units,) and mPEG-SPA (M$_w$ 5.17 kDa). Typically, 20 mg of PLL (corresponding to approximately 116 µmol of lysine monomers) were dissolved in 1 mL of sodium tetraborate buffer (50 mM, pH 8.5, in D$_2$O) in a 2-mL Eppendorf tube. Subsequently, 188 or 324 mg (~36 or 64 µmol, respectively) mPEG-SPA were added and the pH was adjusted to 8.5 with 1 M NaOH. The reaction was monitored by means of $^1$H NMR spectroscopy. The average grafting was calculated via the ratio of “free” lysine (3.0 ppm) and “grafted” lysine (3.2 ppm) using the following formula (Equation 5):

\[
\% \text{Grafting} = \left( \frac{A_{3.2\text{ ppm}}}{A_{3.2\text{ ppm}} + A_{3.0\text{ ppm}}} \right) \cdot 100\%
\]  
Equation 5

After 3 h of stirring, the reaction mixture was diluted to 5 mL with ultra-pure water, transferred to a Float-A-Lyzer G2 dialysis device (5 mL, MWCO 8–10 kDa, Sigma Aldrich,), dialyzed against water for at least 24 h, and lyophilized. Two copolymers with 31% and 61% of lysine residues functionalized with mPEG were obtained (for annotated spectrum see $^1$H NMR, Appendix, Figure S2).
Alkyne groups were introduced into PLL-\(g\)-mPEG using a mixture of alkyne-PEG-NHS and mPEG-SPA in the grafting reaction on PLL. Typically, 20 mg of PLL (corresponding to approximately 116 µmol of lysine monomers) were dissolved in 1 mL of sodium tetraborate buffer (50 mM, pH 8.5, in \(D_2O\)) in a 2-mL Eppendorf tube. Subsequently, 61 mg (~12.5 µmol) of alkyne-PEG-NHS were added followed by 246 mg (~47.5 µmol) of mPEG-SPA. After addition of each component, the pH was adjusted to 8.5 with 1 M NaOH as needed and stirred for 3 h. The product was purified by dialysis in the same way as for PLL-\(g\)-mPEGs and lyophilized. The grafting percentage of the lysine groups was monitored by \(^1\)H NMR spectroscopy after each step. In the final copolymer 7% of lysine side chains were functionalized with alkyne-PEG and 47% with mPEG. Synthesis scheme and annotated \(^1\)H NMR spectra can be found in Appendix, Figure S3.

**Synthesis of fluorescent PLL-g-PEG** In a second step, azido-sulfo-Cy5 was coupled to the alkyne bearing PLL-g-mPEG from above by copper-catalyzed click chemistry. Briefly, 16 mg of copolymer (~0.42 µmol of alkyne groups) were dissolved in 1061 µL of 100 mM phosphate buffer (pH 7.4) followed by addition of a solution of sulfo-Cy5-azide (0.091 mg, 0.114 µM, ~0.27 eq. vs. alkyne groups). Then, 17 µL of a mixture of CuSO\(_4\) (5.7 µL of 20 mM solution) and THPTA (11.3 µL 50 mM solution) was added. Thereafter, 57 µL of a 100 mM solution of sodium ascorbate in water were added, the vial sealed, and placed on an orbital agitator (~500 rpm) for 1 h. Control reactions containing all reagents except the copolymer, or only dye and copolymer without reagents, were run simultaneously. The reaction mixtures were passed through Pierce™ Dye Removal Columns (Thermo Fischer Scientific). The recovered supernatant was blue for the reaction mixture containing all reagents, but colorless for both controls (Appendix, Figure S4). The fluorescent polymer product was further purified by diafiltration (3 × 20 min, 4000 × g) using Amicon-4 filters (MWCO 10 kDa), and dialyzed for 24 h against water with a Float-A-Lyzer (1 mL, MWCO 100 kDa, Sigma Aldrich) in darkness. After that, the polymer was freeze-dried and stored at -20 ºC. The fluorescence emission spectrum of this polymer is shown in Figure 3.9f. This fluorescent polymer is further denoted as PLL-g-PEG-sulfo-Cy5.

**Preparation of PTX NCs** PTX NCs were prepared by wet milling. Briefly, 2 mL of 0.5 wt.% stabilizer solution in ultra-pure water were placed into 20 mL cylindrical vials containing 10 – 40 mg of PTX and 4 mL of yttrium-stabilized zirconium oxide milling beads (~14.7 g, 0.3 mm diameter, Union Process, Akron, OH). The vials were continuously rolled at 6 ºC on a blood roller at 220 rpm for 24 – 48 h. The resulting suspension was separated from the beads by filtration through polyamide sieve fabric (30-µm pores, VWR, Dietikon, Switzerland) and the
beads were washed 4 times with 2 mL of ultrapure water. The resulting suspension was centrifuged at 12,000 × g for 6 min to remove larger aggregates and then purified from the excess of non-adsorbed polymer by 3 cycles of centrifugation at 20,000 × g for 20 min and pellet re-suspension in 1 mL of ultra-pure water. After the third centrifugation step, the pellet was dispersed with a small amount of water (0.5 - 2 mL) and the concentrated dispersion was stored at 6 ºC.

**LbL assembly of the polyelectrolyte multilayer** LbL assembly of polyelectrolytes on particles obtained after milling with PSS70 (NC/PSS70) was performed by sequential mixing of particle suspension with a solution of oppositely charged polyelectrolyte. Briefly, the glass beaker containing solution of polyelectrolyte at 2 mg mL⁻¹ was placed on the sonication bath and an equal volume of diluted NC suspensions (~1 mg mL⁻¹) in water was added and sonicated for 1 min followed by mild stirring for 30 min at room temperature. The excess non-adsorbed polyelectrolyte was removed by 3 centrifugation (20 min, 15,000 × g)/dispersion steps in water. The process was repeated until the desired film architecture was reached. The following combinations of polyelectrolytes were used (polycation/polyanion): poly(allylamine hydrochloride)/PSS70, PLR/dextran sulfate, PLR/hyaluronic acid, and PLR/PSS17. Size and zeta potential were measured at each step by DLS and laser Doppler anemometry, respectively.

**Assembly of top mPEG-b-P(PrMA24-co-MAA55).** For deposition of the mPEG-b-P(PrMA24-co-MAA55), typically 50 µL of the NCs coated with (PLR/PSS17)₂PLR with an outer PLR layer (3 mg mL⁻¹ in water) were added to a 1.5 mL Eppendorf tube containing 0.5 mL of the PEGylated polymer (2 mg mL⁻¹ in PBS, 10 mM phosphate buffer or water) under constant sonication. Afterwards, the suspension was further sonicated for 1 min and incubated on orbital agitator under mild shaking (~250 rpm) for 30 min. Mixing and incubation was performed at room temperature. The non-adsorbed copolymer was removed by centrifugation (20 min, 15,000 × g) and the pellet was dispersed in a respective medium. For titration, small aliquots of a solution of mPEG-b-P(PrMA24-co-MAA55) in water (2 mg mL⁻¹, 7.5 – 75 µL) were placed in Eppendorf tubes. Subsequently, 50 µL of (PLR/PSS17)₂PLR were added under constant sonication and left for another 5 min for adsorption. The mixture was diluted with 0.7 mL of water, and size and zeta potential were measured.

**Assembly of top PLL-g-mPEG layer on NCs** For deposition of the PLL-g-mPEG, typically, 50 µL of the NCs with an outer PSS layer (3 mg mL⁻¹ in water) were added to a 1.5 mL Eppendorf tube containing 150 µL of PLL-g-mPEG (1 mg mL⁻¹ in PBS) under constant sonication. Afterwards, the suspension was further sonicated for 1 min and incubated on orbital agitator.
under mild shaking (~250 rpm) for 1 h. Mixing and incubation was conducted either at room temperature or at 45 °C. The mixture was then diluted with 300 µL of PBS and the non-adsorbed copolymer was removed by three centrifugation (20 min, 15,000 × g)/dispersion steps in PBS. The pellet obtained after every centrifugation was dispersed by sonication in the bath in a low volume of PBS (max. 200 µL for every 50 µL of the initially added NCs suspension).

**Drug release and calculation of half-life of drug release (t½)** Dissolution of the PTX formulations was studied under sink conditions using an adapted protocol from the literature.182, 183 Briefly, a Float-A-Lyzer G2 unit (1 mL, MWCO 100 kDa, Sigma Aldrich,) was filled with 1 mL of the release medium (10 mM phosphate buffer pH 7.4, containing 5 wt.% BSA) and a small volume of concentrated formulation (maximum 50 µL) was added to reach a PTX concentration of 0.025 mg mL⁻¹. The dialysis device was placed inside a conical flask containing 45 mL of the release medium and placed on an orbital agitator (~400 rpm) at 37 °C. At the allotted time points, 30-µL samples were taken from the dialysis device and mixed with 30 µL of docetaxel internal standard solution (0.1 mg mL⁻¹ in acetonitrile), vortexed, and mixed with 200 µL of 0.1 M ZnSO₄ solution and 500 µL of acetonitrile. Samples were vortexed for 1 min to precipitate proteins. Two centrifugation steps (15,000 × g, 10 min) were applied to remove proteins, followed by mixing 200 µL of the supernatant with 100 µL of water, and transfer into HPLC vials for analysis. DDSolver Excel Add-In program was used to fit the release curves in exponential model and calculate release half-life time (t½).

**Colloidal stability of NCs** Approximately 50 µL of ~1 mg mL⁻¹ suspension of NCs were diluted with 0.5 mL of PBS or DMEM supplemented with 10 vol% FBS. The suspensions were incubated at 4 °C (PBS) and 37 °C (PBS and DMEM + 10% FBS) and the size of the NCs was measured by DLS at different time intervals.

**Removal of PTX from coated NCs for FTIR measurements** Suspension of NC/PSS₇₀-(PLR/PSS₁₇)₃ and NC/PSS₇₀-(PLR/PSS₁₇)₃-PEG (~3 mg mL, 200 µL) were mixed with 200 µL of acetonitrile to dissolve the drug, followed by addition of 1 mL of tert-butyl methyl ether to extract the PTX. The sample was vortexed for 1 min, centrifuged for 10 min at 10,000 × g and the upper organic phase containing PTX was discarded. After 3 vortexing/centrifugation steps, the water phase was lyophilized and the FTIR spectra of the remaining residues were recorded.

**Statistical analysis** Average t₁/₂ were compared with one-way Kruskall-Wallis test followed by post hoc Dunn’s test for multiple comparisons with the use of Graphpad Prism software.
3.3. Results and Discussion

3.3.1. Screening of ionic stabilizers for PTX NCs stabilization via wet milling

As discussed in Chapter 2, the preparation of electrostatically-stabilized template drug particles constitutes the initial and crucial step to prepare LbL-coated drug suspensions. In this work, PTX NCs were produced by wet milling. In comparison to other techniques, such as sonication/disintegration or nanoprecipitation, wet milling is a low-energy technique with little batch-to-batch variability that can be scaled to industrial level.\textsuperscript{124, 200} To the best of our knowledge, this is the first report describing the production of NCs via wet milling for subsequent LbL coating assembly. Thus, a series of stabilizers for the template NCs were screened.

Given the negative charge reported for uncoated PTX NCs in aqueous media and the successful use of polycations to generate drug NCs with other top-down methods,\textsuperscript{63, 64, 118} a series of positively charged polyelectrolytes were screened to produce PTX suspension via wet milling. Surprisingly, milling with most of the polycations (poly(allylamine), poly(diallyl dimethylamine), PLL, PLR; all hydrochloride salts) failed to produce a stable dispersion of NCs of the desired size and large flocculates or aggregates were observed during purification (Table 3.1).

It thus appeared that electrostatic interactions were insufficient to promote efficient polyelectrolyte adsorption, at least during the wet milling process of PTX. Only milling with PLHR (M\textsubscript{n} 21 kDa, Figure 3.1a) for 48 h resulted in the formation of a suspension of positively charged NCs of size \(\sim\)200 nm, exhibiting crystalline needle-like structure (Table 3.2, Figure 3.2a). Compared to other polycations, successful particle stabilization with PLHR emerges likely from stronger interplay between the polymer and the surface of the NCs. This is possibly a result of both hydrophobic and electrostatic interactions via the four-membered aliphatic ring and highly charged guanidine moieties, respectively present in the side chain of repeating units of PLHR (Figure 3.1a). Surprisingly, the use of PLHR of higher molecular weight (M\textsubscript{n} 52 kDa) produced larger particles and resulted in lower milling efficiency, presumably due to increased viscosity of the solution of this polymer providing barrier for adsorption (Table 3.2).
Table 3.1. List of polyelectrolytes used in a screening study for PTX nanoparticles preparation by wet milling, which did not lead to production of stable nanosuspensions.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Molecular weight (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Polycations</strong></td>
<td></td>
</tr>
<tr>
<td>Poly(allylamine hydrochloride)</td>
<td>15&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Poly(diallyl dimethylammonium chloride)</td>
<td>8.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>PLR</td>
<td>20.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>PLL</td>
<td>17.3&lt;sup&gt;a&lt;/sup&gt;, 42.9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Polyanions</strong></td>
<td></td>
</tr>
<tr>
<td>Dextran sulfate sodium salt</td>
<td>9 - 21&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Protamine sulfate sodium salt</td>
<td>5.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Poly(L-glutamic acid sodium salt)</td>
<td>15.9&lt;sup&gt;a&lt;/sup&gt;, 30&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> M<sub>n</sub> from manufacturer; <sup>b</sup> M<sub>n</sub> from manufacturer

Figure 3.1. Chemical structures of a) PLHR; b) PSS.

Figure 3.2. Morphological structure of NCs obtained after milling of a) 10 mg PTX with 0.5 % PLHR (48 h); b) 40 mg PTX in presence of 0.5% PSS<sub>70</sub> (48 h).
In the next step, high speed centrifugation was applied to purify the NCs stabilized with PLHR (Mₙ 20 kDa) from excess non-adsorbed polymer. Unfortunately, during this process the NCs exhibited irreversible agglomeration (Table 3.3). It thus seems that the overall strength of those interactions were insufficient to promote efficient long-term polyelectrolyte adsorption, at least for the wet milling process of PTX. Therefore, a more hydrophobic polyelectrolyte with the ability to favorably interact with PTX (via e.g., hydrophobic or aromatic interactions), namely negatively charged PSS, was examined (Figure 3.1b). Indeed, wet milling with high molecular weight PSS₇₀ for 48 h produced a dispersion of nanoparticles (116 ± 1 nm) with a negative zeta potential (Table 3.2) and exhibiting the expected needle-like morphology of PTX NCs (Figure 3.2b). These particles did not aggregate after purification and increased only slightly in size to 173 ± 4 nm. Similar results were obtained in the up-scaled process, where 40 mg of PTX were milled in the presence of 0.5% PSS₇₀ for 48 h (Table 3.3). Thus, PSS₇₀ was selected as the stabilizer for the template PTX NCs used in subsequent experiments.

Interestingly, reducing the molecular weight of PSS to 32 or 17 kDa yielded NCs with similar size characteristics, but the efficiency of the milling (amount of drug recovered) dropped substantially (Table 3.2). The chains of PSS₁₇ and PSS₃₂ are probably not sufficiently long to ensure enough attachment sites and yield strong overall interactions between the polymer and the crystal. Notably, no stabilization was achieved after milling of PTX with a series of polyanions lacking any hydrophobic or aromatic groups in the repeating units (dextran sulfate, protamine sulfate, poly(L-glutamate); all sodium salts) (Table 3.1).

Table 3.2. Mean diameter (D), polydispersity index (PDI), zeta potential of the particles (ζ), and concentrations of PTX in the suspensions obtained after milling for 48 h in the presence of PSS and PLHR) of different molecular weights. Values represented as mean ± SD (n = 3)

<table>
<thead>
<tr>
<th>Polymer</th>
<th>MW (kDa)</th>
<th>Crudeᵃ</th>
<th>Centrifugedᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D (nm)</td>
<td>PDI</td>
<td>D (nm)</td>
</tr>
<tr>
<td>PLHRᶜ</td>
<td>20</td>
<td>494±167</td>
<td>0.32±0.02</td>
</tr>
<tr>
<td></td>
<td>53</td>
<td>596±99</td>
<td>0.22±0.03</td>
</tr>
<tr>
<td>PSSᵈ</td>
<td>17</td>
<td>457±70</td>
<td>0.26±0.01</td>
</tr>
<tr>
<td></td>
<td>32</td>
<td>532±17</td>
<td>0.26±0.05</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>328±36</td>
<td>0.31±0.01</td>
</tr>
</tbody>
</table>

ᵃ suspension obtained only after separation from milling beads;ᵇ supernatant after centrifugation of the crude suspension at 12,000 × g for 6 min;ᶜ Mₙ from manufacturer, d Mₙ from manufacturer
Table 3.3. Mean diameter (D) and zeta potential (ξ) of nanoparticles stabilized by PLHR\textsubscript{20} and PSS\textsubscript{70} after purification of the “centrifuged” suspension.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>MW (kDa)</th>
<th>PTX (mg)</th>
<th>D (nm)</th>
<th>PDI</th>
<th>ξ (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLHR</td>
<td>20</td>
<td>10</td>
<td>506 ± 7</td>
<td>0.32 ± 0.01</td>
<td>n/a</td>
</tr>
<tr>
<td>PSS</td>
<td>70</td>
<td>10</td>
<td>173 ± 4</td>
<td>0.23 ± 0.03</td>
<td>-77±5</td>
</tr>
<tr>
<td>PSS\textsuperscript{a}</td>
<td>70</td>
<td>40\textsuperscript{a}</td>
<td>173 ± 4</td>
<td>0.21 ± 0.02</td>
<td>-58±1</td>
</tr>
</tbody>
</table>

\textsuperscript{a}upscaled milling process; n/a – not available

3.3.2. Coating and choice of the PEGylated polymer

PSS\textsubscript{70}-stabilized NCs (NC/PSS\textsubscript{70}) were then sequentially coated with oppositely-charged polyelectrolytes by the LbL process. Because of their popularity in the LbL field, coatings composed a three bilayers of poly(allylamine hydrochloride)/PSS\textsubscript{70} were initially tested. Their alternate deposition led to a cyclic change of zeta potential between +50 mV and –40 mV. Up to three bilayers were assembled without any measurable change of hydrodynamic size or polydispersity index (Figure 3.3a,b) These resulted in 35% lower drug release \textit{in vitro} within 24 h compared to NC/PSS\textsubscript{70} (Figure 3.4a) However, because of their lack of biodegradability and excretability (at the molecular weights tested), alternatives were sought out. Biodegradable polyanions such as hyaluronate, poly(l-glutamate) or dextran sulfate (both sodium salts) were examined with biodegradable PLR and PLL (the latter only with poly(l-glutamate)). However, these polyanions were excluded due to colloidal instability of the resulting NCs, or lack of effect on drug dissolution kinetics (Figures 3.3 - 3.4). Ultimately, PLR and PSS (17 kDa, PSS\textsubscript{17}) were selected as polycation and polyanion, respectively, because PLR is known to be biodegradable\textsuperscript{172} and the lower molecular weight of PSS\textsubscript{17} would, in principle, make it extractable by renal filtration.\textsuperscript{201} The repetitive adsorption of PLR and PSS\textsubscript{17} led to alternating values potential between +40 mV and –40 mV and no measurable change in hydrodynamic diameter. (Figures 3.3k,l). The drug release after deposition of (PLR/PSS\textsubscript{17})\textsubscript{2}PLR was around 20% lower than from NCs coated with NC/PSS\textsubscript{70} after 24 h. (Figure 3.4b).
Figure 3.3. LbL assembly of polyelectrolytes on PTX NCs. a, c, e) Mean diameter and polydispersity index and b, d, f) zeta potential reversal of NCs during the washless process of shell assembly of: a, b) poly(allylamine)/PSS\textsubscript{70}; c, d) PLR/hyaluronic acid; and e, f) PLR/dextran sulfate. Values represent mean ± SD (n = 3). Figure continued on the next page.
Figure 3.3 cont’d. LbL assembly of polyelectrolytes on PTX NCs. g, i, k) Mean diameter and polydispersity index and h, j, l) zeta potential reversal of NCs during the washless process of shell assembly of g, h) PLR/poly(L-glutamic acid); i, j) PLL/poly(L-glutamic acid); and k, l) PLR/PSS_{17}. For k, l the values represent mean ± SD (n = 3).
Chapter 3: PEGylated LbL-coated PTX NCs – preparation and characterization

3.3.3. PEGylation of LbL-coated NCs

In the next step, the LbL-coated NCs were PEGylated with the aim of introducing the steric stabilization and conferring them stealth properties.

Initially, the positively charged NC-(PLR/PSS\textsubscript{17})\textsubscript{2}PLR were PEGylated with polyanionic mPEG-b-P(PrMA\textsubscript{24-co-MA\textsubscript{55}}) (Figure 3.5a) by incubation with an aqueous solution of the polymer at room temperature. Notably, when the NC-(PLR/PSS\textsubscript{17})\textsubscript{2}PLR were added to the polymer dissolved in PBS (pH 7.4) containing high concentration of salts, immediate irreversible agglomeration of NCs was observed; however, the solution remained stable when pure water or 10 mM phosphate buffer (pH 7.4) were used. Nevertheless, the resulting PEGylated suspensions also agglomerated irreversibly when dispersed in PBS (data not shown). This sensitivity of the NCs to ionic strength variation suggested the absence of steric stabilization by PEG chains that should have been introduced by the adsorption of mPEG-b-P(PrMA\textsubscript{24-co-MA\textsubscript{55}}). The adsorption of mPEG-b-P(PrMA\textsubscript{24-co-MA\textsubscript{55}}) on NC-(PLR/PSS\textsubscript{17})\textsubscript{2}PLR was further characterized by titration of the NCs by mixing with increasing amounts of polymer, accompanied by size and zeta potential measurements (Figure 3.6). The NCs aggregated when the absolute value of zeta potential was below 30 mV, which is reported in the literature as necessary for electrostatic stabilization of the colloidal suspensions in the absence of other stabilization mechanisms.\textsuperscript{202} Overall, these results indicate, that mPEG-b-P(PrMA\textsubscript{24-co-MA\textsubscript{55}}) does not confer steric stabilization of the NC, presumably, due to low density of the PEG chains on the surface of the NC, resulting in poor colloidal stability in physiologically-relevant fluids containing salts (e.g. PBS). This property excludes the use of this polymer for future pharmaceutical applications involving our NC.

![Figure 3.4](image-url)
Figure 3.5. Chemical structures of PEGylated polyelectrolytes investigated in this doctoral work. a) mPEG-b-P(PrMA_{24-co-MAA_{55}}); b) PLL-g-mPEG; c) PLL-g-mPEG(0.47)/alkyne-PEG(0.07). For characterization (\textit{^1}H-NMR spectra) see Appendix Figure S1-S3.

Figure 3.6. Size and zeta potential of NC-(PLR/PSS_{70})_{2}PLR after adsorption of different amounts mPEG-b-P(PrMA_{24-co-MAA_{55}}). Data presented as mass of polymer added per mg of PTX.

To introduce a more dense PEG layer on the surface of the NCs, PEGylated polycation top layer composed of PLL-g-methoxy-PEG (PLL-g-mPEG, Figure 3.5b) was assembled as a top layer on the NCs coated with three bilayers of polyelectrolytes ((NC/PSS_{70})-(PLR/PSS_{17})_{3}). This polymer has notably been described as having excellent protein-repellent properties.\textsuperscript{176, 199, 203-206} PLL-g-mPEG containing different mPEG molar grafting ratios (~30\% or ~60\% of lysine residues, PLL-g-mPEG(0.3) and PLL-g-mPEG(0.6), respectively) were synthesized. The
assembly of the top layer was performed at room temperature in phosphate buffer saline (PBS) pH 7.4. While initially stable, non-PEGylated NCs aggregated upon dilution in PBS. However, both PEGylated NCs maintained their colloidal stability at room temperature and their hydrodynamic size increased only slightly to ca. 200 nm (Figure 3.7). Zeta potential values for the PEGylated NCs were slightly negative (−5 to −10 mV), suggesting incomplete masking of the underlying PSS layer, possibly due to the steric hindrance caused by mPEG. Indeed, despite being relatively stable for over 6 days at 4 ºC in PBS, both PEGylated NCs aggregated within 4 h at 37 ºC (Figure 3.8a,b). While several explanations for this phenomenon are possible, the most plausible is the release of PLL-g-mPEG due to inefficient electrostatic complexation with the underlying PSS layer, again possibly due to the mPEG grafts. However, no obvious difference of mPEG grafting density on colloidal stability was observed, at least for grafting ratios in the 30 – 60% (of lysine residues) range. Surprisingly, greater stability was observed in cell growth medium (DMEM containing 10% FBS at 37 ºC, Figure 3.8c). In this environment, the hydrodynamic diameter of the particles rapidly increased from ca. 200 nm to ~250–450 nm, and the increase was more pronounced at lower mPEG grafting density (i.e. PLL-g-mPEG(0.3)). This was presumably due to adsorption of serum components to exposed charged surfaces formed as a result of insufficient PEG coverage and/or release of the PEGylated copolymer.207, 208

To further investigate this possibility, and to promote better protein repellency of the PLL-g-mPEG(0.6) top layer, the assembly process was carried out at higher temperature (45 ºC). Increasing the temperature has been previously been shown to enhance mPEG polyelectrolyte surface grafting, possibly by providing more energy for the polyelectrolytes to
maximize their mutual electrostatic and non-electrostatic interactions.\textsuperscript{209,210} Indeed, likely due to increase mPEG amount on the NCs coating prepared with PLL-g-mPEG(0.6) and its alkyne-bearing analogue (PLL-g-mPEG(0.47)/alkyne-PEG(0.07), Figure 3.5c) stabilized NCs for at least 24 h at 37 °C in both PBS and cell growth medium supplemented with 10% serum (Figure 3.8b,c).

**Figure 3.8.** Colloidal stability of NC/PSS\textsubscript{70}-(PLR/PSS\textsubscript{17})\textsubscript{3} suspensions coated at different temperatures (room temperature (r.T.) and at 45 °C) with PLL-g-PEG copolymers with various PEG grafting ratios in: a) PBS pH 7.4 at 4 °C; b) PBS pH 7.4 at 37 °C; and c) DMEM cell culture medium containing 10% FBS at 37 °C. Values represent mean ± SD (n = 6 – 15).

To better characterize this system, PEGylation of the NCs was confirmed by Fourier transform infrared (FTIR) spectroscopy, \textit{via} the presence of characteristic peaks of PEG (Figure 3.9a, and Appendix Figure S5 and Table S1). Additionally, the particles were coated at 45 °C with a fluorescent analog of PLL-g-mPEG(0.47)/alkyne-PEG(0.07) bearing sulfo-Cy5 at the extremity of some of the PEG chains (PLL-g-PEG–sulfo-Cy5). Further, fluorescent microscopy of dual-fluorescent NCs containing a Cy5-labeled top layer and a fluorescein isothiocyanate (FITC)-labelled PSS\textsubscript{70} as first stabilizing layer showed co-localization (Figure 3.9b,c,d). The emission spectra of both fluorophores on the NCs matched those of the fluorescent polyelectrolyte building blocks (Figure 3.9e,f). In view of their better stability in serum-containing media, and the potential possibility for attaching active targeting moieties or
labelling agents to the copolymer via click chemistry, NCs with PLL-g-mPEG(0.47)/alkyne-PEG(0.07) top layer assembled at 45 °C were used in all subsequent experiments.

Figure 3.9. Confirmation of the presence of PEG on the surface of NCs. a) FTIR spectra of polyelectrolyte shells after extraction of the drug from NC/PSS70-(PLR/PSS17)3 (black) and NCs/PSS70-(PLR/PSS17)3-PEG (red). Black labels correspond to PLR peaks, blue to PSS17 and green to PLL-g-mPEG(0.47)/alkyne-PEG(0.07). Confirmation of the presence of PEG by fluorescence imaging with the use of NC/PSS70-(PLR/PSS17)3-PEG containing PSS70-FITC as the first stabilizing layer and PLL-g-PEG-sulfo-Cy5 as the outermost shell. b) Imaging of FITC and c) Cy5 in large particles aggregates and d) the overlay of both images. e) Fluorescence emission spectra of particles after FITC excitation at 480 nm and f) sulfo-Cy5 excitation at 620 nm. Spectra of PSS70-FITC, free azide-sulfo-Cy5 and PLL-g-PEG-sulfo-Cy5 are also shown.

3.3.4. Dissolution kinetics of PEGylated PTX NCs

The kinetics of dissolution of several NCs and Abraxane® were evaluated under sink conditions, using phosphate buffer supplemented with albumin (for PTX solubility) as release medium. Release curves were interpreted using an exponential function to obtain the average half-time of drug release ($t_{1/2}$; Figure 3.10, Table 3.1). In comparison to Abraxane®, which rapidly dissolved with a $t_{1/2}$ under 15 min, all PTX NCs dissolved more slowly. PTX NCs possessing only a single stabilizing PSS70 layer (NC/PSS70) had a $t_{1/2}$ of ~11 h, which was unaffected by the addition of a PLL-g-mPEG(0.47)/alkyne-PEG(0.07) second layer (further abbreviated as NC/PSS70-PEG). This suggests that a single polyelectrolyte layer has a negligible effect on slowing dissolution. In support of this, and consistent with the observations of others, 62, 63, 118, 132, 133 the rate of dissolution decreased for NCs possessing thicker polyelectrolyte multilayers.
In particular, PEGylated multilayer coated NCs (NC/PSS\textsubscript{70}(PLR/PSS\textsubscript{17})\textsubscript{3}-PEG) possessed a \(t\textsubscript{1/2}\) of \(~27\) h, which would extend the total release time to over a ca. 2-day period.

**Table 3.1.** Calculated half-time \((t\textsubscript{1/2})\) of PTX release from Abraxane\textsuperscript{®}, NC/PSS\textsubscript{70}, NC/PSS\textsubscript{70}-PEG and NC/PSS\textsubscript{70}(PLR/PSS\textsubscript{17})\textsubscript{3}-PEG (PEGylated NCs with terminal layer of PLL-g-mPEG(0.47)/alkyne-PEG(0.07) deposited at 45 °C).

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Release (t\textsubscript{1/2}) ±SD (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abraxane\textsuperscript{®}</td>
<td>&lt;0.25</td>
</tr>
<tr>
<td>NC/PSS\textsubscript{70}</td>
<td>11.2 ± 2.9</td>
</tr>
<tr>
<td>NC/PSS\textsubscript{70}-PEG</td>
<td>11.9 ± 4.7</td>
</tr>
<tr>
<td>NC/PSS\textsubscript{70}(PLR/PSS\textsubscript{17})\textsubscript{3}-PEG</td>
<td>26.8 ± 17.9</td>
</tr>
</tbody>
</table>

**Figure 3.10.** *In vitro* drug release profiles from NC/PSS\textsubscript{70}, NC/PSS\textsubscript{70}-PEG and NC/PSS\textsubscript{70}(PLR/PSS\textsubscript{17})\textsubscript{3}-PEG (with terminal layer of PLL-g-mPEG(0.47)/alkyne-PEG(0.07) deposited at 45 °C). Each data point represents mean ±SD \((n = 9 - 12\) for particles and \(n = 3\) for Abraxane\textsuperscript{®}).

### 3.4. Conclusions

In summary, the PEGylated LbL-coated PTX NCs prepared in this study have the potential to passively accumulate in tumor tissues via the EPR effect. Considering that drug nanocarriers need to remain in the bloodstream for at least 6 h in order to exploit the EPR effect,\textsuperscript{13} the extended dissolution time of the NCs could, in principle, prolong the circulation time of the PTX *in vivo* in comparison to Abraxane\textsuperscript{®}. Assuming similar dissolution profiles under *in vitro* and *in vivo* conditions, \(~80\)% of the PTX would remain in the form of nanoparticles during the 6 h following the injection of NC, while for Abraxane\textsuperscript{®} all drug would be dissolved. In addition to dissolution time, the overall circulation time of a nanocarrier depends greatly on the extent of its clearance from the bloodstream by the MPS. In principle, the presence of PEG on the NCs should minimize the adsorption of opsonin proteins and substantially reduce their uptake by
macrophages. Moreover, the alkyne groups at the PEG terminus of the PLL-g-PEG would allow for conjugation of specific active targeting moieties (i.e. via click chemistry) to promote the NCs’ uptake by the target cells following their extravasation into the tumor. Notably, these strategies would only be valid if the density of the PEG brush is optimal for protein repellency and, importantly, if the PLL-g-PEG layer does not shed from the surface following the i.v. administration of the NCs.
Chapter 4

PEGylated layer-by-layer coated paclitaxel nanocrystals – cell studies and *in vivo* evaluation

A part of this chapter published:

DOI: 10.1002/smll.201602066R1
4.1. Introduction

Testing the newly developed drugs and drug formulations in complex biological systems (cells and animal models) is crucial to evaluate their safety and efficacy. Cell viability assays are often performed to assess the activity of the cytotoxic drugs and potential toxicity of the excipients in the formulation. Concerning the LbL-coatings, it has been demonstrated that polyelectrolytes complexed in multilayers are much less toxic to the cells, in contrast to their free forms.\textsuperscript{174, 175} Thus, in principle, LbL-coated drug nanoparticles should not illicit cytotoxic response beyond that expected for the drug itself. However, in fact, the cytotoxicity of those systems was not thoroughly investigated. Only few studies demonstrated that the presence of multilayer coating on nanoparticles composed of non-cytotoxic and cytotoxic drugs had no inherent effect on the viability of the cells.\textsuperscript{120, 122, 130, 132} Particles itself can, however, trigger toxicity when they agglomerate and sediment on the surface of the cells and thus limit their accessibility to the nutrients and oxygen and/or are terminated with positively charged polyelectrolytes, which triggers interaction with the negatively charged surface of the cell membrane.\textsuperscript{95, 116}

Moreover, some of the systems were designed with the aim of prolonging the particles’ circulation time following intravenous administration to achieve passive targeting of the tumor tissue via the EPR effect.\textsuperscript{62-64} Namely, LbL-coated drug nanoparticles were decorated with PEG, which, in addition to delayed dissolution of the particle due to multilayer coating, was expected to further prolong their circulation times by reducing the macrophage uptake. However, although several drug nanoparticle-based systems were thoroughly characterized in \textit{vitro} in relation to their colloidal stability and drug release, no information is currently available regarding the stability of polyelectrolyte-coated drug nanoparticles \textit{in vivo}, their circulation time, or drug biodistribution profiles. Thus, to evaluate the potential utility of such systems in cancer treatment, it is of utmost importance to evaluate these parameters in animal models.

As shown in Chapter 3, the PEGylated LbL-coated PTX NCs displayed good colloidal stability in model fluids \textit{in vitro}, and, in comparison to rapidly-dissolving Abraxane\textsuperscript{®}, favorably slow dissolution. Thus, the aim of this chapter was to characterize the formulation regarding its influence on the cell viability, as well as to study the pharmacokinetic and biodistribution profiles in the context of passive tumor targeting in tumor-xenograft bearing mice.
4.2. Experimental

*Materials and equipment* (only those not listed in Chapter 3). Human colon adenocarcinoma HT-29 cells were purchased from ATCC, (Manassas, VA). CellTiter 96® AQeous One Solution Cell Proliferation Assay cell toxicity assay based on (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) was from Promega AG (Dübendorf, Switzerland). Tissuelyser tissue homogenizer and 5 mm stainless beads were from Qiagen (Hombrechtikon, Switzerland).

*Polymer desorption studies.* Briefly, 20 µL of suspension of fluorescent LbL-coated NCs with a PLL-g-PEG–sulfo-Cy5 top layer (2 mg mL⁻¹ deposited at 45 °C) in water were mixed with 180 µL of full mouse serum or phosphate buffer saline (PBS) pre-warmed at 37 °C and incubated for 1 h at this temperature on an orbital agitator at ∼250 rpm. After that, 100 µL of the dispersion were collected and centrifuged at 20,000 × g for 20 min to remove the intact NCs. Fluorescence intensity of sulfo-Cy5 in the supernatant and non-centrifuged controls were recorded (λex/em = 620/674 nm) using microplate reader. The percentage of recovered polymer was calculated from the ratio of fluorescence intensity measured in the supernatant and initial fluorescence intensity in the freshly mixed suspension and medium. Experiments were performed in triplicates.

*Cytotoxicity studies.* HT-29 cells were cultured in growth medium containing DMEM GlutaMAX™ (high glucose) supplemented with 10 vol% fetal bovine serum (FBS), 100 units mL⁻¹ of penicillin and 100 µg mL⁻¹ streptomycin. Cells were maintained at 37 °C under a humidified atmosphere containing 5% CO₂. Cultured cells were tested for mycoplasma contamination on a regular basis. Cells were seeded on 96-well plates at a density of 3500 cells/well and allowed to adhere for 24 h. Growth medium was then replaced by 100 µL of fresh growth medium alone (control) or medium containing PTX at concentrations between 0.1 – 100 nM formulated as Abraxane®, or NCs. After 24, 48 or 72 h of incubation, MTS assay was performed. Briefly, cell growth medium was replaced with medium mixed with the MTS reagent in the ratio 5:1 (v/v). Wells containing only the reagent mixture but no cells were used as blank samples. Following 90 min of incubation at 37 °C, absorbance of the solutions at 490 nm was recorded using microplate reader. Experiments were repeated 3 - 4 times.

*Pharmacokinetic and biodistribution studies.* Pharmacokinetic and biodistribution studies were performed on 6 – 8 week-old female NMRI-nu mice (Janvier Labs, Le Genest-Saint-Isle, France) bearing HT-29 subcutaneous xenografts. Mice were kept under specific opportunistic pathogen-free conditions, and all experiments were performed in accordance to a protocol.
approved by the Cantonal Veterinary Office Zurich (permission number 84/2014). HT-29 cells used for the inoculation were free of specific opportunistic mice pathogens. Animals were inoculated subcutaneously into the left flank with $10 \times 10^6$ cells in 100 µL of PBS. Tumor dimensions were measured every day with a caliper starting from day 6 after cell inoculation, and the volume was calculated according to the modified ellipsoid formula:

$$V(\text{mm}^3) = \text{length} \times \text{width} \times \text{width} \times 1/2.211,212$$

The pharmacokinetic and biodistribution experiments were started on day 10–14 after tumor cells inoculation, when the average tumor volume in the group of mice reached 100 mm$^3$. The animals received 15 mg kg$^{-1}$ body weight PTX via tail vein injection at maximal volume of 0.2 mL kg$^{-1}$ body weight, formulated as Abraxane® or NCs in PBS. After 1, 2, 4, 8 and 24 h following PTX formulation administration, a group of 7 mice was deeply anesthetized with a mixture of ketamine and medetomidine (at least 150 mg kg$^{-1}$ and 2 mg kg$^{-1}$ body weight, respectively, via intraperitoneal injection), and blood was collected via cardiac puncture and transferred into Multivette Hep-Li heparinized tubes (Provet AG, Lyssach, Switzerland). Plasma was isolated by centrifugation at 1500 $\times$ g for 5 min and frozen. Transcardiac perfusion was performed with 30 mL of 150 mM NaCl and the tumor and organs (lungs, heart, liver, spleen, kidneys) were collected. Samples were stored at – 80 ºC. Pharmacokinetic parameters were calculated with the use of Excel Add-In PKSolver Software.

**Quantification of PTX in plasma and tissues.** Organs were weighed, and whole tumors, hearts, kidneys, spleens, and portions of liver and lungs of 70 – 150 mg were homogenized with PBS (0.1 g tissue per mL) at 25 – 30 Hz min$^{-1}$ with the use of 5 mm stainless steel beads and a Tissuelyser homogenizer. Mixtures of 200 µL of tissue homogenate or 100 µL of plasma and 100 µL of ultra-pure water were placed into 2 mL glass borosilicate glass vials. Samples were spiked with 20 µL (for tissue homogenates) or 10 µL (for plasma) of docetaxel internal standard solution in acetonitrile (0.0025 mg mL$^{-1}$), followed by addition of 200 µL of acetonitrile and 0.1 M ZnSO$_4$ solution. Samples were vortexed for 1 min to precipitate proteins and were centrifuged for 10 min at 10,000 $\times$ g, after which 500 µL of supernatant were collected and transferred to clean glass vials. Subsequently, 1.2 mL of tert-butyl methyl ether were added, and drugs were extracted from the water phase by vortexing for 2 min. Samples were centrifuged again for 10 min at 10,000 $\times$ g, and the organic phase was transferred into clean glass vials, followed by solvent evaporation under the stream of nitrogen. The residual was dissolved in 150 µL (for tissue samples) or 75 µL (for plasma) of 40% acetonitrile solution in water and centrifuged at 10,000 $\times$ g for 10 min, and 30 µL of the sample were injected into a HPLC system.
Calibration curves were prepared in triplicates in each tissue matrix by spiking 180 µL of the tissue matrices with 20 µL of PTX standards in acetonitrile yielding concentrations of 50, 100, 500, 1000, 5000, and 10,000 ng mL⁻¹. For plasma, 90 µL of the sample was spiked with 10 µL of PTX standard yielding the same concentrations as for tissues. Samples were further processed as described above. Linear regression line was fitted with the use of least-squares linear regression using a weighing factor 1/x². The method was validated for PTX quantification in a biological matrix. All back-calculated concentrations had to be accurate within ± 15% of their nominal values except at lower limit of quantification where ± 20% was acceptable. These criteria were met for all calibration curves. The correlation coefficient was above 0.99 in all cases (regression parameters are presented in the Appendix, Table S2). The lower limit of quantification was 50 ng mL⁻¹. Two additional quality control samples were prepared on separate days at 50, 500 and 10,000 ng mL⁻¹. The method was validated by calculating the accuracy and precision (coefficient of variation) of the back calculated concentrations (three samples from calibration curves and two quality control samples included in calculations), which had to be within ± 15% and ± 20% for lower limit of quantification. The criteria were met in all cases. The summary of the validation parameters are presented in the Appendix, Table S3).

Statistical analysis. Drug plasma concentrations and tissue contents at each time-point after injection were compared with Mann-Whitney test.

4.3. Results and Discussion

4.3.1. Cytotoxicity of PEGylated PTX NCs

To investigate the release of PTX from PEGylated NCs in a complex environment containing cells, the cytotoxicities of two NCs formulations namely, the one coated with PSS₇₀ and PLL-g-mPEG(0.47)/alkyne-PEG(0.07) (NC/PSS₇₀-PEG) and its LbL-coated version with six inner layers of PLR and PSS₁₇ (NC/PSS₇₀(PLR/PSS₁₇)₃-PEG), were evaluated on the HT-29 cell line in the presence of serum using an MTS cell proliferation assay, and compared to Abraxane®. Exposure of the cells to all three PTX formulations for 24, 48 and 72 h resulted in a similar time- and concentration-dependent cytotoxic effect (Figure 4.1, Table 4.1) and small or no differences were observed between the formulations. After 48 h incubation, the average calculated 70% inhibitory concentrations (IC₇₀) value for NC/PSS₇₀-(PLR/PSS₁₇)₃-PEG was twice higher than for Abraxane®, and the difference between those values was statistically significant, suggesting that less free drug was released from the LbL-coated formulation than
from Abraxane® and was available for the cells. However, after 24 h and 72 h of incubation, no statistically significant differences between the corresponding ICx values between the formulations were observed, which does not allow to confirm the latter hypothesis. These findings rather suggest than the differences between the amounts of free drug released from each formulation have little or no influence on the cell viability at the investigated timepoints and that the most of the drug was released from all formulations within 24 h (in line with the \textit{in vitro} dissolution profile, Figure 3.10 in Chapter 3).

Importantly, there was no additional toxicity induced by the presence of the multilayer polyelectrolyte shell, which is in line with what has been reported for other multilayer encapsulated drug nanocores.\textsuperscript{95, 130, 132, 213} For example, in the work of Zahr \textit{et al.} flow cytometry data revealed the same percentage of MCF-7 cell cycle inhibition at the G2/M phase after their treatment with uncoated PTX nanoparticles and their PAH/PSS-coated version, which suggested that the cells responded to the drug itself and not the polyelectrolyte multilayer.\textsuperscript{130} In other study, cells treated with PAH/PSS-coated nanoparticles of non-cytotoxic drugs (\textit{i.e.} ibuprofen and meloxicam) remained viable in a broad range of drug concentrations.\textsuperscript{95, 213} Similar cytotoxicity profiles for NCs and Abraxane® can also indicate the absence of agglomeration and sedimentation of the former on the cell surface, which may trigger cell death due to their limited access to nutrients and oxygen. This findings are in line with the colloidal stability of PEGylated NCs in cell growth media shown in Chapter 3 (Figure 3.8).

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
\textbf{Incubation time (h)} & \textbf{Abraxane® (nM)} & \textbf{NC/PSS\textsubscript{70}-PEG} (nM) & \textbf{NC/PSS\textsubscript{70}-(PLR/PSS\textsubscript{17})\textsubscript{3}-PEG} (nM) \\
\hline
24 & 80 & 41.4 & 14.3 - 119.5 & 81.3 & 46.7 - 141.3 & 69.0 & 39.3 - 120.0 \\
48 & 70 & 8.2 & 6.8 - 9.9 & 9.9 & 6.8 - 14.6 & 16.8 & 10.1 - 28.0 \\
72 & 50 & 6.3 & 5.7 - 7.1 & 6.0 & 4.3 - 8.3 & 7.7 & 6.3 - 9.5 \\
\hline
\end{tabular}
\caption{Average calculated 80, 70 and 50\% inhibitory concentration values (ICx) for Abraxane®, NC/PSS\textsubscript{70}-PEG and NC/PSS\textsubscript{70}-(PLR/PSS\textsubscript{17})\textsubscript{3}-PEG and their 95\% confidence intervals (CI) (x = 80, 70 and 50 for 24, 48 and 72 h of incubation, respectively).}
\end{table}
Chapter 4: PEGylated LbL-coated PTX NCs - cell studies and in vivo evaluation

4.3.2. In vivo biodistribution and pharmacokinetics

In view of the colloidal stability and dissolution kinetics of NC/PSS\textsubscript{70-}(PLR/PSS\textsubscript{17})\textsubscript{3}-PEG, assessment of its biodistribution and pharmacokinetics was conducted in tumor bearing mice and the data were compared to Abraxane\textsuperscript{®}. Formulations containing equal doses of PTX (15 mg kg\textsuperscript{-1}) were administered by tail vein injection to mice bearing subcutaneous HT-29 tumor xenografts. The choice of this model was based on previous studies, which demonstrated nanoparticle deposition in this tumor type\textsuperscript{,214, 215} Plasma PTX concentrations and tissue distribution (heart, kidneys, livers, lungs, spleen and tumor) were monitored over a 24-h period. While the areas under the time-concentration curves were similar for both formulations (~5000 ng mL\textsuperscript{-1} h), the calculated volume of distribution at steady state was over 10-fold higher for NCs than for Abraxane\textsuperscript{®} (6.89 vs. 0.5 L kg\textsuperscript{-1}) and much greater than the intravascular fluid volume (~0.06 L kg\textsuperscript{-1}) implying that the NCs were mostly distributed in the body tissues/organs in comparison to the plasma compartment (Table 4.2). As shown in Figure 4.2a for Abraxane\textsuperscript{®}, the plasma concentration of PTX dropped rapidly from 1000 ng mL\textsuperscript{-1} to ~10 ng mL\textsuperscript{-1} within the first 8 h. In contrast, the initial plasma PTX concentration observed for the PEGylated NC formulation was much lower (~100 ng mL\textsuperscript{-1}; ~10-fold lower than for Abraxane\textsuperscript{®}), though
remained relatively stable over 24 h. The generally comparable drug concentrations in the lungs for NCs and Abraxane® implied that the particles did not precipitate at the injection site and were not trapped in the lung capillaries (Figures 4.2b). Similar time-dependent biodistribution profiles for the heart and the kidneys were obtained for both formulations (Figure 4.2c,d). However, the substantially higher PTX concentrations which were observed in the liver and spleen compared to Abraxane® at all timepoints (Figure 4.2e,f), indicated that the PEGylated NCs were probably opsonized and cleared from the bloodstream by the MPS within a very short time after injection, resulting in low drug concentrations in the tumor (Figure 4.2g). Further, the sustained and rather high plasma PTX concentration observed long after injection of the NCs could imply gradual dissolution from the NC depots formed in the organs of the MPS, which would continuously release drug into the systemic circulation. In fact, the pharmacokinetic and biodistribution profiles obtained for the NC formulation were typical for non-PEGylated NCs. For example, Hollis et al. prepared hybrid PTX nanocrystals of ~200 nm consisting of partially radiolabeled and fluorescent-dye-labeled drug and prepared without the use of any stabilizers were investigated in a xenograft mouse model. The scintigraphy results revealed extensive accumulation of the nanocrystals in the liver, accompanied by low but steady drug concentrations in plasma, gradually released from the MPS cells. Consequently, only around 1% of the injected dose was detected in the tumor tissue.

Thus, our results suggest in the first place that the PEGylated NCs did not dissolve rapidly upon i.v. injection, which is supported by the in vitro release studies, and secondly that PEGylation did not reduce adsorption of serum components and hence their rapid clearance by the MPS. One additional possibility is that the PEGylated copolymer was shed or displaced by serum components, despite the apparent stability of the coatings in vitro in the presence of 10% FBS. Its release would expose charged or potentially hydrophobic surfaces, which could enhance the adsorption of opsonins.

Table 4.2. Pharmacokinetic parameters after i.v. administration of Abraxane® and NC/PSS70-(PLR/PSS17)3-PEG in mice.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>( t_{1/2}^a ) (h)</th>
<th>( C_0^b ) (ng mL(^{-1}))</th>
<th>( \text{AUC}_0^{\infty}_c ) (ng mL(^{-1}) h)</th>
<th>( \text{CL}^d ) (L h(^{-1}) kg(^{-1}))</th>
<th>( \text{Vd}_{ss}^e ) (L kg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abraxane®</td>
<td>3</td>
<td>4820</td>
<td>5468</td>
<td>0.27</td>
<td>0.50</td>
</tr>
<tr>
<td>NC/PSS70-(PLR/PSS17)3-PEG</td>
<td>16</td>
<td>115</td>
<td>5002</td>
<td>0.30</td>
<td>6.89</td>
</tr>
</tbody>
</table>

\(^a\) elimination half-life; \(^b\) extrapolated peak plasma concentration; \(^c\) area under the plasma concentration–time curve; \(^d\) total body clearance; \(^e\) volume of distribution at steady state
Chapter 4: PEGylated LbL-coated PTX NCs - cell studies and *in vivo* evaluation

Figure 4.2 *In vivo* study. Plasma pharmacokinetics and tissue distribution after injection of Abraxane® and NC/PSS70-(PLR/PSS47)3-PEG. a) Plasma PTX concentration as a function of time post-injection. PTX concentration in: b) lungs; c) heart; d) kidney; e) liver; f) spleen and g) tumor. Data expressed as mean ± S.D (n = 4 - 7 mice). Values marked with “×” were below lower limit of quantification. The differences between drug concentrations after injection of Abraxane® and NCs were statistically significant at every timepoint (p < 0.05), except in plasma after 2 and 4 h after injection, lungs after 1 h, heart after 1 and 4 h and kidneys after 2 h. The exact levels of significance are presented in the Appendix, Table S4. (Figure continued on the next page).
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Figure 4.2 cont’d. PTX concentration in: g) tumor. Data expressed as mean ± S.D ($n = 4$ - 7 mice). Values marked with “×” were below lower limit of quantification. The differences between drug concentrations after injection of Abraxane® and NCs were statistically significant at every timepoint ($p < 0.05$. The exact levels of significance are presented in the Appendix, Table S4. h) Desorption of PLL-g-PEG-sulfo-Cy5 from NC/PSS$_{70}$-(PLR/PSS$_{17}$)$_3$-PEG after incubation in PBS or mouse serum at 37 °C. The results are presented as percentage of fluorescent polymer recovered in the supernatant after centrifugation. Data expressed as mean ± S.D ($n = 3$).

To further investigate this possibility, the release of fluorescent PLL-g-PEG-sulfo-Cy5 from NC/PSS$_{70}$-(PLR/PSS$_{17}$)$_3$ was measured at 37 °C after 1 h of incubation either in PBS or full mouse serum. This experiment revealed that in PBS, only ~9% of the fluorescent polymer was shed off from the surface of NCs in contrast to ~55% in mouse serum (Figure 4.2h). Thus, these data suggest that serum components trigger the shedding of (at least) the PEGylated copolymer top layer. For instance, the copolymer could be rapidly displaced by a protein of higher affinity in a process similar to the Vroman effect described for protein exchange in a protein corona.219 However, this finding contrasts with previous studies that reported the stability of PLL-g-mPEG adsorbed to silicon/titanium oxide surface upon incubation in undiluted serum.206

4.4 Conclusions

The results provided in this chapter suggest that the presence of the polyelectrolyte multilayer shell on the surface of the NCs did not affect the cell viability. However, they also indicate that electrostatic complexation alone was not sufficient to retain the PEGylated polyelectrolyte multilayer on the NCs in blood. In the case of PEGylated PTX NCs, serum components led to rapid destabilization and shedding of the outermost PEGylated polyelectrolyte layer. This process was verified ex vivo in full mouse serum. Pharmacokinetics and tissue distribution results were consistent with delayed dissolution of the NCs (> 24 h), probably due to inner polyelectrolyte multilayer coating maintaining its integrity over an extended period of time.150
Uncontrolled shedding of the PEGylated stabilizer, as observed in this study, is undesirable because of the loss of stealth properties of the NCs as well as of any attached targeting/internalizing agents.\textsuperscript{53}
Chapter 5

Conclusion and Outlook
Side effects related to systemic exposure to cytotoxic drugs represent one of the major hurdles in cancer chemotherapy. The inherent ability of these drugs to kill not only the tumor cells but also other rapidly proliferating cells, e.g. hair follicles, bone marrow, digestive tract cells results in an endless list of side effects that the patients receiving chemotherapy may suffer from, including alopecia, myelosuppression or mucositis. Moreover, many of the potent anticancer drugs are poorly water soluble, which imposes the use of potentially harmful solubilizing excipients causing additional dangerous events, for example hypersensitivity reactions. The side effects substantially lower the quality of the patients’ lives and extends the time required for their recovery after treatment. Accordingly, lower doses of the chemotherapeutics must be administered, which in turn, compromises their efficacy. Nanotechnology may partially solve these problems. Drug nanocarriers have a great potential to deliver hydrophobic drugs more specifically to the tumor tissue and less to healthy organs by extravasating through the fenestrations often found in the tumoral vasculature (passive targeting via the EPR effect). They can also be preferentially taken up by the tumoral tissue following their surface functionalization with a targeting moiety binding to a molecule overexpressed on tumor cells or their vicinity (active targeting).

The aim of this doctoral work was to develop a PTX drug delivery nanosystem which would be able to exploit the passive tumor targeting mechanism. Among a plethora of available nanocarriers, a strategy based on drug NCs was selected. In principle, this type of formulation would enable deposition of large quantity of a hydrophobic drug at the tumor site and minimize the amount of non-drug material in the formulation. Given that efficient accumulation of the nanosystems in the tumor tissue necessitates their sufficient circulation time after i.v. administration, the NCs were coated with a multilayer of LbL-assembled polyelectrolytes and PEGylated, to avoid rapid dissolution and reduce their uptake by the macrophages. The in vivo and in vivo performance of the NCs was compared to Abraxane®, a marketed PTX formulation, comprising of amorphous drug nanoparticles stabilized with albumin, a natural excipient considered as safe and generally not causing side effects. However, these nanoparticles cannot exploit the EPR effect and deliver high drug payload to the tumor due to their immediate dissolution into small soluble albumin-drug complexes after i.v. administration.

The obtained PEGylated LbL-coated NCs exhibited good colloidal stability in physiologically – relevant media, which is crucial for i.v. administered pharmaceutical formulations in order to avoid embolization of blood capillaries. The colloidal stability emerged from the electrostatic adsorption of PLL-g-PEG as the topmost layer which introduced high density of the steric stabilizer PEG on the surface of the NCs. The presence of the PEGylated
multilayer coating did not illicit an additional cytotoxicity, as evidenced by the similar toxicity profiles obtained for the formulation and Abraxane®. Moreover, the favorable characteristics of the PEGylated LbL-coated PTX NCs namely longer time required for their dissolution and presence of PEG on the surface which should, in principle, reduce the MPS clearance, could facilitate their long circulation after i.v. administration and thus efficient tumor accumulation (Figure 5.1).

Unfortunately the pharmacokinetics and biodistribution experiments performed in tumor-xenograft bearing mice failed to confirm the latter hypothesis. Notably, the in vivo profile was consistent with delayed dissolution of the NCs compared to Abraxane®, but not with the expected stealth properties of their surface. Namely, the NCs indeed did not dissolve immediately after injection. However, they accumulated extensively in the MPS organs (i.e. liver and spleen) and consequently, little drug was found in the tumor tissue. The relatively steady concentration of the drug in the plasma could be ascribed to the gradual dissolution of the drug particles in the MPS organs and its release back to the systemic circulation. This in vivo behavior was probably a consequence of the rapid destabilization and shedding of the outermost PEGylated polyelectrolyte layer (PLL-g-PEG) in the presence of serum, which was confirmed in vitro. (Figure 5.1)

![Graphical representation of the outcomes of this doctoral work.](image)

**Figure 5.1.** Graphical representation of the outcomes of this doctoral work.

These results suggests that, at least in certain cases, electrostatic complexation alone may be insufficient to retain PEGylated polyelectrolytes on NCs in the body. Uncontrolled release of
the PEGylated stabilizer is undesirable because it causes the loss of stealth properties of the surface as well as any attached targeting/internalizing agents.\textsuperscript{53} Thus, the strategy to retain the PEG in \textit{in vivo} conditions poses a great challenge.

Taking under consideration the results of our previous study, where the non-sheddable PEGylated coatings were obtained by the covalent crosslinking of the hydrophobic part of the amphiphilic PEG polymer directly on the surface of the NC,\textsuperscript{61} a similar strategy could be applied in the LbL system. For example, crosslinkable (\textit{e.g.} alkyne) groups could be introduced in the side chains and the reaction could be performed after electrostatic adsorption of the polymer on the surface of the NC. Direct crosslinking of the amine groups in the lysine’s side chain (\textit{i.e.} with glutaraldehyde) could also be an option. Nevertheless, one should consider that any post-assembly procedures to introduce covalent bonds increase of the complexity of this already sophisticated drug delivery system, and pose a risk of toxicity caused by the remaining traces of the crosslinking reagents. Another strategy that would not require post-assembly modifications of the multilayers could take advantage from the interactions that are intrinsically stable in the physiological conditions, \textit{e.g.} biotin – streptavidin pair complexation. For instance, a fraction of lysine residues in PLL-g-PEG could be modified with biotin and deposited on the surface of the NC terminated either with a polymer conjugated to streptavidin, or with the pure protein adsorbed electrostatically. Similar design has already been shown for fabrication of PEGylated multilayer, where biotin-PLL-g-PEG or biotin-PLL were assembled alternatively with streptavidin on a sacrificial templates which was subsequently removed to obtain hollow spheres.\textsuperscript{221} However, the use of streptavidin may entail a risk of immune reactions \textit{in vivo}. Moreover, an approach based on interactions of cyclodextrins with small hydrophobic compounds, such as adamantane can be employed. Indeed, PEG-adamantane was used to PEGylate particles composed of cyclodextrin-conjugated poly(ethylene imine) complexed to DNA, which improved their colloidal stability at physiologically-relevant salt concentrations and \textit{in vivo} conditions.\textsuperscript{222} All these strategies should be thoroughly evaluated \textit{in vitro} in terms of retaining the stealth properties \textit{e.g.} by performing PEG shedding experiments after incubation of the coated NCs in plasma before pursuing \textit{in vivo} studies.

Another important aspect that needs to be considered for multilayer-coated nanosystems is the fate and toxicity of the coatings in the body. Although, the complexed polyelectrolyte used in our study did not affect the cell viability in the \textit{in vitro} assays, their free forms (especially positively charged PLR) may trigger toxicity. Given that the polymers comprising the coating would, in principle, be biodegradable (PLR) or extractable (PSS\textsubscript{17}) by the renal filtration, their rapid elimination should occur in a case of their release in the body.
Nevertheless, the particle safety upon single and repeated exposure to the multilayer coated NCs should be more thoroughly evaluated by measuring for instance the complement activation-related pseudoallergy (CARPA), biochemical markers and performing histopathological analysis of important organs (liver, spleen, lungs, etc.).

Importantly, the alternatives for the stabilizer for the first coating layer should be sought out since PSS$_{70}$ is neither biodegradable nor excretable. For example, random copolymers of amino acids containing both hydrophobic and ionic building blocks, which would interact with the surface of the NC and provide electrostatic charge, respectively could be a promising option to produce template PTX NCs for further LbL assembly.$^{223}$

Nevertheless, even in the case of a successful development of the PTX drug delivery system based on PEGylated LbL-coated NCs and confirmation of their passive accumulation in the tumor in the pre-clinical models in the future, its translation into humans will not be straightforward. The heterogeneities that occur among tumor types and patients makes the initially supposed wide prevalence of the EPR effect in human questionable.$^{17}$ Future strategies for maximizing the benefits of the passive targeting in humans may involve personalized approach including pre-treatment diagnostics to establish the occurrence of the EPR effect in a certain tumor type in an individual patient.$^{224}$ Treatment of patients bearing tumors that are susceptible to EPR effect with long circulating NCs would result in delivery of a large drug payload to the tumor, which is envisaged to translate into increased antitumor efficacies. Therefore, strategies that could help retain the stealth properties of the LbL-coated drug NCs surface and enable their long circulation times could pave the way for new potent targeted anti-cancer drug formulations.
Figure S1. General structure of mPEG-b-P(PrMA-co-MAA) and its annotated $^1$HNMR spectrum, recorded in D$_2$O.
Figure S2. a) General structure of PLL copolymer with methoxy-polyethylene glycol (PLL-g-mPEG) \((x + y = 100)\). Annotated \(^1\)H NMR spectra of copolymer of PLL with: a) 31% and b) 61% of lysine residues grafted with mPEG. Spectra recorded in 50 mM sodium tetraborate buffer pH 8.5, prepared in D\(_2\)O.
Figure S3. a) Two-step synthesis of PLL-g-mPEG(0.47)/alkyne-PEG(0.07). b) Annotated $^1$H NMR spectrum of PLL with 7% of lysine residues grafted with alkyne-PEG-NHS c) Spectrum recorded in 50 mM 50 mM sodium tetraborate buffer (STBB), pH 8.5, prepared in D$_2$O.
Appendix

Figure S3 con’d. c) Annotated $^1$H NMR spectrum of the same polymer after additional mPEG-NHS grafting. The final grafting percentage lysine backbone is 54% (7% of alkyne-PEG and 47% mPEG). Spectrum recorded in 50 mM STBB, pH 8.5, prepared in D$_2$O.

Figure S4. Confirmation of coupling of azide-sulfo-Cy5 to PLL-g-mPEG(0.47)/alkyne-PEG(0.07) by copper catalyzed click chemistry (CuAAC). Reaction mixture contained alkyne-copolymer, azide-sulfo-Cy5 and click reagents (CuSO$_4$, THPTA and sodium ascorbate). Control 1 contained only alkyne-copolymer and azide-sulfo-Cy5. Control 2 contained only azide-sulfo-Cy5 and click reagents. Mixtures were incubated for 1 h and passed through dye removal column. The column resin retains only free uncoupled dye hence blue filtrate in the case of reaction mixture and transparent in case of controls confirmed the successful coupling of the azide-sulfo-Cy5 to PLL-g-mPEG(0.47)/alkyne-PEG(0.07).
Figure S5. FT-IR spectra of a) PLR; b) PSS<sub>15</sub>; c) PLL-g-mPEG(0.47)/alkyne-PEG(0.07) used for the LbL deposition on NCs.


### Table S1.
Assignment of main bands in FT-IR spectra of the PLR, PSS\textsubscript{17} and PLL-g-mPEG(0.47)/alkyne-PEG(0.07).

<table>
<thead>
<tr>
<th>Peak (cm\textsuperscript{-1})</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>a) PLR</strong></td>
<td></td>
</tr>
<tr>
<td>3278</td>
<td>N-H stretching amide and guanidine</td>
</tr>
<tr>
<td>3155</td>
<td>N-H stretching in guanidine</td>
</tr>
<tr>
<td>1648</td>
<td>amide I (C=O stretching) and C=N stretching</td>
</tr>
<tr>
<td>1543</td>
<td>amide II (CNH stretch-bend)</td>
</tr>
<tr>
<td>1177; 1130</td>
<td>C-N stretching</td>
</tr>
<tr>
<td><strong>b) PSS\textsubscript{17}</strong></td>
<td></td>
</tr>
<tr>
<td>3441</td>
<td>O-H stretch</td>
</tr>
<tr>
<td>2923</td>
<td>alkyl C-H asymmetrical stretching</td>
</tr>
<tr>
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<td>C-C in plane stretching of aromatic ring</td>
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<tr>
<td>1498; 1452; 1411</td>
<td>overtones</td>
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<tr>
<td>1636</td>
<td>O-H bend</td>
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<tr>
<td>1179</td>
<td>S=O asymmetric stretch</td>
</tr>
<tr>
<td>1125; 1009</td>
<td>C-H in plane bending in aromatic ring</td>
</tr>
<tr>
<td>1038</td>
<td>S=O symmetric stretch</td>
</tr>
<tr>
<td>831</td>
<td>out of plane vibrations in para position</td>
</tr>
<tr>
<td>776</td>
<td>C-H out of plane bending in aromatic ring</td>
</tr>
<tr>
<td>673</td>
<td>phenyl out of plane bending</td>
</tr>
<tr>
<td>619</td>
<td>ring in-plane deformation</td>
</tr>
<tr>
<td><strong>c) PLL-g-mPEG(0.47)/alkyne-PEG(0.07)</strong></td>
<td></td>
</tr>
<tr>
<td>2881</td>
<td>PEG-CH\textsubscript{2} symmetric stretching</td>
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<tr>
<td>1652</td>
<td>PLL amide I (C=O stretching)</td>
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<tr>
<td>1546</td>
<td>PLL amide II (CNH stretch-bend)</td>
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<td>1467</td>
<td>PEG CH\textsubscript{2} scissoring</td>
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<td>1279; 1241</td>
<td>PEG CH\textsubscript{2} twisting</td>
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<td>PEG C-O, C-C stretching</td>
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<tr>
<td>963; 841</td>
<td>PEG CH\textsubscript{2} rocking</td>
</tr>
</tbody>
</table>

### Table S2.
Linear regression parameters of analytical assay for paclitaxel extracted from various organs ($n=3$ for organs, $n=2$ for plasma).

<table>
<thead>
<tr>
<th>Organ</th>
<th>Linearity range (ng mL\textsuperscript{-1})</th>
<th>Regression data ($y=a + bx$)</th>
<th>LOQ (ng mL\textsuperscript{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>a</td>
<td>S\textsubscript{a}</td>
</tr>
<tr>
<td>liver</td>
<td></td>
<td>0.00694</td>
<td>0.00084</td>
</tr>
<tr>
<td>kidney</td>
<td></td>
<td>0.00742</td>
<td>0.00054</td>
</tr>
<tr>
<td>tumor</td>
<td></td>
<td>0.00722</td>
<td>0.00023</td>
</tr>
<tr>
<td>spleen</td>
<td>50 - 10000</td>
<td>0.00714</td>
<td>0.00022</td>
</tr>
<tr>
<td>heart</td>
<td></td>
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<td>0.00050</td>
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<tr>
<td>lungs</td>
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<td>0.00038</td>
</tr>
<tr>
<td>plasma</td>
<td></td>
<td>0.00700</td>
<td>0.00047</td>
</tr>
</tbody>
</table>

LOQ: limit of quantification
**Table S3.** Validation of analytical HPLC assay for quantification of paclitaxel extracted from mouse organs. Inter-day precision and accuracy of HPLC assay for paclitaxel from mouse organs (n = 5, 3 samples from calibration curve included + 2 quality control samples prepared on separate days).

<table>
<thead>
<tr>
<th>Organ</th>
<th>Spiked conc. (ng mL⁻¹)</th>
<th>Mean measured conc. (ng mL⁻¹)</th>
<th>% COV* (precision)</th>
<th>% Deviation (accuracy)</th>
</tr>
</thead>
<tbody>
<tr>
<td>liver</td>
<td>50</td>
<td>58.7 ± 6.0</td>
<td>10.3</td>
<td>17.4</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>470.8 ± 36.3</td>
<td>7.7</td>
<td>-5.8</td>
</tr>
<tr>
<td></td>
<td>10,000</td>
<td>10,174 ± 822</td>
<td>8.1</td>
<td>1.7</td>
</tr>
<tr>
<td>kidney</td>
<td>50</td>
<td>58.6 ± 3.2</td>
<td>5.5</td>
<td>-17.1</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>474.4 ± 29.2</td>
<td>6.2</td>
<td>5.1</td>
</tr>
<tr>
<td></td>
<td>10,000</td>
<td>10,076 ± 1010</td>
<td>10.0</td>
<td>-0.8</td>
</tr>
<tr>
<td>tumor</td>
<td>50</td>
<td>50.8 ± 7.0</td>
<td>13.8</td>
<td>-1.6</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>461.1 ± 24.1</td>
<td>5.2</td>
<td>7.8</td>
</tr>
<tr>
<td></td>
<td>10,000</td>
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*COV=coefficient of variation; b n = 4 (2 samples from calibration curve + 2 quality controls )

**Table S4.** Significance levels for Mann-Whitney test performed for statistical difference between the PTX tissue content after injection of Abraxane® and NC/PSS₇₀-(PLR/PSS₁₇)₃-PEG. (ns = not significant) p > 0.05, (*) p < 0.05, (**) p < 0.01, (***) p< 0.001.

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60. Hollis, C. P.; Weiss, H. L.; Leggas, M.; Evers, B. M.; Gemeinhart, R. A.; Li, T.; Biodistribution and Bioimaging Studies of Hybrid Paclitaxel Nanocrystals: Lessons Learned of the Epr Effect and Image-Guided Drug Delivery. *J. Controlled Release* 2013, 172, 12-21.


LIST OF ABBREVIATIONS
List of Abbreviations

alkyne-PEG-NHS - propargylacetamido-poly(ethylene glycol) succinimidyl butanoate

BSA – bovine serum albumin

DMEM – Dulbecco’s Modified Eagle Medium

DLS – dynamic light scattering

EMA – European Medicinal Agency

EPR – enhanced permeability and retention

FBS – fetal bovine serum

FDA – Food and Drug Administration Agency

FITC – fluorescein isothiocyanate

FTIR – fourier transform infrared spectroscopy

$^1$H NMR – proton nuclear magnetic resonance

HPLC – high pressure liquid chromatography

HT-29 – human colon adenocarcinoma cell line

i.v. – intravenous

MAA – methacrylic acid

mPEG- methoxy-poly(ethylene glycol)

mPEG-SPA – methoxy-poly(ethylene glycol) succinimidyl propionate

MPS – mononuclear phagocyte system

NC – nanocrystal

NSAID - non steroidal antinflammatory drug

PAH – poly(allylamine hydrochloride)

PrMA - propylmethacrylate

PBS – phosphate buffer saline

PDDEA – poly(diallyldimethylammonium chloride)

PEG – poly(ethylene glycol)
<table>
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<th>Abbreviation</th>
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<tr>
<td>PLHR</td>
<td>poly(l-homoarginine)</td>
</tr>
<tr>
<td>PLL</td>
<td>poly(l-lysine hydrochloride)</td>
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<tr>
<td>PLL-g-PEG</td>
<td>poly(l-lysine)-graft-poly(ethylene glycol)</td>
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<tr>
<td>PLR</td>
<td>poly(l-arginine hydrochloride)</td>
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<tr>
<td>PSS</td>
<td>poly(styrene sulfonate) sodium salt</td>
</tr>
<tr>
<td>PTX</td>
<td>paclitaxel</td>
</tr>
<tr>
<td>PVA</td>
<td>poly(vinyl alcohol)</td>
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<tr>
<td>PVP</td>
<td>poly(vinyl pyrrolidone)</td>
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<td>LbL</td>
<td>layer-by-layer</td>
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<td>TEM</td>
<td>transmission electron microscopy</td>
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CURRICULUM VITAE

& SCIENTIFIC CONTRIBUTIONS
Anna Karolina Polomska

Born 18 May 1987 in Cracow, Poland
polomska.ania@gmail.com

EDUCATION AND PROFESSIONAL EXPERIENCE

05/2012 – 09/2016  Doctoral Studies | ETH Zürich, Institute of Pharmaceutical Sciences | Drug Formulation and Delivery | Zurich, Switzerland
Doctoral Project: Polyelectrolyte multilayer-stabilized paclitaxel nanocrystals.
Supervisor: Prof. Jean-Christophe Leroux

Teaching assistant | Galenic pharmacy | ETH Zürich, Institute of Pharmaceutical Sciences

09/2011- 04/2012  Research Assistant | University of Geneva, Faculty of Sciences, Laboratory of Colloid and Surface Chemistry | Geneva, Switzerland
Research Project: Stability and aggregation latex particles in presence of multivalent ions.
Supervisor: Dr. Istvan Szilagyi

10/2006 – 06/2011  M.Sc. in Chemistry (specialized in Biological Chemistry) | Jagiellonian University, Faculty of Chemistry | Cracow, Poland
Master Project: Synthesis of new model photosynthetic pigments in application for photodynamic therapy.
Supervisor: Prof. Leszek Fiedor, Faculty of Biochemistry, Biophysics and Biotechnology

09/2009 – 10/2010  B.Sc. Hons in Medicinal Chemistry (First Class) | School of Science, University of the West of Scotland | Paisley, Scotland
Bachelor Project: Chiral Analysis of Common Amino Acids Present in Teeth Using Reversed-Phase High Performance Liquid Chromatography
Supervisor: Dr. Calum Morrison

TRAININGS AND INDEPENDENT COURSES

03/2016  Regulatory Affairs | ETH Zürich
10-11/2013  Presenting Science | ETH Zürich
06/2012  LTK Module 1 | Introductory Course in Laboratory Animal Science | University of Zürich

LANGUAGES

Polish  mother tongue
English  fluent | full professional proficiency
German  intermediate | limited professional proficiency
ORIGINAL PUBLICATIONS


ORAL PRESENTATIONS


POSTER PRESENTATIONS


ACKNOWLEDGEMENTS
In the first place, I would like to thank prof. Jean-Christophe Leroux for the great opportunity to pursue my Doctoral studies in his research group. I greatly appreciate his scientific support, encouragement, motivation and patience throughout my project.

I would like to thank prof. Bruno Gander for accepting to be my co-examiner.

Moreover, I would like to sincerely acknowledge Dr. Davide Brambilla for his supervision and help in writing the review article. Special thanks to prof. Marc A. Gauthier for the scientific input and support in writing the research article.

I would like to thank Dr Jong Ah Kim for English proofreading of the publications and the thesis.

Many thanks to Dr. Thomas Weber and the whole RCHCI team, as well Dr. Franziska Bootz and Samuele Cazamalli from the Neri group for the support in the animal facility. Many thanks also to Dr. Annamarie Alitalo and Dr. Maike Heimann for the assistance in writing the animal protocol. Dr. Davide Brambilla, Maurizio Roveri, Athanasia Dasargyri, Rea Signorell and Dr. Soo Hyeon Lee are greatly acknowledged for help during the in vivo experiments.

Peter Tiefenböck is acknowledged for taking the fluorescent microscopy images and the Scientific Center for Optical and Electron Microscopy ScopeM of the ETHZ for the TEM imaging.

I greatly acknowledge the Swiss National Science Foundation (SNF 310030-138342) for the financial support of this project.

Of course, I would like to acknowledge present and past members of the Drug Formulation and Delivery group. I really appreciated working in such a supportive, friendly and collaborative atmosphere and spending amazing time with many of them outside the lab. In the first place, I would like to thank Elena Moroz and Athanasia Dasargyri, with whom I shared the first and the last days of my doctoral studies. I am really glad that we could experience together all the beautiful moments of our PhD and support each other in difficult times. Special thanks go also to Dr. Kathrin Furhmann, who introduced me in the field of drug nanosuspensions at the beginning of my PhD. I would like to thank Xiangang Huang, Sofia Bisso, Aaron Schmidt, Dr. Estelle Durantie, Dr. Yuhui Gong and Sandhya Ananta for the amazing atmosphere in the office. Special thanks also go to Dr. Jessica Schulz, Diana Andina, Dr. Jong-Ah Kim, Maurizio Roveri, Dr Soo Hyeon Lee, Dr. Valentina Agostoni, Dr. Paola Luciani, Dr. Bastien Castagner, Dr. Lorinne Brülisauer, Virginie Rusca, Nadine Häni-Doëlly, Anna Pratsinis Dr. Arnaud Felber, Dr. Matthias Ivarsson and Dr. Vincent Forster for their friendship.

I would like to also thank Dawid Kedracki, and my family for their constant support.