

Theranostic biodegradable particles for image-guided  
intraperitoneal drug delivery:  
from synthesis to preclinical validation



Doctoral thesis  
for  
the award of the doctoral degree  
of the Faculty of Mathematics and Natural Sciences  
of the University of Cologne

submitted by  
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2025

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Date of Submission:

24.08.2025

Date of Examination:

24.10.2025

*“I place one experiment above a thousand opinions born only of imagination.”*

***Mikhail Lomonosov***

## Abstract

Peritoneal carcinomatosis (PC) results from the spread of tumor cells to the peritoneal surface in the abdomen. While intraperitoneal (i.p.) chemotherapy enables high local drug concentrations, its effectiveness is limited by rapid clearance. Significant gaps remain in understanding the routes of drug absorption (lymphatic versus blood) and pharmacokinetics following i.p. administration, particularly when complex delivery systems such as nano- and microparticles (NPs and MPs) are involved. This thesis explores novel biodegradable drug delivery systems (DDS) designed for sustained i.p. release and reduced systemic toxicity. The *in vivo* behavior of NPs and MPs was studied, focusing on how physicochemical properties such as size, charge, and composition influence absorption into lymph, peritoneal organ uptake, as well as lymphatic clearance and pharmacokinetics, following i.p. administration. By integrating advanced imaging modalities (e.g., magnetic resonance imaging (MRI), computer tomography (CT), single-photon emission CT (SPECT)), the biodistribution and degradation of DDS were quantitatively monitored.

The work centers on two chemotherapeutic agents, paclitaxel (PTX) and *cis*-diamminedichloroplatinum (cisplatin, CDDP), representing hydrophobic and hydrophilic drugs, respectively. Poly(lactic-co-glycolic acid) (PLGA)-based core-shell particles loaded with PTX and containing either perfluorooctylbromide (PFOB) for  $^{19}\text{F}$ -MRI or iodinated oil octan-2-yl 2,3,5-triodobenzoate (TIBO) for dual-energy CT (DECT) imaging were synthesized and optimized for controlled size and drug loading. A novel  $^{19}\text{F}$ -MRI acquisition technique (Balanced Spiral Spectroscopic Imaging, BaSSI) enabled sensitive *in vivo* tracking of fluorinated DDS, while DECT provided complementary visualization and quantification of iodinated particles.  $^{19}\text{F}$ -MRI of an animal injected with PFOB capsules revealed prolonged peritoneal retention and gradual degradation near the injection site. TIBO capsules, detectable by DECT due to their iodine content, exhibited higher PTX loading and slow release kinetics with similar biodistribution patterns. Both systems demonstrated particle aggregation at injection sites with significant retention up to 18 days and particle presence in tumor-prone regions.

Additionally, collagen-poly(glutamic acid) NPs were developed for complexation and sustained release of platinum-based drugs. Hydrophilic platinum-based drugs such as CDDP were complexed with free functional groups abundant in the NPs which enabled



high CDDP loading with sustained release. Radiolabeling with [ $^{111}\text{In}$ ]InCl<sub>3</sub> facilitated sensitive SPECT/CT imaging, to longitudinally assess NP biodistribution and clearance, highlighting the potential for lymphatic targeting. Notable retention in the peritoneal cavity and uptake in metastatic target organs were observed.

Overall, this work provides a versatile platform combining advanced imaging and tailored DDS to optimize i.p. drug delivery in a theranostic approach. The findings emphasize the importance of balancing particle size, surface properties, and drug loading to enhance anti-tumor effectiveness, biodistribution and peritoneal retention through the careful selection of DDS. Such optimization could improve treatment outcomes for PC. Future studies employing imageable tumor models will be beneficial to validate therapeutic efficacy and translate these systems into clinical application.

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## 1 Introduction

### 1.1 Clinical question

Worldwide, cancer is currently the leading cause of death accounting for approximately 10 million casualties per year.[1,2] Due to the demographic change towards an aging society, prevalence of cancer will further increase. Thus, more efficient and cost-effective therapies will be needed to offer improved treatments while simultaneously reducing the burden on healthcare systems. Over the past decades, therapeutic outcome and survival rates improved for most cancers, especially when diagnosed early enough. However, once cancer is disseminated with distant metastases present, complete cure is often out of reach and therapy is considered palliative requiring close management of the patient. A particular problematic medical condition is found for cancers that originate from abdominal organs, such as stomach, pancreas, uterus, kidney or colon. Here, cancer cells often disseminate within the peritoneum covering the peritoneal surface with metastases. Peritoneal metastases (PMs) frequently cause intestinal obstruction and lead to a malignant ascites, a disease stage commonly denoted as PC. The reported incidence of PMs depends on the origin of the primary tumor. For example, reported incidence for gastric cancer is in the range of 15–43%, [3] for colorectal cancer, the incidence amounts to 4–25%. [4,5] Incidence of PMs from ovarian cancer is particularly high with 60–70% whereas it is less than 10% for other gynecological malignancies. [6] The large variations are on the one hand a consequence of different subtypes of cancer having different tendencies to form PMs but are on the other hand also affected by the healthcare system. Here, regular screening programs that allow early detection of cancer which strongly reduces the prevalence of PMs. Once PMs are present, the onset of ascites is an additional indicator for a poor prognosis. For example, the 5-year survival rate for ovarian cancer decreases from over 90% to only 25–29%. [7–9]

PC is characterized by small, scattered tumor nodules throughout the peritoneal cavity. Current treatment options comprise cytoreductive surgery (CRS) removing all macroscopically visible tumors, followed by either hyperthermic intraperitoneal chemotherapy (HIPEC) or pressurized intraperitoneal aerosol chemotherapy (PIPAC), both exposing the residual cancer cells within the peritoneal cavity to a locally high drug concentration. In a HIPEC procedure, the peritoneal cavity is incubated and

washed with a solution of cytostatic drugs at hyperthermic temperatures to eradicate remaining cancer cells and micro-lesions. This approach builds on the synergistic effects of hyperthermia with chemotherapy to enhance drug distribution and uptake. During PIPAC, chemotherapy is administered in form of aerosol droplets, which distribute under pressure (12 mm Hg) across the cavity and provide enhanced drug coverage and permeability, particularly in cases of unresectable peritoneal cancer. This method helps to overcome tumor interstitial pressure and increases drug exposure. A recently introduced innovative approach is electrostatic precipitation PIPAC (ePIPAC) which further optimizes the aerosol distribution. By generating an external electrostatic field, ePIPAC charges the aerosol droplets negatively while the patient's tissue becomes slightly positively charged, creating an electrostatic attraction that counters gravity and drag. ePIPAC has been shown to further improve aerosol distribution and facilitate deeper tissue penetration of therapeutics (Fig. 1).[10–16] Depending on clinical guidelines for the respective primary tumor, HIPEC, PIPAC or ePIPAC are followed up with multiple cycles of intravenous (i.v.) administered chemotherapy.

However, as peritoneal tumors are typically poorly perfused, drug uptake from i.v. administered chemotherapy is limited leading to a poor therapeutic response. In contrast, i.p. administered chemotherapy as used in HIPEC or PIPAC provides a high drug concentration directly at the peritoneal surface improving treatment of residual microscopic cancer tissue. Yet, a drawback of i.p. administered chemotherapy is the rapid elimination of small cytostatic molecules from the peritoneal cavity, which limits the overall exposure of cancer cells to chemotherapy. Thus, residual cancer cells remaining in the abdomen typically cause recurrence. In conclusion, all current treatment options of PMs, including HIPEC, PIPAC and ePIPAC are considered palliative care and only allow to extend mean time of survival.

This clinical challenge highlights the need for a more effective drug delivery method that can improve retention at the peritoneal surface with increased drug uptake in order to provide higher therapeutic efficacy for patients.

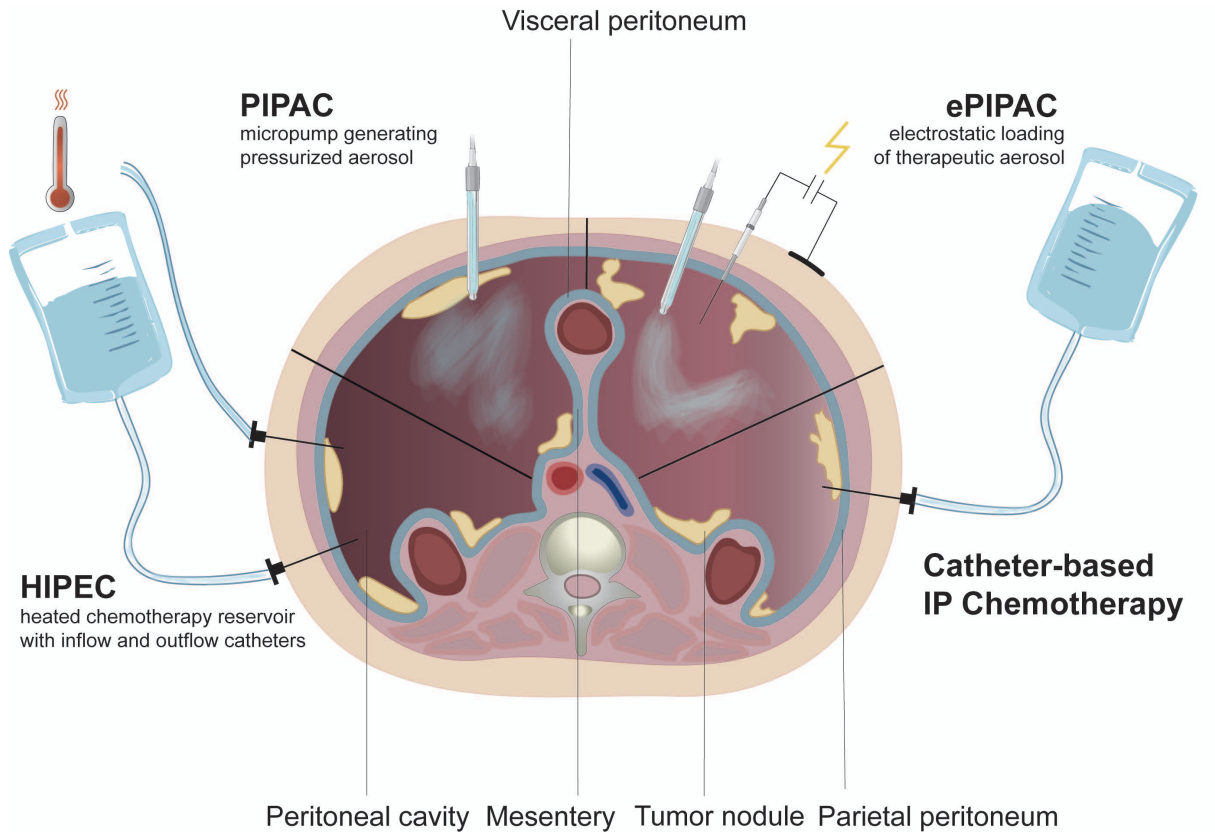


Fig. 1: Current approaches to i.p. chemotherapy. Based on a figure by Kakchekeeva et al.[10]

## 1.2 Cytostatic drugs

An essential requirement in PM treatment is the careful selection of a chemotherapeutic drug with cytotoxic activity being highest for the primary cancer. For instance, mitomycin C is commonly used for colorectal and appendicular PMs, while taxanes such as PTX and docetaxel (DTX) are preferred for gastric and ovarian PMs. Additionally, irinotecan and 5-fluorouracil (5-FU) are typically used for colorectal PMs, and platinum-based agents such as [(1*R*,2*R*)-cyclohexane-1,2-diamine](ethanedioato-*O*,*O'*)platinum(II) (oxPt) are indicated for colorectal, gastric, and ovarian PMs. Additionally, important pharmacokinetic variables must be considered including drug dose, volume, carrier solution, and molecular weight.[17–19] Commonly used cytotoxic drugs in HIPEC are summarized below with their respective treatment regimens (Table 1).

Table 1: Commonly used cytotoxic drugs for HIPEC.[17–20]

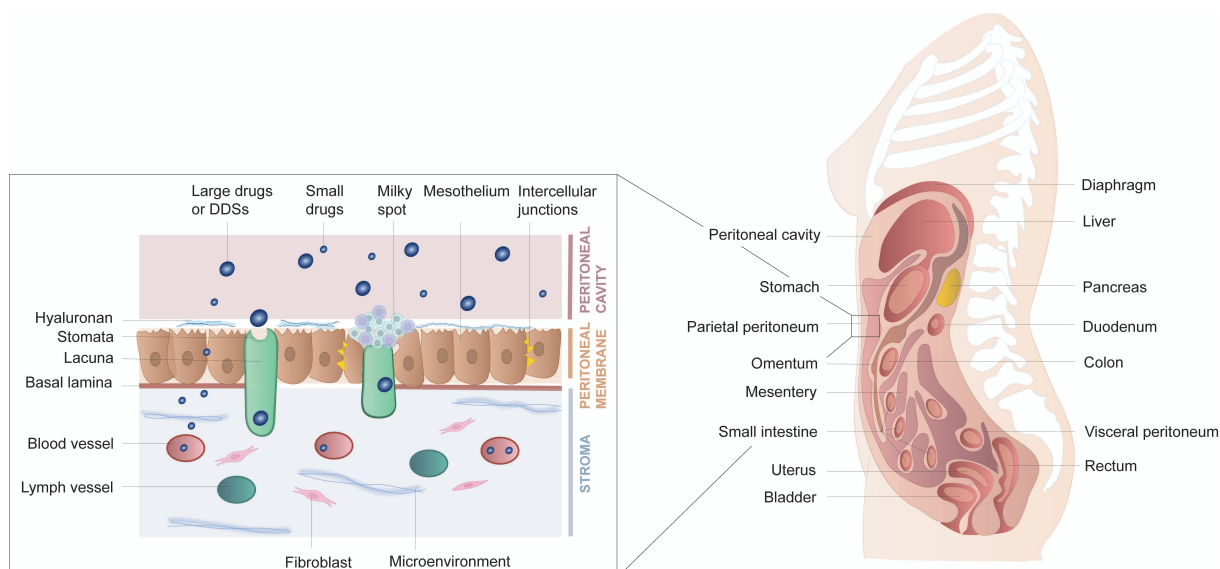
| Cytostatic        | Biological function                                                                                                                                                                                                                                                                                                | Carrier solution                           | Cancer type                                                                                                    |
|-------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------|----------------------------------------------------------------------------------------------------------------|
| 5-Fluorouracil    | thymidylate synthase inhibitor binds covalently with enzyme and prevents formation of thymidine monophosphate, DNA nucleoside precursor; 5-FU by its metabolites 5-fluorouridine diphosphate and 5-fluorouridine triphosphate gets incorporated in RNA, resulting in second cytotoxic pathway; cell cycle specific | Hypertonic, high molecular weight solution | appendiceal and other gastrointestinal tumors                                                                  |
| Carboplatin       | cross-links DNA, interfering with DNA replication inhibiting cancer cell growth                                                                                                                                                                                                                                    | NaCl solution                              | appendiceal tumors                                                                                             |
| CDDP              | alkylating agent that causes apoptotic cell death by formation of DNA adducts                                                                                                                                                                                                                                      | NaCl solution                              | appendiceal tumors, malignant peritoneal mesothelioma (MPM), ovarian cancer, gastric cancer                    |
| oxPt              | forms inter- and intra-strand cross-links in DNA, which prevent DNA replication and transcription[21]                                                                                                                                                                                                              | Dextrose solution                          | colorectal and appendiceal neoplasms                                                                           |
| Doxorubicin (DOX) | intercalates into DNA and inhibits topoisomerase II, preventing DNA replication by stabilizing the cleaved DNA-topoisomerase complex and blocking strand re-ligation                                                                                                                                               | Dextrose solution                          | appendiceal tumors, malignant peritoneal mesothelioma (MPM), sclerosing effect in malignant ascites            |
| Mitomycin C       | alkylating tumor antibiotic; most important mechanism of action through DNA cross-linking                                                                                                                                                                                                                          | NaCl solution                              | colorectal cancer, appendiceal cancer, ovarian cancer, gastric cancer, malignant peritoneal mesothelioma (MPM) |
| DTX               | taxanes stabilize microtubule against depolymerization, thereby disrupting normal microtubule dynamics; evidence supporting additional mechanisms of action; cell cycle specific                                                                                                                                   | NaCl solution                              | gastric cancer, diffuse-type adenocarcinoma, ovarian cancer                                                    |
| PTX               | taxanes stabilize microtubule against depolymerization, thereby disrupting normal microtubule dynamics; evidence supporting additional mechanisms of action; cell cycle specific                                                                                                                                   | NaCl solution                              | gastric cancer, diffuse peritoneal mesothelioma, ovarian cancer or in treatment of malignant ascites           |
| Irinotecan        | inhibition of topoisomerase leading to inhibition of DNA replication and transcription                                                                                                                                                                                                                             | NaCl solution                              | colorectal cancer                                                                                              |

### 1.3 Anatomical structure of healthy peritoneum

The peritoneum is the largest serous membrane in the human body, with a surface area of approximately 1.5–2.0 m<sup>2</sup>. [22,23] It plays an important role in the function and health of the abdominal organs. The peritoneum consists of two primary layers: the parietal peritoneum, which lines the abdominal wall, and the visceral peritoneum, which envelops the outer serosal layers of the abdominal organs. The space between these two layers is called the peritoneal cavity. Additionally, peritoneal folds give rise to important anatomical structures such as the ligaments, omenta, and mesenteries (Fig. 2). [24] Of particular significance is the mesentery which is a common site for cancer metastasis with its rich vascular and lymphatic network. [25]

Anatomically, the peritoneum is composed of three distinct layers. The mesothelium, the outermost layer, is composed of a single layer of flat, squamous cells approximately 25 µm in diameter. [26] Mesothelial cells are tightly connected through intercellular junctions such as tight junctions, adherent junctions, gap junctions, and desmosomes (Fig. 2). [27] The apical surface of these cells is covered with microvilli and glycocalyx, which traps fluid forming a protective fluid layer. Additionally, this layer is rich in proteoglycans and glycosaminoglycans, establishing a negative polarity that maintains the semi-permeable barrier function of the peritoneum. [23] Beneath the mesothelium lies the basal lamina, which separates the mesothelium from the underlying submesothelial stroma. The submesothelial stroma consists mainly of collagen, adipocytes, lymphocytes, fibroblasts and macrophages. Furthermore, blood vessels and lymphatics are found here.





*Fig. 2: Anatomical structure of the abdominal cavity showing both the parietal and visceral layers of the peritoneum, which together delineate the peritoneal cavity. An ultrastructural perspective of the peritoneum reveals a surface mesothelial cell layer with lymphatic stomata and lymphoid tissue (milky spots), which drain into lymphatic vessels. Below lies stroma matrix with both blood and lymphatic vessels. While small therapeutic molecules can trespass the mesothelium to enter systemic circulation via blood vessels, larger drug molecules or carrier systems are cleared through stomata into the lymphatic pathway. Based on a figure by Sarfarazi et al.[24]*

The peritoneum is supplied by two distinct blood circulatory pathways. The parietal peritoneum receives blood from the abdominal wall and parietal pelvic arteries, draining into systemic veins. In contrast, the visceral peritoneum, along with the mesentery and omentum, is supplied by the mesenteric, coeliac, and visceral pelvic arteries, with drainage into the portal veins.[28,29]

Physiologically, the peritoneum serves several functions. It aids lubrication, reducing friction and allowing free movement of abdominal organs. Furthermore, the capillary perfusion within the peritoneum facilitates drug-plasma exchange.

Peritoneal fluid is a liquid that is continuously renewed, approximately every 1–2 hours.[30] In healthy humans, the volume of peritoneal fluid ranges from 10–100 ml, whereas in mice, it is significantly smaller, ranging from 0.02–0.1 ml.[31,32] It typically maintains a pH 7.5–8.0, demonstrating a buffering capacity.[33] This fluid contains a mixture of water, electrolytes, proteins, and various immune cells, including macrophages, natural killer cells, and lymphocytes. Physiologically, peritoneal fluid acts as a lubricant, and is involved in nutrient exchange.[34]

The peritoneal-plasma barrier regulates the exchange of substances between the peritoneal cavity and plasma. It is composed of peritoneal fluid, mesothelium, basal lamina, interstitium, and the vascular wall. This barrier plays an essential role in

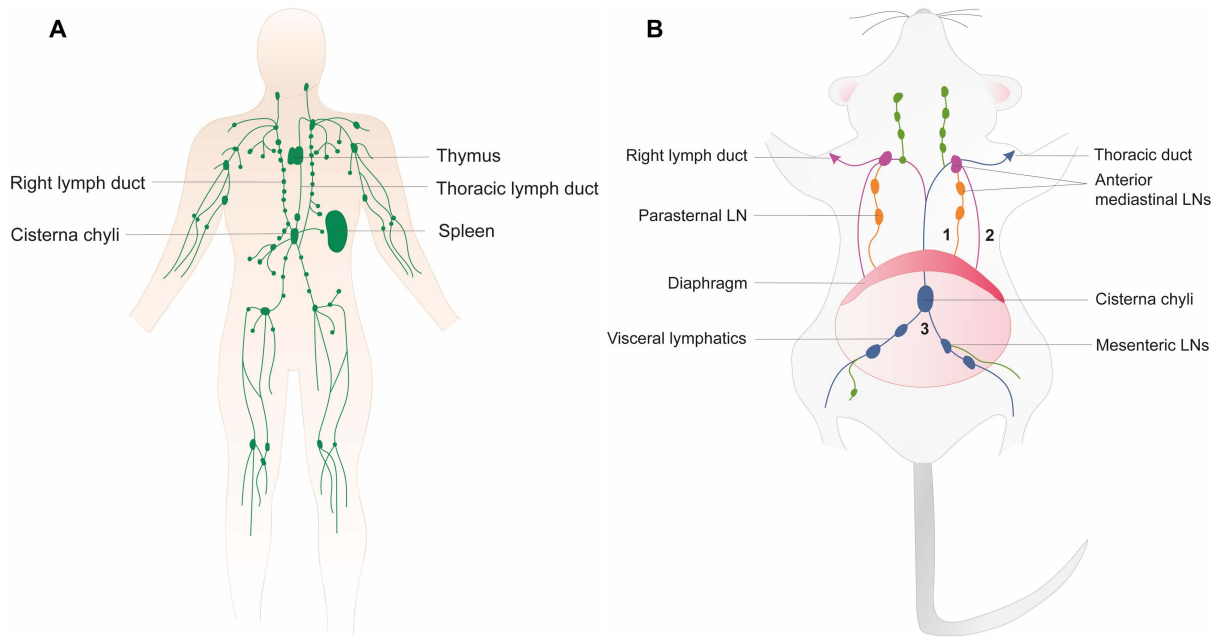
maintaining a concentration gradient of cytotoxic drugs between the peritoneal cavity and the plasma compartment.[35] Following i.v. administration of cytotoxic drugs, the peritoneal-plasma barrier inhibits efficient drug transport from the plasma to the peritoneal tumor nodules and ascites, limiting their effectiveness in treating peritoneal lesions. As a result, i.v. infusion of these drugs yields only limited results for patients with PC. In comparison, after administration of chemotherapy directly into the peritoneal cavity through the i.p. route, the peritoneal-plasma barrier slows drug clearance from the cavity. This leads to high concentrations of the therapeutic agent within the peritoneal cavity, offering a significant advantage for treating PM and ascites.[36]

Clearance from the peritoneal cavity occurs mainly via the abdominal lymphatic system comprising several key components, such as lymph capillaries, collecting vessels, lymph nodes (LNs), lymphoid tissues, and lymphatic organs. Its primary functions include maintaining fluid balance by returning tissue fluid to the heart, conducting immune surveillance, and assisting in the transport of macromolecules into the bloodstream. Beyond its physiological functions, the lymphatic system is also a critical pathway for the spread of cancer cells. Tumor cells from the peritoneal cavity can be transported to distant sites via this network.[24,37] The presence of stomata (physiological openings that connect the peritoneum to the lymphatic vessels) facilitates disease spread. Stomata, which are located near milky spots containing lymphocytes and macrophages, are mainly found in the peripheral muscular regions of the diaphragm. Stomata only allow entrance into the lymphatic system of particles with diameters below a certain cut-off that varies between species. For example, in mice, the stomata diameter measures 3.6  $\mu\text{m}$ , [38] in rats the cut-off is around 2.7  $\mu\text{m}$  [39] while in humans, it ranges from 1.6–1.8  $\mu\text{m}$ . [40] Small molecules can directly enter the blood stream from the peritoneal cavity, all larger molecules (> 20 kDa) or particles with diameters above > 20 nm, are cleared via the lymphatic system.[41,42]

The lymphatic vessel network begins with the lymphatic capillaries, which serve as the smallest vessels and the initial point of entry for lymph. These capillaries are found in several regions, including the parasternal, paravertebral, mediastinal, intercostal, and retroperitoneal areas, and form lymphatic vessels that connect to regional lymph nodes (LNs). LNs, which are small, bean-shaped structures distributed along the lymphatic vessels, play a central role in cancer metastasis. The human body contains

approximately 800 LNs, strategically located in regions such as the neck, abdomen, axilla, thorax, and groin (Fig. 3A). Peritoneal lymphatic drainage is primarily associated with the parasternal, mediastinal, and mesenteric LNs. As lymph gradually flows from the capillaries into larger vessels, trunks, and finally the ducts, it eventually drains into the venous circulation. The thoracic duct, the largest of these ducts, drains lymph into the junction of the left subclavian vein and left internal jugular vein, while the right lymphatic duct empties into the venous circulation between the right subclavian vein and the right internal jugular vein.[43,44]

The lymphatic system in mice shares fundamental anatomical and functional similarities with that of humans, facilitating lymph transport and immune cell trafficking. However, a notable distinction lies in the significantly reduced number of lymph nodes in mice compared to humans. Three major lymphatic drainage routes from the peritoneum have been identified. The principal option is the parasternal lymphatic pathway. It originates at the diaphragmatic stomata on the ventral side, draining into lymphatic vessels aligned with the internal thoracic artery. These vessels then lead to parasternal LNs, followed by mediastinal LNs, and ultimately to the right or thoracic lymphatic vessels. The second pathway, known as the thoracic cavity lymphatic route, also begins at the diaphragmatic stomata but drains into lymphatic vessels along the posterolateral aspect of the thoracic cavity, eventually reaching the mediastinal LNs. Lastly, the visceral abdominal lymphatic pathway connects the peritoneum to the cisterna chyli and thoracic ducts (Fig. 3B). Studies have shown that the majority of peritoneal fluid drains into the lymphatic system, with approximately 20–30% entering the thoracic duct, while the remaining 70–80% is returned to the venous circulation via the right lymphatic duct.[45–48]



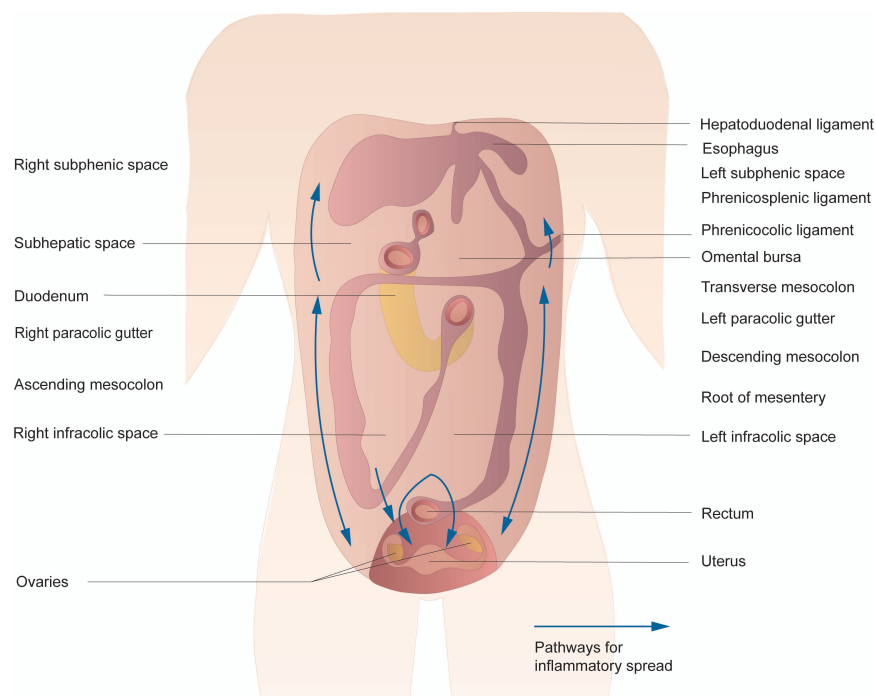
*Fig. 3: A) The human lymphatic system begins with blind-ended lymphatic capillaries within tissues, which join into pre-collecting and subsequently collecting lymphatic vessels. Lymph passes through one or more lymph nodes before joining into either the thoracic duct or right lymphatic duct. Ultimately, lymph is returned to the bloodstream at the junction of the internal jugular and subclavian veins. Based on a figure by Sarfarazi et al.[24] B) Lymphatic system within mice with three main routes of lymphatic drainage from the peritoneum. First pathway (orange) is the parasternal lymphatic pathway, second pathway (purple) depicts the thoracic cavity lymphatic pathway and the third pathway (blue) follows the visceral lymphatics pathway. Based on a figure by Lee et al.[49]*

#### 1.4 Anatomical structure of pathological peritoneum

In pathological conditions, peritoneal fluid can accumulate to volumes of 200 ml and beyond, a pathological condition referred to as ascites.[50] The formation of ascites is a result of an imbalance between the secretion and drainage of peritoneal fluid. Increased microvascular permeability results in higher protein content in the peritoneal cavity, while the secretion of cytokines and chemokines raises osmotic pressure. Furthermore, reduced lymphatic drainage magnifies fluid retention.[51] However, ascites is commonly associated with PC, where it serves as an indicator of poor prognosis. In ovarian carcinoma, the ascitic fluid contains tumor cells, lymphocytes, and macrophages.[52] Notably, analysis of ascitic fluid from ovarian and gastric cancers reveal elevated levels of pro-tumorigenic factors such as interleukins (IL-6, IL-10), which contribute to the tumor microenvironment and promote cancer progression.[53,54]

As illustrated in Fig. 4, malignant ascites facilitates tumor dissemination to different intraperitoneal sites. Fluid within the peritoneal cavity tends to accumulate at specific

sites, gravitating toward posterior regions when the patient is supine and toward lower abdominal sites in the upright position. Awareness of these predilection sites facilitates accurate identification of fluid collections in their respective compartments. In the presence of disease, cancer cells may migrate along these pathways in the peritoneal cavity, facilitating the spread of infection or cancer. Fluid redistribution occurs with changes in patient positioning, consistently moving toward the lowest gravitational point. Common sites for intra-abdominal fluid accumulation include the right and left perihepatic spaces, the right subhepatic space (Morison's pouch), subphrenic and perisplenic spaces, the omental bursa, perivesical space, and the interloop spaces between intestinal segments, often causing bowel loop separation.[55,56]



*Fig. 4: Pathways of inflammatory disease spread with flow of peritoneal fluid. Adapted from Dietrich et al.[55]*

One of the major obstacles to the success of i.p. chemotherapy is the limited penetration of therapeutic agents into large tumors. Transportation from the peritoneal cavity to tumors occurs via three pathways: convection, systemic circulation, and lymphatic networks. Convective forces facilitate surface delivery, while diffusive forces enable deeper penetration. Convection depends on pressure gradients and is influenced by tumor tissue's hydraulic conductivity, and stromal architecture. Diffusion rate is affected by temperature, therapeutic's properties, and tumor matrix structure.[57–59]

The tumor microenvironment (TME) serves as the primary physical barrier for effective drug penetration (Fig. 5). Its pathological characteristics differentiate from healthy tissues, featuring hypoxia, an acidic environment, an abnormal vascular network, elevated interstitial fluid pressure (IFP), and an overgrowth of the extracellular matrix (ECM), which is highly infiltrated by cancer-associated fibroblasts (CAFs) and marked by desmoplastic reactions.[60]

Among the key barriers to drug delivery, tumor solid stress plays a crucial role. The increased density of cancer cells, fibroblasts, collagen, and hyaluronan leads to an elevation in solid stress within the tumor. This stress not only hampers drug diffusion but also contributes to the remodeling of the vascular and lymphatic structures, further increasing IFP. High IFP, resulting from tumor vasculature leakage and obstructed lymphatic circulation, creates pressure gradients within and around the tumor, which significantly inhibits the diffusion of NPs and other therapeutic agents. Additionally, the tumor stroma, composed primarily of the ECM, is another significant barrier. The ECM in tumors contains a higher concentration of collagen and exhibits increased mechanical stiffness and rigidity compared to healthy tissues. The ECM's negative net charge, combined with the dense and interconnected network of collagen fibers, makes the tumor environment even less permeable to therapeutic compounds.[61,62] Typically, drug delivery by passive diffusion can only reach up to 3 mm depth in bulky tumors.[57–59] As drug uptake is mainly driven by concentration gradients, past research aimed to develop DDS that provide high local concentrations and sustained drug exposure.

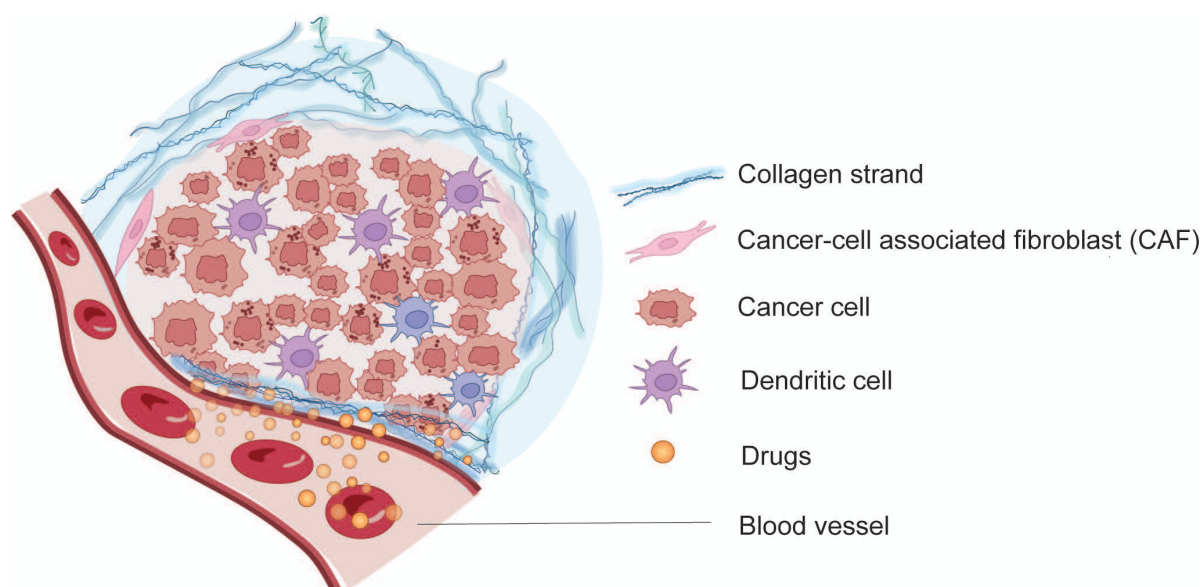
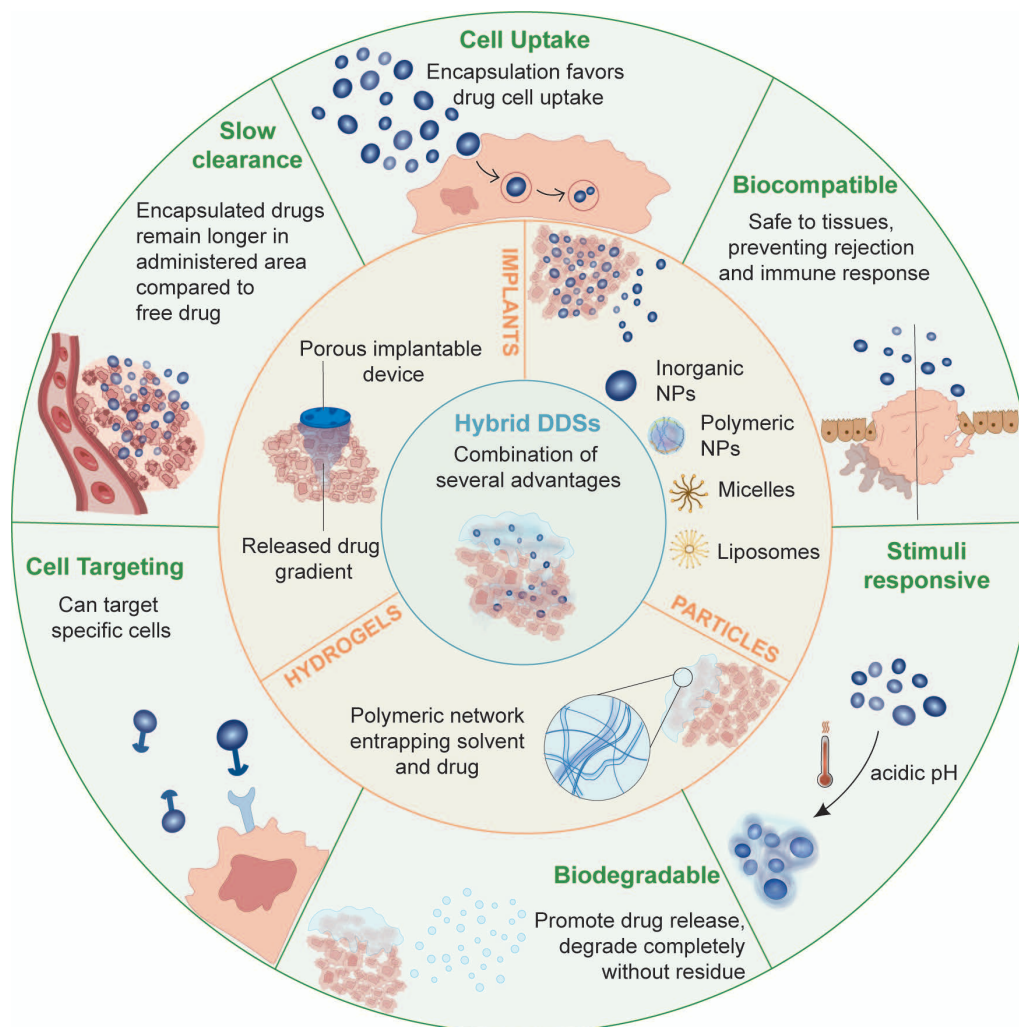


Fig. 5: Tumor microenvironment with drug diffusion barrier.



## 1.5 Types of DDS



*Fig. 6: Overview of DDS types according to their leading advantages and properties. These formulations can be combined to form hybrid DDS which implement advantages of the original formulations. Figure adapted from Perelló-Trias et al.[63]*

For optimal clinical application, DDS must be tailored to the specific needs of i.p. administration. The ideal distribution of NPs in i.p. therapy requires a high and sustained concentration within the peritoneal cavity over an extended period of time. This localized drug release should minimize systemic toxicity through rapid clearance after entering systemic circulation. This requires careful consideration of pharmacokinetics and physiology, as well as the advantages and limitations of each DDS type (Table 2). Such tailored systems improve therapeutic outcomes, ensuring a more precise and effective treatment strategy for peritoneal cancers.

DDS can be categorized according to various aspects, such as chemical composition, formulation, their cytostatic loading, and morphology. In the context of i.p. chemotherapy, several DDS types have been developed, including hydrogels,

implants, NPs, and hybrid systems (Fig. 6). These approaches allow for localized administration of chemotherapeutic agents, offering sustained release that pays respect to the unique physiology of the peritoneum and peritoneal cavity, thus enhancing drug concentration at the targeted site. Below the different DDS types as well as commonly used cytostatic drugs for i.p. chemotherapy are summarized.

*Table 2: Suitability of DDS for use in i.p. chemotherapy. Table adapted from Perelló-Trias et al.[63]*

| DDS       | Advantages                                                                                                                                  | Disadvantages                                                                                                                                                                                                                                                                                                                                                               |
|-----------|---------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Hydrogels | <ul style="list-style-type: none"> <li>• Prevention of postsurgical adhesions</li> <li>• Easily injectable</li> </ul>                       | <ul style="list-style-type: none"> <li>• Heterogeneous drug distribution</li> <li>• Viscosity issues (low viscous hydrogels may fail in providing delayed drug release, while high viscous hydrogels may present difficulties in administration)</li> <li>• Macrophage uptake</li> <li>• Remain mainly near injection site, limited diffusion through peritoneum</li> </ul> |
| Implants  | <ul style="list-style-type: none"> <li>• Can be fixed near tumor side</li> <li>• Increased and more localized drug delivery</li> </ul>      | <ul style="list-style-type: none"> <li>• Invasive</li> <li>• Surgical expertise needed for implantation</li> <li>• Increased tissue damage</li> <li>• Remain near injection site, no diffusion through peritoneum</li> </ul>                                                                                                                                                |
| Particles | <ul style="list-style-type: none"> <li>• High tumor nodule penetration and cell uptake</li> <li>• Extensive peritoneal diffusion</li> </ul> | <ul style="list-style-type: none"> <li>• Rapid peritoneal clearance through lymphatic system (depending on particle size)</li> </ul>                                                                                                                                                                                                                                        |



### 1.5.1 Non-particulate DDS

#### *Hydrogels*

Hydrogels are three-dimensional hydrophilic polymer networks characterized by a high-water content and an affinity for biological fluids, which allow them to retain various substances in their porous structure. Their deformability, moldability, and injectable nature make them particularly suitable for minimally invasive applications. Furthermore, their properties, such as pore size, gelation time, degradability, swelling capacity, and release rate, can be finely tuned during synthesis to optimize performance. Hydrogels generally exhibit bi-phasic release profiles: an initial diffusion phase followed by sustained release through degradation or erosion.

A key challenge in hydrogel-based DDS is viscosity control. Viscosity must be carefully balanced to ensure proper injectability and distribution within the peritoneal cavity. Hydrogels with low viscosity may diffuse too quickly, while those with high viscosity may be difficult to inject. Smart hydrogels, such as pH or temperature-sensitive versions, can address these issues by adapting their properties in response to environmental conditions. Thermosensitive hydrogels, for example, are liquid at room temperature (rt), facilitating easy administration, but solidify into a gel upon reaching body temperature, forming an *in situ* drug depot. Such examples are based on poly(D,L-lactide)-poly(ethylene glycol)-poly(D,L-lactide) (PDLLA-PEG-PDLLA) and poly(lactic-co-glycolic acid)-poly(ethylene glycol)-poly(lactic-co-glycolic acid) (PLGA-PEG-PLGA), which are gaining attention for their ability to undergo sol-gel transitions in response to temperature variations. This property not only facilitates easy injection but also ensures high mechanical strength and stability, making them promising candidates for i.p. DDS. In contrast, supramolecular hydrogels, driven by pH-responsive sol-gel transitions, combine the unique properties of PEG chains modified with ureido-pyrimidinone (UPy) units. When used with pressurized PIPAC, these hydrogels achieve uniform distribution within the peritoneal cavity through gelation due to acid stimulation provided by carbon dioxide, enhancing therapeutic efficacy. Furthermore, hyaluronic acid (HA)-based hydrogels, formed through covalent cross-linking, offer extended and more stable retention in the peritoneal cavity compared to their physically cross-linked counterparts, thus prolonging drug residence time.[64–67]

While hydrogels offer many advantages, they also present challenges. Their high water content and large pore sizes may lead to an initial burst effect, where the drug is

released rapidly before complete hydrogel degradation occurs. This can limit sustained drug delivery. Additionally, a notable research gap exists in the development of enzyme-responsive hydrogels for i.p. therapy. Enzymes represent promising triggers for the controlled release of anticancer drugs, offering the potential for more precise and effective drug delivery. Further exploration in this area could significantly enhance the therapeutic outcomes of i.p. chemotherapy. Optimizing the balance between adhesiveness, injectability, and controlled release is crucial to ensuring the therapeutic efficacy of these systems.[37,63,68]

### *Implants*

By implanting biocompatible depots directly at the tumor site, precise drug release is enabled, while off-target effects and systemic toxicity are minimized. One of the key advantages of implantable DDS is their ability to deliver drugs over an extended period, offering sustained therapeutic effects while allowing the adjustment of drug release rates based on degradation time.

PLGA is a preferred polymer in these systems due to its biodegradability and the ability to fine-tune its degradation rate to control drug release. The lactic acid:glycolic acid (LA:GA) ratio in PLGA-loaded materials is an effective parameter for controlling the degradation time and drug release rate. A higher GA content corresponds to a faster degradation rate. PLGA implants exhibit faster drug release in acidic environments, which aligns with slightly acidic TME, making them particularly effective. While initial burst release remains a challenge, a biphasic release pattern may provide a balance between achieving an effective initial dose and maintaining prolonged therapeutic action.[69]

A significant hurdle in cancer treatment, particularly in PC, is the challenge of multi-drug resistance (MDR), which can cause cancer recurrence even after apparent remission. To combat this, metronomic chemotherapy has emerged as a promising strategy. This approach involves the frequent, low-dose administration of chemotherapeutic agents, ensuring sustained local drug exposure, enhancing anti-tumor effects, and reducing resistance. Implants, such as electrospun polydioxanone nanotextiles incorporating PTX for sustained i.p. drug release, may provide a solution. In this study, approximately 55% of PTX was released within one week, with 70% released over 28 days.[70–72]

Despite their potential, implantable DDS face challenges, including tissue damage, adhesion complications, and the need for invasive insertion by healthcare professionals. Moreover, current implants face limitations in tumor cell penetration. However, combining implants with NPs could significantly enhance their therapeutic efficacy.[37,63]

### 1.5.2 Particulate DDS

#### *NPs and MPs*

NPs and MPs are a versatile biomaterial for DDS, offering controlled and targeted release of therapeutic agents. Their size influences release dynamics, residence time in the peritoneal cavity, and systemic circulation. Small particles evade immune detection longer and penetrate tumors deeper. Due to their small size, they are rapidly cleared from the intraperitoneal space via the lymphatic system, and subsequently re-enter systemic circulation through draining into the venous system. Eventually, most particles are cleared by reticuloendothelial system with main uptake organs being liver and spleen.

Inorganic NPs, synthesized from materials such as silica, gold, and iron oxides, have been explored for drug delivery applications due to their versatile properties. One of the key advantages of inorganic NPs is their ability to be easily functionalized. Mesoporous silica NPs (MSNs) administered via i.p. injection demonstrate tumor-specific accumulation and sustained drug release. While current research points to a "collagen matrix-based interaction" as a key targeting strategy, the precise intracellular transport mechanisms of MSNs remain unclear. PEG-tetramethylsilane (PEG-TMS)-coated MSNs ranging from 25 to 150 nm in size demonstrated biodistribution across the abdomen within 30 minutes of i.p. injection. Notably, these NPs exhibited significant accumulation in the mediastinal LNs. Over time, the distribution pattern evolved to resemble that of i.v. administration, with NPs transitioning from lymphatic vessels to systemic circulation, ultimately accumulating in organs such as the liver and spleen. This dynamic distribution demonstrates the potential of PEG-TMS-coated MSNs for targeted drug delivery and their ability to navigate the lymphatic system, offering insights into their therapeutic applications. Additionally, inorganic particles can generate heat in response to external stimuli, which has significant implications for therapeutic strategies such as hyperthermia. Gold NPs (AuNPs) with a layer of free electrons on their surface generate heat under near-infrared light (NIR), enhancing the

anti-tumor effects of chemotherapeutic agents like 5-FU. Additionally, magnetic NPs (MNPs) can induce localized heat, similar to HIPEC, and offer magnetic targeting for precise tumor localization, improving treatment efficacy. While magnetic NPs (MNPs) have shown potential to induce localized hyperthermia, similar to HIPEC, their clinical efficacy remains under investigation. Claims of enhanced tumor targeting and treatment precision through magnetic guidance are promising but currently limited by challenges such as uneven heat distribution, limited penetration depth, and difficulties in achieving consistent targeting *in vivo*. Magnetization is one of the most common methods used with inorganic NPs in cancer treatment. However, their potential toxicity, stability issues in physiological conditions, possible immune responses, and limited biodegradability remain significant challenges for their clinical use.[37,73–77]

Polymeric NPs, composed of one or more polymers, offer versatile strategies for drug encapsulation. Drugs can be either conjugated to the polymer network, encapsulated within, or attached to the surface of these particles, allowing for controlled delivery. One notable feature of polymeric NPs is their ability to exhibit stimuli-dependent release. For example, expandable NPs release drugs in response to environmental triggers, with significant release of PTX at pH 4 after 24 hours, in contrast to the slower release observed at physiological pH 7. The size of the particles plays a crucial role in their distribution and retention, particularly in the peritoneal cavity. *Lu et al.* demonstrated that 4  $\mu\text{m}$  particles exhibit better diffusion and retention compared to larger 30  $\mu\text{m}$  particles, which are more readily recognized and cleared by the immune system. Thus, overly large particles can face limitations in retention due to immune surveillance and hindered tissue access.[78,79]

Polymeric NPs also offer the advantage of reducing drug toxicity by improving their solubility, enabling the administration of higher dosages with reduced cytotoxicity to healthy tissue, thereby maximizing therapeutic effects. PLGA's biocompatibility and biodegradability make it an ideal material for this purpose, ensuring minimal toxicity and safe systemic clearance.[80] *Dwivedi et al.* reported curcumin-loaded PLGA-based MPs with tunable core-shell architecture and high encapsulation efficiency amounting over 70%. The resulting 20  $\mu\text{m}$  particles exhibited sustained release over 14 days and enhanced stability. i.p. administration in rats demonstrated prolonged peritoneal retention and superior pharmacokinetics compared to free drug.[81]

Biopolymers are preferred over synthetic polymers in drug delivery due to their biocompatibility, biodegradability, and non-toxicity, which reduce possible immune reactions and systemic toxicity. Their ability to degrade naturally due to enzymes in the body allows for controlled, sustained drug release. Biopolymers also offer versatility in design through possible modification, enhancing drug loading and targeting specific tissues. Additionally, they are environmentally sustainable, derived from renewable resources. The degradation rate of polymeric NPs is another critical factor influencing drug release. Rapid degradation can lead to accelerated drug release. Natural biopolymers, such as HA or collagen, degrade quickly, whereas modifying the degradation rate can help control drug release. As mentioned earlier, PEGylation is one approach to enhancing the bioavailability of drugs by preventing aggregation and phagocytosis. Additionally, modulating polymer concentration has been shown to influence the degradation rate; for instance, *Yamashita et al.* found that decreasing gelatin concentration accelerated degradation.[63,82]

However, a common challenge for polymeric NPs is their limited drug-loading efficiency, which hampers their clinical applicability. For example, *cis*-diamminedichloroplatinum (CDDP), commonly known as cisplatin, is a potent chemotherapeutic agent, yet faces significant challenges in drug delivery due to its poor solubility in both aqueous and organic solvents. The entrapment of CDDP in polymeric NPs is hindered by low encapsulation efficiency and suboptimal release profiles.[83,84] To address these issues, self-assembly strategies utilizing macromolecules such as gelatin as templates have been explored. Gelatin-poly(acrylic acid) (Gel-PAA) NPs, synthesized via polymerization of acrylic acid (AA) in a gelatin aqueous solution, exhibit stable core-shell structures with multiple functional groups. CDDP is incorporated into these NPs through platinum-carboxyl interactions. The release of CDDP from Gel-PAA NPs demonstrates a controlled release profile with an initial burst of only 13–28% over 8 hours. Furthermore, the high encapsulation efficiency (up to 70%) is attributed to the abundance of carboxyl groups from both gelatin and PAA, facilitating significant drug loading and controlled release kinetics.[85,86]

### **Micelles**

Micelles composed of amphiphilic block copolymers have emerged as promising carriers for the loading and delivery of hydrophobic drugs, such as DTX. In this regard,

*Hang et al.* developed a micellar system based on methoxy-poly(ethylene glycol)-block-poly(*D,L*-lactide) (mPEG-PDLLA), which demonstrated an exceptionally high encapsulation efficiency of  $99.2 \pm 0.6\%$ . This high encapsulation capacity, coupled with the physical entrapment of the drug within the micellar structure, contributed to a slower and more controlled drug release profile. An option for CDDP incorporation is its chemical complexation by amphiphilic block copolymers containing carboxylic groups such as poly (*L*-glutamic acid)-*b*-poly (2-methacryloyloxyethyl phosphorylcholine) (PLG-*b*-PMPC) obtaining micellar structures with polymer-metal complexes with an average size of 91 nm which prevent initial burst release and increase drug loading to 15%. [87,88]

### *Liposomes*

Lipid-based nanocarriers have garnered significant attention in DDS due to their biocompatibility and biodegradability. Among these, liposomes composed of amphipathic phospholipids are a prominent class. Liposomes can be classified into four types based on their structure: unilamellar vesicles (ULVs), oligolamellar vesicles, multilamellar vesicles, and multivesicular liposomes (MVLs). ULVs, in turn, can be further categorized by size, including small, large, and giant particles, offering flexibility in design depending on therapeutic needs. [89]

Anionic liposomes, which are formed by lipids carrying a negative charge, exhibit a strong binding affinity for certain drugs, such as mitoxantrone. For instance, cardiolipin liposomes have been shown to possess the strongest binding affinity for mitoxantrone, enhancing the delivery of this chemotherapeutic agent. Liposomes are typically limited by their aqueous core, which restricts them to loading hydrophilic chemotherapeutic agents. However, while hydrophobic drugs have been shown to be incorporated into the lipid bilayer of liposomes, their release kinetics differ significantly from those of hydrophilic agents, potentially affecting therapeutic outcomes. [90–93] Additionally, liposomes can be engineered to respond to specific environmental conditions. pH-sensitive liposomes, for example, are designed to release their drug payload in acidic TME, which is beneficial for localized delivery and improved drug retention within peritoneum. [63,94–96]

Furthermore, liposomes with their high surface lipophilicity with diameters exceeding 500 nm show reduced clearance when drained through the lymphatic system, resulting in significantly prolonged retention compared to free drugs. However, even cationic

liposomes, which are known for their extended retention properties, remain in the peritoneal cavity for a maximum of only three days.[97,98]

### 1.5.3 Hybrid DDS

Hybrid DDS combine the advantages of simple DDS, such as hydrogels and NPs, resulting in new characteristics that favor controlled drug release and improved drug retention at the target site. One of the most widely studied configurations involves embedding e.g. NPs, or micelles within injectable hydrogels. This combination enables slower drug release while preventing rapid clearance, which is a common challenge in conventional delivery methods.[99,100]

Hybrid DDS are often based on copolymer combinations, offering a range of formulations tailored to specific needs. For example, alginate-based nanogels have been widely used due to their biocompatibility and versatility. Thermosensitive hydrogels are particularly favored for these systems because they can gelate at the injection site, limiting drug dispersion and enhancing the localized drug delivery. In contrast, non-thermosensitive, chemically cross-linked hydrogels are gaining attention due to their resistance to dilution after injection, ensuring better retention and control over drug release. One study highlights the superior anticancer efficacy of a gelatin-based hydrogel containing CDDP-loaded Gel/PAA NPs administered via peritumoral implantation, compared to i.v. delivery in a murine hepatoma model. Strikingly, five out of six mice receiving the hydrogel implant showed complete tumor inhibition within 15 days, with a tumor volume doubling time extended to 22.5 days, over four times longer than in the i.v. group (5.3 days). Biodistribution analysis revealed significantly higher intratumoral CDDP concentrations, up to threefold compared to i.v. administration, even at later time points, while reducing off-target organ exposure. Immunohistochemical studies confirmed that the NPs infiltrated tumor tissue via both passive diffusion and local vasculature, enabling deeper and more uniform drug penetration.[100–103]

Overall, hybrid DDS offer substantial benefits for the treatment of PC through i.p. drug delivery. These systems enable the development of tunable, controlled drug release strategies, making them a promising avenue for overcoming the limitations of traditional drug delivery methods.

## 1.6 Peritoneal anatomy and physicochemical properties impacting NP *in vivo* fate in intraperitoneal chemotherapy

The advantages of employing i.p. drug delivery include prolonged localized drug retention in the peritoneum due to the peritoneal-plasma barrier which makes it an effective approach for targeting peritoneal lesions compared to systemic treatment. Additionally, the ability of NPs to exploit lymphatic pathways to enter common sites of metastasis such as lymph nodes holds potential for improved treatment outcomes. For application and translation of i.p. drug delivery, several key factors are crucial (Table 3).

As explained above, the peritoneal cavity has a size-dependent clearance pathway for NPs. As particles smaller than 20 nm can enter the blood stream directly, their clearance is considered too fast for i.p. drug delivery. Particles larger than 20 nm are drained through the lymphatic system, eventually re-entering the circulatory system. While NPs smaller than 50 nm can pass through LNs, particles exceeding 500 nm tend to be retained in these nodes. Liposomes sized at 1000 nm have been identified as optimal with respect to retention in the peritoneal cavity.[104] Interestingly, studies have shown no clear correlation between particle size and peritoneal clearance when liposomes are smaller than the stomata size of the peritoneal cavity.[105] However, the use of particles with diameters larger than the stomata size presents several challenges. Particles can cause peritoneal adhesion and inflammation, show inhomogeneous distribution within the cavity, and poorly penetrate tumor tissue.[78,104,106,107] Furthermore, larger particles also tend to be localized near the injection site in the lower abdomen, limiting their therapeutic effectiveness.[78] Particle size also plays a crucial role in tumor penetration. Liposomes sized around 100 nm typically enter tumors through the recirculation pathway, while those larger than 1000 nm utilize convection. Larger particles, such as 3000 nm liposomes, tend to deposit on the outer surface of abdominal organs, limiting their penetration. NPs with a hydrodynamic diameter smaller than 30–50 nm are generally more likely to penetrate solid tumors. Ultimately, the balance between particle size for optimal peritoneal retention and efficient tumor penetration is to some extent contradictory and still topic of research.[42,108]

The physiological characteristics of the peritoneum with its anionic surface charge and rapid drug-plasma exchange strongly determines NP behavior. The material properties



of NPs, such as surface charge and size consequently influence their peritoneal retention, organ distribution, protein adsorption, macrophage uptake, lymphatic drainage, and tumor accumulation and penetration. For example, electrostatic interaction of NPs with a positive surface charge with the negatively charged peritoneal membrane leads to a prolonged retention. However, this increased retention is accompanied by reduced lymphatic and LN recovery. This may be attributed to the increased interaction of positively charged liposomes with antigen-presenting cells, which remain in or traffic to LNs. In contrast, negatively charged liposomes are cleared more rapidly from the peritoneal cavity but show higher uptake by peritoneal macrophages. Furthermore, positively charged liposomes show overall a lower lymphatic uptake with the proportion that enters lymph showing higher retention in visceral lymphatics and mesenteric LNs.[49] In contrast, negatively and neutrally charged liposomes tend to drain through diaphragmatic lymphatics, leading to their retention in mediastinal LNs.[49] Regarding size and charge interactions, liposomes with diameters of 100 nm and 1000 nm exhibit different behaviors based on their charge. Positively charged liposomes demonstrated the highest peritoneal accumulation after 48 hours. Consequently, the retention of negatively charged liposomes decreased significantly upon the incorporation of negatively charged distearoylphosphatidylglycerol (DSPG). Additionally, macrophage uptake of negatively charged liposomes is more pronounced with increasing particle size. The surface charge also dictates protein adsorption, with positively charged NPs predominantly binding proteins such as albumin ( $pI \leq 5.5$ ), while negatively charged NPs preferentially adsorb proteins like IgG ( $pI \geq 5.5$ ). In summary, surface charge is a more significant factor than particle size below the cut-off of stomata in influencing peritoneal retention and lymphatic drainage. Despite a growing body of evidence suggesting that positively charged particles exhibit longer peritoneal retention, the impact of surface charge on intratumoral penetration and cellular uptake remains poorly understood, which warrants further research to optimize NP-based drug delivery strategies.[109–112]

Additionally, the rich capillary network in the peritoneum facilitates drug-plasma exchange, which typically leads to decreased retention time for small-molecule drugs, limiting their therapeutic effectiveness. However, frequent peritoneal and/or ascitic fluid turnover may also accelerate NP excretion. Moreover, pathological changes in peritoneal fluid composition may affect NP behavior *in vivo*. In detail, NPs undergo

interactions with biological components upon entering the physiological environment, forming a "protein corona" that alters their properties. The protein corona's composition is influenced by NP characteristics and factors like body fluids, temperature, pH, and disease conditions. The protein composition in ascites resembles that in blood, with ascitic fluid containing approximately half the protein concentration of human serum with more than 93% of identified proteins also present in plasma. The protein corona can increase NP size, influence surface modifications, weakening their targeting efficiency, stability, and causing rapid clearance by the mononuclear phagocytic system (MPS). However, in the peritoneal cavity, the interaction between NPs and the protein corona may also offer certain benefits, such as prolonging the retention time of NPs within the peritoneum. NP size increase from 200 nm up to 1000 nm after 36 incubation with ascites has been shown to lead to extended retention up to 120 hours.[113] Furthermore, PEGylation can reduce aggregation and enhance stability.[97,114] The dynamics of NP behavior in ascites also influence their pharmacokinetics, with some studies showing hindered initial release, presumably as a result of hindrance by outer protein corona layer,[113] while others demonstrate burst release due to competition between charged proteins and encapsulated drugs.[114,115] Ascites can also reduce NP cellular uptake, affecting their therapeutic efficacy. Macrophages, key components of the MPS, make up about half of the cellular composition in ascites and play a crucial role in the clearance and targeting of NPs. The extent of NP uptake by macrophages depends on particle size and surface properties, with larger NPs being more easily phagocytosed. Negatively charged liposomes are preferentially internalized compared to neutral or positively charged due to receptors on macrophage surface for negatively charged phospholipids. Additionally, the size of NPs significantly influences their accumulation in the liver and spleen. As larger particles are more readily taken up by the MPS, their clearance and accumulation are accelerated in these organs. Studies have demonstrated that liposomes around 400 nm exhibit the highest redistribution to the liver and spleen compared to smaller or larger particles.[104,113–120]

To reduce macrophage uptake, PEGylation is commonly used. PEG forms a hydration layer around NPs, neutralizing their charge and preventing macrophage phagocytosis. PEGylation of anionic liposomes did not alter DOX levels in the peritoneal cavity but increased concentrations in plasma and tumors, especially for 150 nm liposomes. This may result from the stealth effect of PEGylation, which reduces phagocytosis by

macrophages in the peritoneal fluid and, for negatively charged particles, reduces the repulsion of peritoneal mesothelial cells. However, for larger liposomes ( $\geq 1000$  nm), PEGylation has been shown to increase macrophage opsonization and phagocytosis, leading to reduced peritoneal retention and increased splenic localization. PEG also reduced aggregation, decreasing retention time for positively charged liposomes. PEGylation might interfere with specific tumor interactions, such as collagen in the ECM, potentially reducing tumor accumulation. Thus, the overall effectiveness of using PEGylated liposomes in i.p. therapy remains inconclusive.[97,109,121,122]

On top of that, TME presents several challenges to i.p. chemotherapy. Particularly, solid stress, high IFP, and the dense tumor stroma, are crucial factors to address penetration depth in tumors. Collagen fiber density and geometry within the ECM also impact NP diffusion. In i.p. administration, NPs diffuse into the tumor from its exterior, but increased IFP creates outward convection, limiting NP penetration. Additionally, particles preferentially accumulate at the tumor periphery through distribution of the lymphatic network.[31,123]

In addition to tumor's physiological structure, NP tumor accumulation also depends on the physicochemical properties of the NPs. Superior anti-tumor effects at low dosages were found with 10 nm NPs compared to 200 nm NPs. This effect is attributed to differences in tumor penetration, with the 10 nm size being more beneficial for effective tumor uptake.[124]

When considering tumor accumulation and penetration, the impact of surface charge remains inconclusive. Positively charged particles may be trapped at the surface of tumors due to the negatively charged membranes of cancer cells, although some studies have suggested that positively charged particles penetrate tumors better than their negatively charged or neutral counterparts. In contrast, negatively charged particles have been shown to exhibit superior tumor penetration in some studies.[125–127]

Active targeting strategies, where NPs are conjugated with ligands that specifically bind to overexpressed receptors on tumor cells, should induce receptor-mediated endocytosis. However, several challenges remain with targeting approaches. Tumor heterogeneity can render single-target therapies ineffective, as resistance may develop. Moreover, modifying NPs with targeting ligands can lead to the "binding site barrier" effect, where rapid binding of high-affinity ligands to tumor cells limits NP

permeability and prevents deep tissue penetration.[128] For instance, folic acid-modified mesoporous silica NPs (MSNs) demonstrated enhanced accumulation *in vitro*, yet there were no significant differences *in vivo* compared to unmodified MSNs or PEGylated MSNs. This outcome suggests that tumor ECM, rather than the targeting modification itself, plays a more substantial role in NP accumulation at the tumor site.[37,73,129]

Yet, the biodistribution pattern of NPs is also closely linked to toxicity. To a large extent, NPs ( $d < \text{cut-off}$ ) translocate into the lymphatic vasculature, accumulate within LNs, and subsequently drain into the venous circulation via the thoracic duct, facilitating their systemic distribution. Macrophage-mediated phagocytosis in the peritoneal cavity often leads to higher NP accumulation in the spleen and liver.[104,109,130,131]

To improve therapeutic outcomes, several advanced sustained-release systems that can act as repositories for chemotherapeutic drugs have been explored. Physical methods like ultrasound, radiation, and hyperthermia are being explored to enhance their tumor penetration. Localized hyperthermia increases cell membrane permeability, boosting the influx of chemotherapeutic agents. For this reason, thermosensitive liposomes (TSLs), used with hyperthermia, have been shown to improve drug delivery. Furthermore, the combination of nanocarriers with HIPEC, PIPAC, ePIPAC or high-intensity focused ultrasound (HIFU) can help overcome tumor penetration barriers and has shown promising synergistic effects, improving drug distribution and therapeutic efficacy.[13,132–138]

*Table 1: In vivo fate of DDS in dependence of their physicochemical properties after i.p. administration. Adapted from Pan et al.[37]*

|                                          | Peritoneal retention                                                                                                                                                                                                                                                                                            | Tissue distribution                                                                                                                                                                                             | Interactions in peritoneum                                                                                                                                | Tumor penetration                                                                                                                                                                               |
|------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Particle size                            | <ul style="list-style-type: none"> <li>• &lt;20 nm: blood clearance [24,139]</li> <li>• &gt;20 nm: lymphatic drainage [24,139]</li> <li>• &lt;100 nm: lymph to blood in 2–24 h[104]</li> <li>• 500 nm: LN accumulation[104]</li> <li>• 1000 nm: optimal[104]</li> <li>• 3000 nm: organ surface [104]</li> </ul> | <ul style="list-style-type: none"> <li>• 400 nm: highest uptake in liver and spleen[104]</li> </ul>                                                                                                             | <ul style="list-style-type: none"> <li>• Larger particles (&lt;1 <math>\mu</math>m): increased MPS uptake[76]</li> </ul>                                  | <ul style="list-style-type: none"> <li>• &lt;30–50 nm: penetration into solid tumors[107]</li> </ul>                                                                                            |
| Surface charge                           | <ul style="list-style-type: none"> <li>• Positive: higher peritoneal level, longer retention time, higher uptake through visceral lymphatics[49,109]</li> <li>• Negative: higher uptake through diaphragmatic lymphatics[49]</li> </ul>                                                                         | <ul style="list-style-type: none"> <li>• 100 nm positive: high spleen levels[109]</li> <li>• Positive: overall reduced lymphatic uptake with higher proportion entering lymph accumulated in LNs[49]</li> </ul> | <ul style="list-style-type: none"> <li>• Negative: macrophage uptake, IgG adsorption[111,112]</li> <li>• Positive: albumin adsorption[111,112]</li> </ul> | <ul style="list-style-type: none"> <li>• Positive: high tumor accumulation[127]</li> </ul>                                                                                                      |
| PEGylation                               | <ul style="list-style-type: none"> <li>• No change or increase in peritoneal retention[109,121]</li> <li>• For 1000 nm: may cause instability and decreased i.p. retention levels[109]</li> </ul>                                                                                                               | <ul style="list-style-type: none"> <li>• Reduced levels in gastrointestinal tract, spleen and liver[109]</li> </ul>                                                                                             | <ul style="list-style-type: none"> <li>• Stealth effect: reduces phagocytosis and repulsion by peritoneal mesothelial cells[37,109]</li> </ul>            | <ul style="list-style-type: none"> <li>• PEGylation of negative liposomes: higher drug concentration in tumors[121]</li> <li>• PEGylation of positive liposomes: no benefit [97,121]</li> </ul> |
| Ligands targeting tumor-specific markers | –                                                                                                                                                                                                                                                                                                               | –                                                                                                                                                                                                               | –                                                                                                                                                         | <ul style="list-style-type: none"> <li>• Higher tumor accumulation or no effect[73]</li> <li>• “Binding site barrier” effect[128]</li> </ul>                                                    |

## 1.7 Translation into clinic

To optimize DDS performance, rigorous safety and efficacy testing in *in vitro* and *in vivo* models is essential to ensure biocompatibility and prevent adverse events. Clinical translation requires a multidisciplinary approach, involving biology, material science, chemistry, engineering, and medicine. Research on DDS formulations for PC are mostly driven from chemistry and material science aspects using generic chemotherapeutics for a proof of concept with subsequent *in vivo* testing in animal models. Many times, the choice of drug and animal model does not match the final clinical application, hampering further development. Another significant challenge is the variation across experimental studies, with no standardized experimental setting. Differences in the types of animal models used (e.g. mice or rats),[38,81,113] tumor models (e.g. colorectal, gastric, ovarian or pancreatic cancer)[78,82,113,140] and treatment regimens, such as single injections, multiple injections, or continuous dosing are common.[141–145] The lack of standardized protocols complicates direct comparison of results and hinders the identification of optimal treatment strategies. To address this, collaboration between researchers and clinicians is necessary to ensure clinical relevance, and a more systematic approach to DDS design and drug selection. Recent clinical studies highlight the potential of DDS in various cancer types, demonstrating the promise of DDS for advancing cancer therapy from preclinical research to clinical application.[106,136,146]

The development of DDS is an intricate and resource-intensive process, requiring extensive time, investment, and rigorous testing before clinical trials can commence. Preclinical studies play a critical role in evaluating the safety and efficacy of DDS, necessitating the careful selection of animal models that closely mimic clinical conditions. However, current models, particularly those for i.p. chemotherapy targeting PC, are unable to replicate key disease mechanisms, including tumor detachment and angiogenesis. The human peritoneal environment, marked by dynamic interactions and fluid pressures, is challenging to simulate in rodents, underscoring the need for advanced models.[63,147] A key difference is that most animal models rely on the injection of cancer cells to form metastases, whereas human tumors typically originate from a primary tumor that sheds cancer cells into the intraperitoneal space, which subsequently form metastases. In this respect, while patient-derived xenografts capture the genetic variability of patients' tumors, they fail to replicate the natural tumor progression and microenvironment. Additionally, the use of immune-deficient mice

complicates handling, experimental set-up and removes the possibility to assess any immune reactions. Potentially, the use of genetically engineered animals may be advantageous. The animals are genetically modified to spontaneously develop tumors through oncogene activation or tumor suppressor gene loss. Because tumors arise naturally in immunocompetent hosts, this model may provide a more realistic tumor microenvironment while preserving immune interactions, better mimicking natural tumor development and progression.

Patent data reveals that particulate DDS, including polymeric NPs, liposomes, and micelles, dominate the landscape, with 28 patent families identified. Despite promising preclinical results, the translation of DDS into clinical settings is often hindered by pharmacokinetic variability and reduced efficacy in human tumors. Notably, clinical trials exploring i.p. formulations of PTX have demonstrated promising initial findings, yet these studies remain limited in number. Recent clinical research in i.p. drug delivery has primarily focused on albumin NPs, a natural compound (Table 4). Among the promising candidates, NanoTax<sup>®</sup>, a sterile PTX powder designed for i.p. administration, has demonstrated significant potential. When administered, PTX concentrations in peritoneal fluid were found to be 450–2900 times higher than those in plasma, with minimal systemic exposure and reduced toxicity. Clinical results from a study involving 21 patients with peritoneal solid tumors revealed varied responses: 4 patients remained stable, 12 showed disease progression, and 5 survived for more than 400 days.[63,146]

Also albumin-based NPs loaded with PTX (nab-PTX<sup>®</sup>) is currently investigated in combination with PIPAC technology and CDDP. In clinical trials, i.p. administered nab-PTX<sup>®</sup> exhibited a 147-fold higher peritoneal-to-plasma area under the curve (AUC) ratio compared to standard treatments. Among 27 patients, 8 experienced progression-free survival for over six months, 1 achieved a complete response, and 6 maintained stable disease. Aerosolized nab-PTX<sup>®</sup> also showed promising anticancer efficacy, with a median overall survival of 10 months and a one-year survival rate of 50%, dropping to 20% after two years.[136,148,149]

One study aimed to assess the safety and pharmacokinetics of i.p. administration of Paclimer<sup>®</sup> microspheres (polylactofate/PTX 10% w/w) in ovarian cancer patients. This Phase I trial demonstrated that the treatment was well tolerated up to 1200 mg Paclimer<sup>®</sup>/m<sup>2</sup>, with no dose-limiting toxicity observed. However, inflammatory

responses and bowel obstruction were observed, most likely contributing to the decision by the manufacturer (Guilford Pharmaceuticals, Inc.) to terminate the study before a maximum tolerated dose could be defined (Guilford Pharmaceuticals, Inc.).[106] In contrast, an ongoing study is investigating the pharmacokinetics and antitumor effects of PTX-loaded tumor-penetrating MPs (TPMPs) in a Phase I trial. The target population consists of patients with PC who are not eligible for standard-of-care therapies. Patient recruitment is currently underway, with the study expected to conclude in January 2026.[150]

Recently, nanoliposomal irinotecan (nal-IRI) has been approved by the Food and Drug Administration (FDA) and European Medicines Agency (EMA) for treatment of metastatic pancreatic adenocarcinoma in 2024 (clinical trial ID: NCT04083235).[151,152] This formulation has also gained attention for use in HIPEC procedures. This combination has been shown to reduce toxicity and adverse events compared to systemic administration. Clinical trials have reported that 83% of patients maintained a disease-free status during a six-month follow-up.[153]

A promising new study is investigating the efficacy, tolerability, and safety of a single i.p. injection of the investigational drug *Prospidelong* (prospidium chloride as active ingredient) in patients with disseminated gastric cancer. This novel DDS is based on a pH-sensitive hydrogel derived from dextran phosphate. Patient recruitment is ongoing, and the study is estimated to finish by December 2025.[154]

Translational research faces several hurdles, including protocol inconsistencies, patient population heterogeneity, and high failure rates in clinical trials. To improve success rates, refining hypotheses, integrating data from preclinical and clinical studies, and fostering collaborations with pharmaceutical industries are essential strategies. Leveraging artificial intelligence and repurposing existing DDS can also enhance research efficiency. Furthermore, the use of human tissues for biomarker discovery and precision medicine could provide more relevant disease models. The clinical implementation of novel DDS for i.p. chemotherapy is limited by a lack of long-term safety and efficacy data, despite promising preclinical results. To overcome these barriers, continuous research and technological innovation are essential. Key measures include establishing consistent research protocols and promoting transparent reporting of results (including negative findings). These efforts can improve decision-making in clinical practice and foster collaboration among researchers,



clinical institutions, and industry partners, accelerating the development and clinical integration of DDS for enhanced cancer therapy.

Table 4: Clinical trials of DDS for i.p. chemotherapy. Adapted from Perelló-Trias et al. [63].

| Formulation, indication                                                                           | Physicochemical properties                                        | Status                | Enrolled patients | Outcome                                                                                                                                                                                                                          | Identifier       |
|---------------------------------------------------------------------------------------------------|-------------------------------------------------------------------|-----------------------|-------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------|
| <b>Nanotax<sup>®</sup></b><br>peritoneal malignancies, solid tumors confined to peritoneal cavity | 600–700 nm, rod-shaped, nanoparticulate PTX powder for suspension | Phase I, completed    | 22                | PTX levels in peritoneal fluid reached 450 to 2900 times higher than peak drug plasma levels without increased toxicity                                                                                                          | NCT0066991[146]  |
| <b>Nab-PTX</b><br>advanced cancer confined to peritoneal cavity                                   | 130 nm                                                            | Phase I, completed    | 27                | 8 of 27 patients achieved progression free survival of at least 6 months                                                                                                                                                         | NCT00825201[148] |
| <b>Nab-PTX</b><br>PIPAC: PM from ovarian, breast, gastric, hepatobiliary, pancreatic origin       | 130 nm                                                            | Phase I, completed    | 23                | After median follow up of 12 months, overall survival was 10 months with survival rate of 50% after 1 year and 20% after 2 years                                                                                                 | NCT03304210[136] |
| <b>Nab-PTX, CDDP</b><br>PIPAC: PC of gastric, pancreatic, ovarian origin                          | Nab-PTX: 130 nm                                                   | Phase I, ongoing      | 36                | –                                                                                                                                                                                                                                | NCT04000906[149] |
| <b>Nal-IRI</b><br>HIPEC: PC from appendiceal, colorectal neoplasms                                | 110 nm                                                            | Phase I, completed    | 18                | During 6-month follow-up: 83% (n = 15) of patients remained disease-free                                                                                                                                                         | NCT04088786[153] |
| <b>Paclimer<sup>®</sup> microspheres</b><br>ovarian, primary PC                                   | 20 to 200 µm, polylactofate/PTX 10% w/w                           | Phase I, discontinued | 13                | Well tolerated up to 1200 mg Paclimer <sup>®</sup> /m <sup>2</sup> without dose-limiting toxicities; discontinued by manufacturer (Guilford Pharmaceuticals, Inc) before determining maximum tolerated dose, reasons undisclosed | NCT00005046[106] |

## Introduction

|                                                                                                               |                                      |                           |                    |   |                                  |
|---------------------------------------------------------------------------------------------------------------|--------------------------------------|---------------------------|--------------------|---|----------------------------------|
| <b>PTX-loaded tumor penetrating MPs (TPMPs)</b><br>PC                                                         | Polymeric MPs                        | Phase I, ongoing          | 30 (estimated)     | – | NCT05159050                      |
| <b>nab-PTX, gemcitabine, CDDP</b><br>PIPAC: biliary tract cancer, PM                                          | 130 nm                               | Phase I, ongoing          | 12 (estimated)     | – | NCT05285358[155]                 |
| <b>Nab-PTX, PTX, Ramucirumab</b><br>PIPAC: stomach cancer with PM                                             | 130 nm                               | Phase I, ongoing          | 30 (estimated)     | – | NCT06675136                      |
| <b>Nab-PTX</b><br>Combined PIPAC with systemic Nab-PTX-<br>Gemcitabine for PM from pancreatic cancer          | 130 nm                               | Phase 2, ongoing          | 38 (estimated)     | – | NCT05371223[156]                 |
| <b>Sintilimab, S-1/Oxaliplatin, Nab-PTX</b><br>gastric/gastroesophageal adenocarcinoma with malignant ascites | 130 nm                               | Phase 2, ongoing          | 35; 38 (estimated) | – | NCT06046963;<br>NCT04781413[157] |
| <b>Prospidelong</b><br>disseminated gastric cancer                                                            | Dextran phosphate hydrogel drug[154] | Phase 2, Phase 3, ongoing | 120 (estimated)    | – | NCT06232434                      |

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## 2 Motivation and Aims

This thesis explores new DDS for treatment of peritoneal carcinomatosis (PC) using a theranostic approach. The focus is on creating DDS based on biodegradable and biocompatible materials, which could potentially also be translated into clinical use. From regulatory perspective, materials are avoided that are not biocompatible or biodegradable. Upon introduction into the peritoneal cavity, the particles should ideally distribute and preferentially accumulate at common metastatic sites through passive targeting. This ensures that cancer cells are exposed to the cytostatic drug over an extended time period, as the drug is gradually released from the particles.

The main objective is to combine these DDS with advanced imaging techniques to enable longitudinal, quantitative visualization of biodistribution. This combined approach of image-guided drug delivery, often termed theranostics, will allow for real-time tracking of the DDS and its drug payload, thereby providing essential insights into their behavior *in vivo*. This thesis will investigate the impact of key factors, including the size and surface charge of the DDS, as well as the incorporation of different cytostatic drugs, both hydrophilic and hydrophobic, to ensure that the DDS can be applied to PC originating from various primary tumor types. The primary focus will be on PTX and CDDP, two widely used chemotherapeutic drugs for treatment of various abdominal cancers.

The research will encompass the synthesis of biocompatible polymer-based DDS, their drug loading, *in vitro* testing, and imaging based on quantification of biodistribution in mice over several days. The goal is to identify critical factors that influence biodistribution and ultimately establish a comprehensive DDS platform for cancer therapy, providing a systematic approach to improving treatment outcomes in PC. The aim of this thesis is to investigate several particulate DDS, as illustrated in Fig. 1.

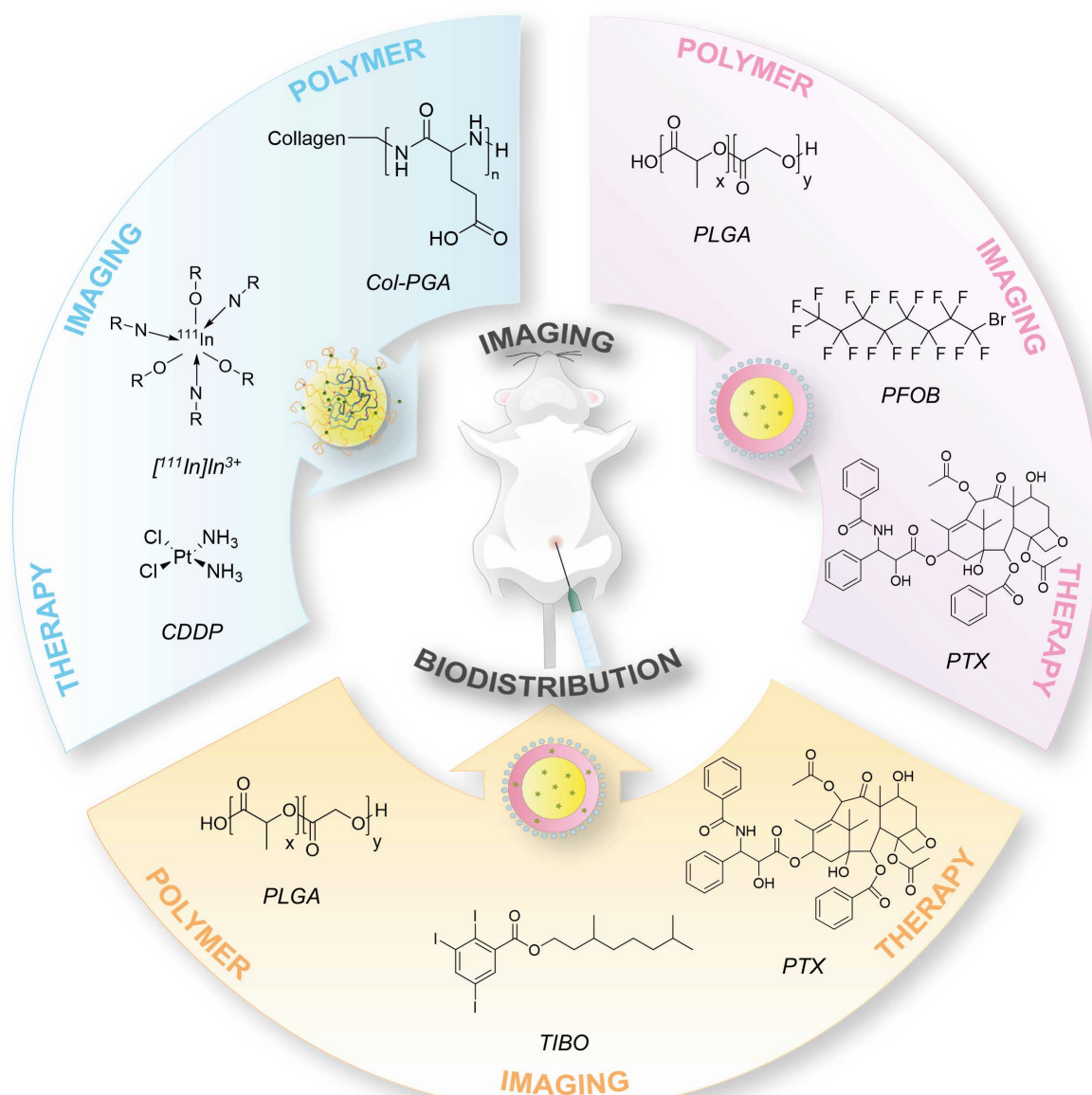


Fig. 1: Schematic overview of aim of thesis with three distinct projects leading to imageable biodistribution studies in mice after i.p. administration.

In **chapter 3.1**, PLGA-based particles filled with PFOB are produced with defined diameters and loaded with PTX, optimized for longitudinal  $^{19}\text{F}$ -MRI imaging of biodistribution. Particle size is precisely controlled through formulation parameters to yield core-shell particles across a range of diameters. Following comprehensive *in vitro* characterization, *in vivo* studies utilize the novel BaSSI sequence to enable sensitive detection and spatial mapping of minuscule fluorine amounts on a clinical MRI platform. This strategy allows for sensitive, longitudinal assessment of the biodistribution of PTX-loaded, fluorinated PLGA particles, advancing non-invasive tracking of drug carriers at very low fluorine concentrations.



In **chapter 3.2**, the first objective is the development of PLGA-based particles encapsulating TIBO and PTX with tunable particle diameters in a solvent-emulsion-evaporation process. The iodinated oil TIBO acts as a contrast agent in CT imaging and as a solvent for PTX. The particles are characterized *in vitro* with respect to cancer cell toxicity and drug release profiles. Subsequently, *in vivo* studies are conducted to enable longitudinal assessment of biodistribution using DECT imaging on a clinical modality. This approach offers a quantitative framework for monitoring of particle distribution *in vivo*, facilitating evaluation of size-dependent pharmacokinetics and tissue distribution over time.

In **chapter 3.3**, NPs based on biocompatible collagen-poly(glutamic acid) are developed for efficient loading with low soluble platinum-based chemotherapeutics. Cell toxicity and drug release are studied *in vitro*. The *in vivo* fate of these NPs is explored in animal studies employing  $^{111}\text{In}$ -radiolabeled NPs for SPECT imaging. The imaging study provides quantitative, longitudinal assessment of pharmacokinetic behavior and biodistribution, opening the possibility to link material properties to detailed pharmacokinetic and pharmacodynamic (PKPD) information.

Finally, this thesis concludes with an overall discussion of the obtained experimental results, which systematically investigate the influence of key parameters such as particle size, surface charge, and drug loading on the release profiles, and biodistribution of the developed DDS. Critical factors for optimizing future therapeutic strategies for PC are identified.

### 3 Results

#### 3.1 Fluorine-19 MRI BaSSI spectroscopic imaging employing fluorinated drug-loaded particles for image-guided intraperitoneal delivery

Peritoneal carcinomatosis is a late-stage cancer with poor prognosis, often treated with intraperitoneal chemotherapy to achieve high local drug concentrations. However, conventional formulations are rapidly cleared from the peritoneal cavity. To address this, perfluorocarbon-loaded poly(lactic-co-glycolic acid) (PLGA) capsules were developed for sustained intraperitoneal drug delivery and non-invasive tracking. Paclitaxel was encapsulated demonstrating slow release kinetics. Capsules were optimized for imaging and therapeutic performance.  $^{19}\text{F}$ -MRI using the BaSSI sequence enabled longitudinal *in vivo* monitoring, revealing gradual degradation and site-specific retention over seven days. Histology confirmed accumulation at tumor-prone areas, supporting their potential for targeted treatment.

The concept of the following publication was developed by Prof. Dr. Holger Gröll and Xenia Kovalyk. The synthesis and formulation of the drug delivery systems were carried out by Xenia Kovalyk. Optimization of the physicochemical properties and release profiles was performed by Xenia Kovalyk. Preclinical imaging experiments and data acquisition were conducted by Xenia Kovalyk, Dr. Muhammed Yildirim, Dr. Johannes Lindemeyer, Sven Saniternik and Dr. Markus Schütz. MRI data analysis and quantification were performed by Xenia Kovalyk and Dr. Muhammed Yildirim. Katharina Schwab conducted SEM measurements. The manuscript was written by Xenia Kovalyk, with extensive review by Prof. Dr. Holger Gröll and scientific input from all co-authors.

This chapter is based on: Kovalyk X, Yildirim M, Saniternik S, Schwab K, Schütz MB, Schmidt AM, Lindemeyer J, Gröll H. Fluorine-19 MRI BaSSI spectroscopic imaging employing fluorinated drug-loaded particles for image-guided intraperitoneal delivery. Manuscript in preparation.

## 3.1.1 Abstract

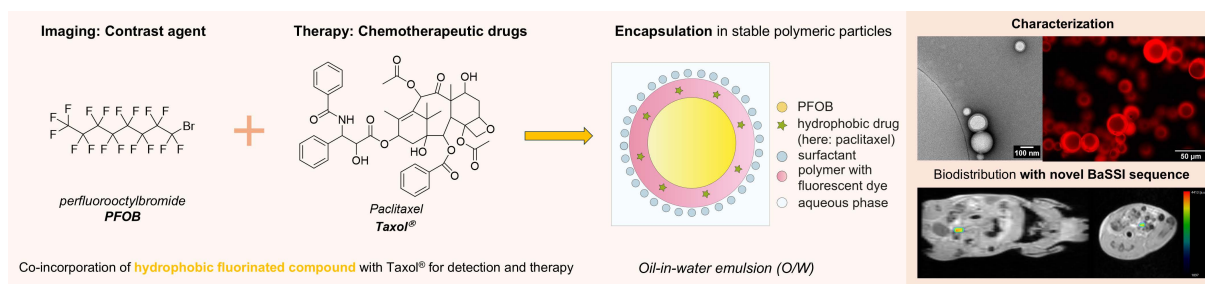


Fig. 1: Graphical abstract.

Perfluorocarbon-containing particles were synthesized to investigate their use in image-guided intraperitoneal (i.p.) drug delivery for treatment of peritoneal carcinomatosis. These capsules possess a shell of PLGA and a liquid core of perfluorooctylbromide (PFOB) to serve as a contrast agent for  $^{19}\text{F}$ -MR imaging. Additionally, paclitaxel was incorporated as chemotherapeutic drug. Variation of the different reaction parameters during synthesis identified the most beneficial conditions for obtaining capsules of well-defined size and drug incorporation. Kinetic of drug release depended on capsule size and provided sustained drug levels over seven days. With an intended use as drug delivery system (DDS) for treatment of intraperitoneal metastases, particle distribution in the peritoneal space was imaged using  $^{19}\text{F}$ -magnetic resonance imaging (MRI). The latter employed a new spectroscopic imaging sequence using Balanced Spiral Spectroscopic Imaging (BaSSI) in comparison to the current gold standard of 3D balanced UTE SSFP imaging. *In vivo*  $^{19}\text{F}$ -MRI shows that the capsules remain primarily located in the vicinity of the injection site and slowly disintegrate over the course of seven days. *Ex vivo* histological analysis has shown particle accumulation at common tumor sites such as small intestines and pancreas. This accumulation leads to a promising opportunity for cancer treatment in the future.

### 3.1.2 Introduction

MRI is the imaging modality of choice when it comes to high-resolution anatomical images. MRI provides a variety of different images based on the magnetic properties of water protons and their different spin-lattice ( $T_1$ ) or spin-spin relaxation ( $T_2$ ) times, their local density, or also water diffusion. As hydrogen nuclei are ubiquitously present in soft tissue, the intrinsically low sensitivity of MRI is compensated by the large proton concentration.[1] The relaxation times can be further shortened using contrast agents (CAs), leading to local signal enhancements or signal voids depending on the type of CA and the used sequence.[2,3] Pathologies, which alter the normal pharmacokinetic behavior of CAs are mainly identified on images acquired before and after CA injection. MRI has also been used for theranostic approaches to track DDS *in vivo*. To this aim, DDS, which are typically nanoparticles in the size range of 50–500 nm diameter, are functionalized with numerous contrast agents such as gadolinium-based CA or superparamagnetic iron oxide nanoparticles, which renders them visible on MR images.[4,5] However, localization and quantification of the DDS using pre- and post-injection scans remains challenging, especially with increasing time between two image acquisitions. Particularly challenging is MR imaging of the abdomen, where imaging is further complicated by breathing and bowel motion or also local susceptibility changes e.g. induced by air pockets in the bowel.[6] Tracking of DDS over longer time spans using co-registered images is largely impossible.

An alternative method is  $^{19}\text{F}$ -MR imaging. The fluorine isotope  $^{19}\text{F}$  has a high gyromagnetic ratio and as an exogeneous nucleus has no natural background in the body. Thus,  $^{19}\text{F}$  provides hot-spot images comparable to nuclear imaging methods but without any ionizing radiation or half-life limitations.[7] The potential of  $^{19}\text{F}$ -MRI for diagnostic or theranostic applications has been recognized for many years and was investigated accordingly. MRI's low intrinsic sensitivity is compensated using mostly nanoparticles carrying a high number of fluorine atoms. In particular, fluorinated oils such as PFOB, perfluorodecalin or perfluorocrownethers were formulated into emulsions or polymer-stabilized capsules providing such a high number of fluorine atoms per particle that the detection limit in particles is in the range of nanomole per liter.[7,8] From all fluorinated oils, PFOB has the most favorable properties for *in vivo* use. A PFOB-based emulsion is FDA approved (though discontinued) as blood substitute underlining its remarkable non-toxicity. Clearance occurs mainly via liver uptake and subsequent exhalation via the breath.[9,10] However, the fluorine atoms in

PFOB are not chemically identical leading to different chemical shifts. The latter translates into ghost images when fast phase-encoding sequences are used. Consequently, new MR methods using spectroscopic imaging methods were explored using e.g. the multi-spin-echo based fluorine ultrafast turbo spectroscopic imaging (F-uTSI) sequence with radial k-space trajectories.[11] F-uTSI appeared superior to standard MR approaches but was later outperformed by 3D ultrashort-echo-time balanced steady-state free precession (3D UTE-SSFP) sequence.[12] A new approach for spectral imaging was recently published employing the BaSSI sequence. The sequence utilizes an all-phase-encoded pseudo-spiral k-space trajectory, enabling the acquisition of broadband (80 ppm) fluorine spectra free from chemical shift ghosting artefacts. BaSSI significantly outperforms FuTSI as well as other typical MRSI sequences used in hydrogen-1 ( $^1\text{H}$ ) or phosphorus-31 ( $^{31}\text{P}$ ) imaging. Results showed that BaSSI was able to detect the hotspot of 0.05  $\mu\text{l}$  PFOB with only 2 minutes scan time on a clinical 3T MRI scanner.[13]

In most  $^{19}\text{F}$ -MRI studies, the contrast agents were intravenously (i.v.) injected which comes with limitations on size, osmolarity and dose.[7,8] Furthermore, surface modifications are required to achieve long circulation time, prevent rapid clearance by the reticuloendothelial system, and to facilitate uptake in the target tissue. Here, we explore the use of  $^{19}\text{F}$ -MRI for image-guided drug delivery in peritoneal carcinomatosis (PC), which comes with less restrictions and offers a promising area of application.

PC is the metastatic dissemination of malignant cells within the peritoneal cavity, a frequent manifestation of advanced-stage gastrointestinal cancers such as ovarian cancer, gastric cancer, pancreatic cancer, and colorectal malignancies.[14] Its diagnosis is associated with poor prognosis, as it reflects widespread dissemination with limited curative options. Current treatment comprises cytoreductive surgery combined with hyperthermic intraperitoneal chemotherapy (HIPEC) or pressurized intraperitoneal aerosol chemotherapy (PIPAC).[15–17] Treatment is further complemented with i.v. chemotherapy to treat residual cancer cells. A major limitation of systemic chemotherapy lies in the poor perfusion of peritoneal tumor nodules. These often non-vascularized lesions exhibit elevated interstitial pressure and dense stromal barriers, which significantly hinder the extravasation and diffusion of i.v. administered drugs. Consequently, achieving therapeutic drug concentrations at the site of disease via i.v. injection is challenging. In contrast, i.p. drug administration offers direct exposure of tumors to therapeutic agents and prolongs residence time within the cavity,

allowing for improved local efficacy and reduced systemic toxicity. However, the clinical application of i.p. chemotherapy is in need of improvement, largely due to rapid clearance of free drug.[18–22]

To overcome these limitations, DDS that provide sustained release have been explored but little is known about their distribution, decomposition and drug release within the peritoneal space. Due to limited systematic research the optimal particle size and design for i.p. administration remain unclear, with studies exploring carriers ranging from nanometers to several microns. While smaller particles offer better penetration into tumors, they are more prone to rapid clearance. Larger particles and microparticles, on the other hand, offer sustained release but may suffer from poor distribution within the peritoneal cavity and limited tumor uptake.[23–25]

PLGA-based nano- and microparticles have emerged as promising candidates as sustained release drug carriers also for i.p. use. PLGA is an FDA-approved, biocompatible, and biodegradable polymer with encapsulation properties offering enhanced stability, improved drug-loading capacity, and has previously been used as carrier for PFOB.[26] These particles have also been employed to encapsulate chemotherapeutic agents such as PTX, a widely used drug in the treatment of ovarian and gastric cancers.[27–29] It has been shown that their physicochemical properties, including particle size, morphology, and surface characteristics, can be finely tuned during synthesis.[26,30] Notably, PLGA-based PTX formulations circumvent the need for Cremophor<sup>®</sup> EL, a toxic solvent present in commercial paclitaxel products like Taxol<sup>®</sup>, which is associated with hypersensitivity reactions and other systemic toxicities. PLGA stabilized capsules filled with PFOB were already employed as theranostic systems but for i.v. use. Thus, synthesis procedures mainly focused on sub-micron sized particles.

*Boissenot et al.* have shown that encapsulating PTX within PLGA-poly(ethylene glycol) (PEG)/PFOB theranostic nanocapsules presents a significant formulation challenge, given the system's complexity involving four distinct components and the dynamic nature of the emulsion-solvent evaporation process. Two primary obstacles were encountered: retaining the volatile, fluorinated imaging core (PFOB) during particle formation, and achieving efficient incorporation of the highly hydrophobic, crystallization-prone PTX within the polymeric shell.[8]

So far, preclinical studies demonstrated successful  $^{19}\text{F}$ -MRI signal detection from PFOB-loaded particles after i.v. injection, but longitudinal imaging over several days after i.p. administration is lacking.[7,8] Imaging of particle biodistribution is crucial to optimize size and surface properties of particles for i.p. drug delivery application.

Here, we investigated the potential of  $^{19}\text{F}$ -MRI for tracking PLGA-based microcapsules loaded with PFOB as i.p. DDS. To this aim, monodisperse PFOB-capsules loaded with paclitaxel (PTX) ranging from 100 nm to 25  $\mu\text{m}$  were fabricated using an optimized emulsion-solvent evaporation technique. Stability, PFOB loading, drug-loading efficiency, and imaging signal were assessed *in vitro*. Finally, after i.p. injection in mice, longitudinal  $^{19}\text{F}$ -MRI measurements were performed using the BaSSI sequence over seven days giving insights on crucial parameters that are important for design of particles for i.p. drug delivery. This work establishes a platform for real-time, non-invasive assessment of i.p. DDS performance, but also lays the foundation for rational design of image-guided therapies for PC.

### 3.1.3 Experimental section

#### *General/Materials*

All solvents were obtained from Sigma-Aldrich. PLGA (LA:GA: 75:25,  $M_w=66,000$ – $107,000$  g/mol, ester-terminated) was purchased from Sigma-Aldrich. PLGA (Resomer<sup>®</sup> RG 756 S: LA:GA (75:25),  $M_w=76000$ – $115000$  g/mol, Resomer<sup>®</sup> RG 752 H: LA:GA (75:25),  $M_w=4,000$ – $15,000$  g/mol, Resomer<sup>®</sup> RG 503 H: LA:GA (50:50),  $M_w=24,000$ – $38,000$  g/mol, ester-terminated, Resomer<sup>®</sup> RG 653 H: LA:GA (65:35),  $M_w=24,000$ – $38,000$  g/mol, acid-terminated, Resomer<sup>®</sup> RG 858 S: LA:GA (85:15),  $M_w=190,000$ – $240,000$  g/mol, ester-terminated) was kindly provided by Evonik Operations GmbH (Essen, Germany). 9-(diethylamino)-5H-benzo[a]phenoxazin-5-one (Nile Red), poly(vinyl alcohol) (PVA) ( $M_w=67000$  g/mol) and sodium cholate were purchased from Sigma-Aldrich (St Louis, MO, USA). All commercial reagents were used as received without further purification. Water was purified using a Milli-Q system by Sartorius (Göttingen, Germany). A Hereaus Multifuge 3 SR Plus centrifuge (Hanau, Germany) was used for centrifugation. For emulsification, an IKA Ultra Turrax T25 (Staufen im Breisgau, Germany) and a microtip probe sonicator by GEX-600 Cole Palmer (Saint Neots, United Kingdom) were used.

### *Particle Synthesis*

PFOB was encapsulated in PLGA capsules according to a protocol established by *Pisani et al.*[26] The polymer PLGA (100 mg) was dissolved under sonication in 4 ml of dichloromethane (DCM). PFOB (60  $\mu$ l) was added. For fluorescent or confocal microscopy, a Nile Red solution in acetone (0.08 mg/ml, 60  $\mu$ l) was added to the organic solution prior to emulsification. For drug-loaded particles, PTX was added to the organic solution. The mixture was stirred for 10 minutes at room temperature (rt). The organic solution was poured into an ice-cooled beaker containing a 1.5% (w/v) sodium cholate aqueous solution (20 ml). The mixture was emulsified for 1 minute at 24000 rpm and 0 °C with an Ultraturrax-T18. For emulsification speeds below 4000 rpm PVA was chosen instead of sodium cholate. The reaction mixture was stirred for 3 hours at rt for the evaporation of the organic solvent. The suspension was filled up to 20 ml with double distilled water. 1% (w/v) PVA solution (20 ml) was added. The mixture was centrifuged (2000 rpm, 4 °C, 20 minutes). The supernatant was decanted. The precipitated particles were redispersed in 5 ml of purified water via Vortex and stored at 4 °C.

For nano-sized emulsions an ultrasonic tip and PVA as emulsifier were used.

### *In vitro stability*

For stability studies particles were dispersed in 1.5 ml of human peritoneal ascitic fluid (PF, pH=8) or phosphate-buffered saline (PBS, pH=7) as control and incubated at 37 °C under shaking. At determined time intervals, a sample of 5  $\mu$ l was taken and analyzed by Coulter Counter as described below. Studies were carried out in triplicate. PF was anonymously provided by patients of gastroenterology department of the University Hospital of Cologne in accordance with the ethical committee.

Chemotherapeutic-loaded particles (0.05 mg of PTX) were dispersed in 1.5 ml of fetal bovine serum (FBS) and incubated at 37 °C under shaking. At determined time intervals, the vials were centrifuged at 4500 rpm for 20 minutes. 0.75 ml of supernatant was taken and analyzed by HPLC as described below. Equal volume of FBS was replaced. Studies were carried out in triplicate.



## *Characterization*

### *Size distribution*

The hydrodynamic radius and the particles with sizes  $> 1.5 \mu\text{m}$  radius was measured as a function of time with dynamic light scattering (DLS) and Coulter Counter, respectively. No further filtrations of the particles were performed throughout the entire experiment. In between measurements the samples were stored at rt.

The hydrodynamic radius and polydispersity of the particles was determined on a DLS/SLS goniometer system at a scattering angle of 90 degrees, 633 nm wavelength, 20 °C (*ALV GmbH*, Reutlingen, Germany) at rt. The samples were dispersed in purified water. The diffusion coefficient ( $D$ ) was obtained from cumulant fits of the intensity correlation function using ALV software. All reported hydrodynamic radii were calculated using the Stokes-Einstein equation  $r_h = kT/(6\pi\eta D)$ , where  $k$  is the Boltzmann constant,  $T$  is the absolute temperature and  $\eta$  is the solvent viscosity. Measurements were repeated 5 times and calculations were performed on the averaged correlation function of the 5 measurements.

Size distribution of micron-sized particles were recorded on a Coulter Counter instrument (Multisizer 3, *Beckmann Coulter*, Brea, CA, USA) at rt. The mean diameter of particles was measured using a 50  $\mu\text{m}$  aperture tube. For each measurement, 10  $\mu\text{l}$  of the sample was mixed for with 20 ml of sodium chloride (0.9%) was analyzed in triplicate.

### *Microscopy*

TEM was used to study the size and morphology of the particles. TEM images were recorded on a side-entry electron microscope (Talos L120C, *ThermoFisher*, Waltham, USA). Samples for TEM were prepared by placing the emulsion (3  $\mu\text{l}$ ) on a 300-mesh carbon-coated copper grid and letting it dry at rt. Grids were previously hydrophilized with Alcian blue.

SEM images are taken with a Zeiss Neon 40 microscope (*Carl Zeiss AG*, Oberkochen, Germany) at 5 kV. For preparation, the dried capsules were attached to sample holders and coated with a thin layer of gold under vacuum before measurement.

For fluorescence and light microscopy, measurements were recorded at rt on a Leica DM6000B instrument (*Leica*, Wetzlar, Germany). The samples were dispersed in

purified water and deposited on a glass microscope slide covered with a glass coverslip. 63X and 40X objectives were used.

### *NMR spectroscopy*

<sup>19</sup>F-NMR spectra were recorded on a Bruker Avance II spectrometer (*Bruker Corporation*, Billerica, Massachusetts, USA) operating at 300 MHz. PFOB content was measured in reference to trifluoroacetic acid (TFA) with a coaxial inlet tube. The precise concentration of PFOB directly in the aqueous particle dispersion was determined. The total amount of PFOB in the suspension was determined after integration of the peak at -83.1 ppm corresponding to the CF<sub>3</sub> group and normalization by the area of the TFA peak at -75.6 ppm.

### *High-performance liquid chromatography (HPLC)*

The drug loading efficiency was determined in triplicate by HPLC (Agilent 1100 series, *Agilent Technologies*, Diegem, Belgium). The mobile phase consisted of acetonitrile/0.1% trifluoroacetic acid (TFA) (v/v) in water (50:50 v/v). The reverse phase column was a Phenomenex Luna C18(2). The flow rate was set at 1.0 ml/min, and the detection wavelength was 227 nm. Particles were dissolved in acetonitrile and vigorously stirred to get a clear solution. Docetaxel was used as reference substance and added directly to the particle sample. The encapsulation efficiency was defined by the ratio of measured and initial weight of PTX encapsulated in nanoparticles.

$$\text{Incorporation efficiency [\%]} = \frac{\text{Weight of PTX in particles}}{\text{Initial weight of PTX}} \times 100 \quad \text{Eq. 1}$$

The drug loading was defined as the weight of incorporated drug divided by the polymer particle weight.

$$\text{Drug loading efficiency [\%]} = \frac{\text{Weight of PTX in particles}}{\text{Weight of particles}} \times 100 \quad \text{Eq. 2}$$

### *Hard- & Software Environment*

3.0 T clinical MRI system (Achieva®, *Philips Healthcare*, Best, The Netherlands) equipped with a dual tuned <sup>1</sup>H/<sup>19</sup>F small animal coil was used. Parameters: (FOV=32 mm x 32 mm x 24 mm, voxel size: 1 mm x 1 mm x 2 mm, 16 averages, T<sub>E</sub>=0.621 ms, T<sub>R</sub>=3 ms, FA=30°). The offset-frequency was set to target the CF<sub>2</sub> group of PFOB, images were generated by integrating the CF<sub>2</sub> peak over a narrow window (~1 kHz) to maximize signal to noise ratio (SNR). For anatomic overview <sup>1</sup>H-MRI with an RF-spoiled gradient echo sequence was used: (FOV=32 mm x 32 mm x 24 mm,

voxel size: 0.5 mm x 0.5 mm x 2 mm,  $T_E=10.345$ ,  $T_R=75.000$ ,  $FA=30^\circ$ ). Images were evaluated with Imalytics Preclinical® (*Gremse-IT*, Aachen, Germany).[31]

### *In vivo experiments for biodistribution*

Animal experiments were approved by the State Agency for Consumer Protection and Nutrition of North-Rhine Westphalia (LAVE, Animal Ethical Proposal no.: 81-02.04.2020.A320) and were performed by authorized personnel. A healthy BALB/c mouse was injected i.p. with PFOB-filled particles (5.5 mg PFOB@PLGA-MP-8 in 0.1 ml sodium chloride 0.9%). To investigate the subsequent biodistribution,  $^1H/^{19}F$ -MRI measurements were conducted for seven days post injection (p.i.). The organ tissues (mesenterium, small intestines, colon, peritoneum, spleen, pancreas, stomach, bladder, uterus, kidneys, liver) of the mouse were explanted for histology on the last day after treatment. The slices obtained were examined by microscopy.

## 3.1.4 Results

### *Particle synthesis and influence of parameter variation*

The aim of this study was the synthesis of PLGA-shelled nano- and microparticles filled with PFOB in the range of 100 nm–25  $\mu m$  for potential theranostic application in i.p. drug delivery.  $^{19}F$ -MRI potentially allows to monitor the size-dependent particle distribution in the peritoneum and follow the degradation kinetics for different sizes and shell thickness. As the dose for i.p. injection is only limited by potential toxicity, we expect that the administered amount of fluorine exceeds the lower detection limit of  $^{19}F$ -MRI. Biocompatible and biodegradable polymer particles with solid shells and liquid fluorinated cores were synthesized and characterized extensively for application as fluorine contrast agents for *in vivo* visualization using  $^{19}F$ -MRI.

First, capsules of different diameters were synthesized using a solvent-emulsion-evaporation technique which was originally introduced by *Loxley and Vincent et al.*[32] and later improved by *Pisani et al* (Fig.).[26] Previous work demonstrated a versatile process which allows for controlled particle synthesis with diameters ranging from 100 nm to 50  $\mu m$ . Additionally, variation of PFOB/PLGA ratio led to controlled capsule shell thickness.[26,30,33] Influence of surfactant and solvent has been extensively studied by *Pisani et al.* and *Diou et al.*[7,26,30,33] One of the critical steps for encapsulation is the evaporation, which has been found to be more favorable under a fume-hood compared to evaporation under reduced pressure.[8]

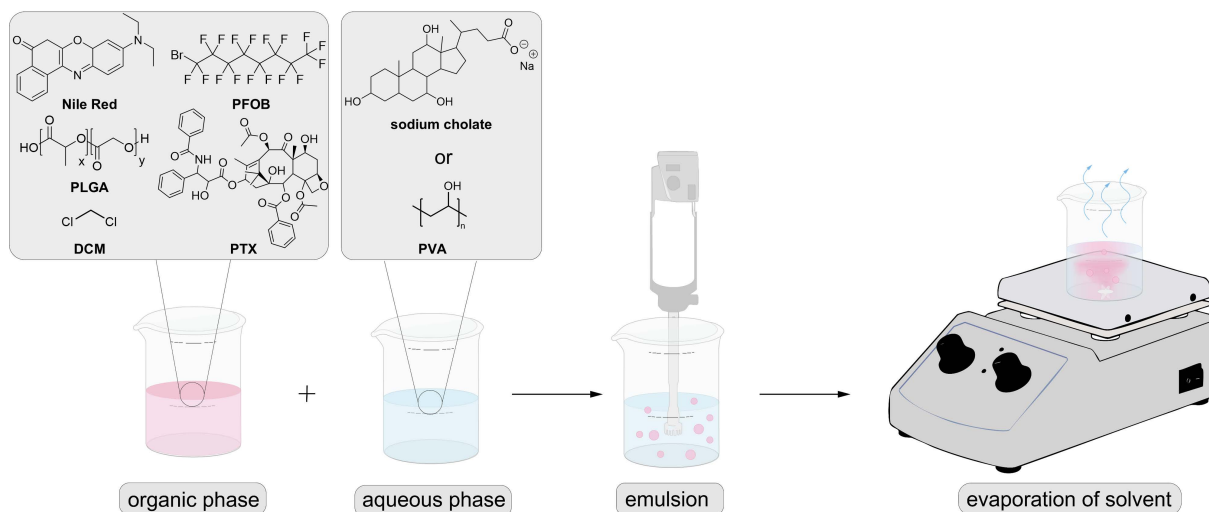


Fig. 2: Schematic overview of the synthesis process for PLGA-shelled nano and microcapsules containing PFOB and PTX.

To visualize the core-shell structure of the capsules, fluorescent dye Nile Red was added to the organic phase, which only accumulates in the polymer shell. Here, we have focused on studying the molecular weight (thus, polymer chain length) on particle diameter as well as the ratio of lactic to glycolic acid, ratio of PFOB to PLGA, and polymer termination on PFOB encapsulation.

Capsule diameter is mainly governed by the organic emulsion droplets obtained after emulsification and the volume fractions of PLGA and PFOB in the organic phase. Consequently, particle diameters can be controlled by varying speed used for emulsification as already reported by *Pisani et al.*[26] Further size reduction for synthesis of nanocapsules is possible by using an ultrasound tip for the emulsification (Fig. 2). PFOB content in capsules was quantified using NMR spectroscopy. In Fig. 3, an exemplary NMR spectrum of microparticles dispersed in water with a TFA reference in water is displayed.

## Results

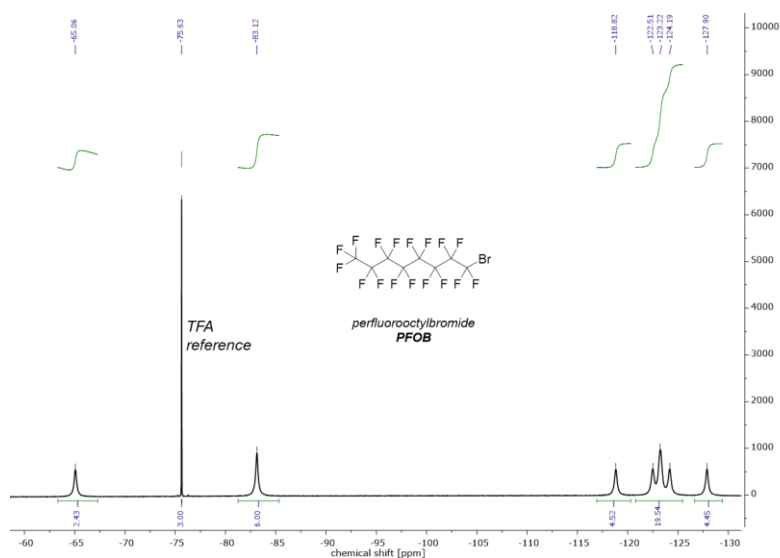


Fig. 3: NMR spectrum of PFOB@PLGA-MP-1 and TFA reference.

Micro-sized particles exhibited higher PFOB loading (around 1.5 times higher) relative to their nanoparticle counterparts. As expected, when comparing smaller micron-sized particles (approximately 3  $\mu\text{m}$  diameter) to larger particles (around 22  $\mu\text{m}$  diameter), there was no substantial difference in PFOB loading (Table 1).

## Results

*Table 1: Overview of samples and corresponding reaction parameters with respect to varying parameters. PLGA polymer is used in all experiments. PFOB content expressed as PFOB per mg particle.*

|                 | Sample         | Polymer<br>(LA:GA,<br>M <sub>w</sub> [g/mmol],<br>termination) | Emulsification | PFOB<br>volume<br>[μl] | Diameter<br>[μm] /<br>Zeta [mV] | PFOB<br>content<br>[μl/mg] |
|-----------------|----------------|----------------------------------------------------------------|----------------|------------------------|---------------------------------|----------------------------|
| Emulsification  | PFOB@PLGA-MP-1 | LA:GA: 75:25,<br>M <sub>w</sub> : 66–107,<br>ester             | 24000 rpm      | 60                     | 2.8±2.4                         | 0.392                      |
|                 | PFOB@PLGA-MP-2 |                                                                | 3200 rpm       |                        | 21.6±7.8                        | 0.397                      |
|                 | PFOB@PLGA-NP-1 |                                                                | Ultrasonic tip |                        | 0.284<br>PDI: 0.234             | 0.246                      |
| PFOB/PLGA ratio | PFOB@PLGA-MP-3 | LA:GA: 75:25,<br>M <sub>w</sub> : 76–115,<br>ester             | 24000 rpm      | 45                     | 2.4±1.0 /<br>–33.4              | 0.247                      |
|                 | PFOB@PLGA-MP-4 |                                                                |                | 60                     | 2.7±2.1 /<br>–43.9              | 0.267                      |
|                 | PFOB@PLGA-MP-5 |                                                                |                | 75                     | 2.8±1.6 /<br>–29.4              | 0.257                      |
| LA/GA ratio     | PFOB@PLGA-MP-6 | LA:GA: 50:50,<br>M <sub>w</sub> : 24–38,<br>acid               | 24000 rpm      | 60                     | 2.3±1.3                         | 0.275                      |
|                 | PFOB@PLGA-MP-7 | LA:GA: 65:35,<br>M <sub>w</sub> : 24–38,<br>acid               |                |                        | 2.1±1.6                         | 0.278                      |
| Termination     | PFOB@PLGA-NP-2 | LA:GA: 50:50,<br>M <sub>w</sub> : 24–38,<br>ester              | Ultrasonic tip | 60                     | 0.154<br>PDI: 0.371             | 0.156                      |
|                 | PFOB@PLGA-NP-3 | LA:GA: 50:50,<br>M <sub>w</sub> : 24–38,<br>acid               |                |                        | 0.108<br>PDI: 0.214             | 0.117                      |
| M <sub>w</sub>  | PFOB@PLGA-MP-8 | LA:GA: 85:15,<br>M <sub>w</sub> : 190–240,<br>ester            | 24000 rpm      | 60                     | 3.0±1.6                         | 0.306                      |

Fluorescent imaging of the particles revealed a distinct core-shell morphology, which was well-defined in both micro-sized and nanoparticulate systems. The shell thickness in the larger micro-sized particles (d=21.6 μm) was approximately 3.8 μm, which is notably thicker than the shell observed in nanoparticles, with a shell thickness of approximately 17 nm (Fig. 4). The shell thickness in microparticles with a diameter of 2.8 μm was around 0.5 μm.

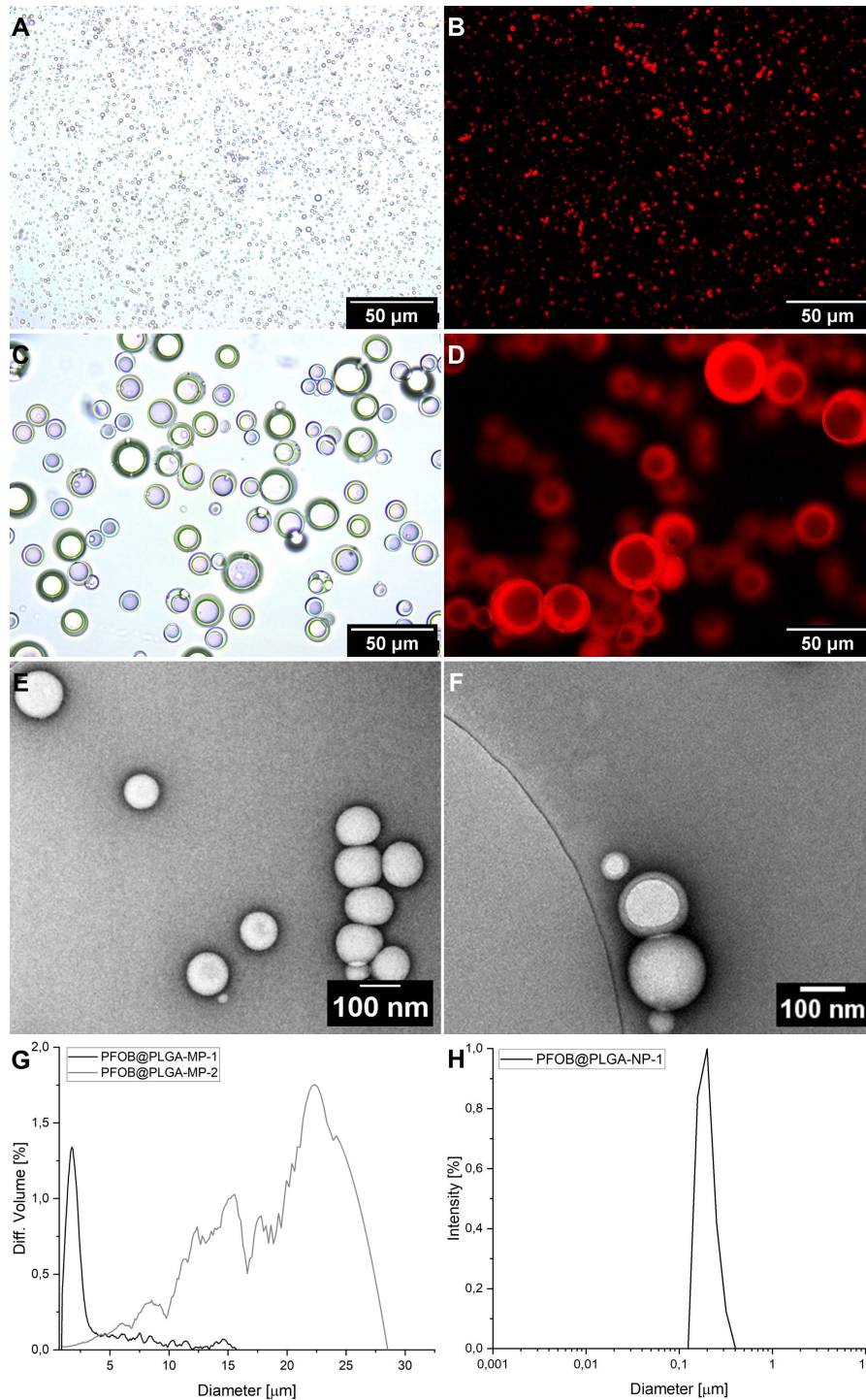


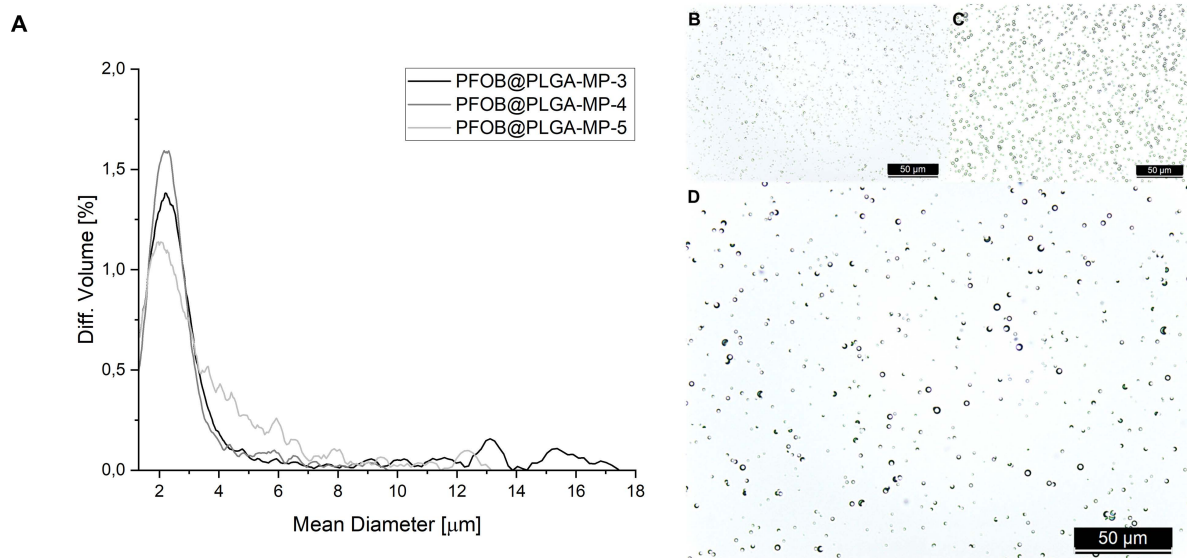
Fig. 4: A) Optical microscope image of PFOB@PLGA-MP-1. B) Fluorescent microscope image of PFOB@PLGA-MP-1. C) Optical microscope image of PFOB@PLGA-MP-2. D) Fluorescent microscope image of PFOB@PLGA-MP-2. E), F) TEM images of PFOB@PLGA-NP-1. Size distribution of G) PFOB@PLGA-MP-1 and PFOB@PLGA-MP-2 by Coulter Counter, and H) PFOB@PLGA-NP-1 by DLS.

While nanoparticles showed a narrow size distribution with a polydispersity index (PDI) of 0.234, the size distributions broadened with increasing particle diameters.

The second set of experiments, the amount of polymer in the organic solvent is fixed (100 mg) and the volume of PFOB varied between 45 and 75  $\mu\text{l}$  (Fig. 5). Particles with

similar diameters within the standard deviation and similar PFOB loading were observed.

All samples exhibited strongly negative surface potentials without correlation found between surface potential and PFOB content or particle size. For this reason, surface potential measurements were discarded in future measurements.



*Fig. 5: A) Size distribution of PFOB@PLGA-MP-3, PFOB@PLGA-MP-4 and PFOB@PLGA-MP-5 by Coulter Counter. B) Optical microscope image of PFOB@PLGA-MP-3. C) Optical microscope image of PFOB@PLGA-MP-4. D) Optical microscope image of PFOB@PLGA-MP-5.*

The ratio of the acids in PLGA may influence polymer shell degradation in biological media resulting in tunable drug release behavior. This was studied by varying the ratio of LA:GA from 65:35 to 50:50. Both samples exhibited very similar particle diameters and PFOB content in the range of the standard deviation (Table 1). The polymer termination was varied from an ester to an acid. Both samples exhibited very similar particle diameters and PFOB content in the range of the standard deviation (Fig. 6).



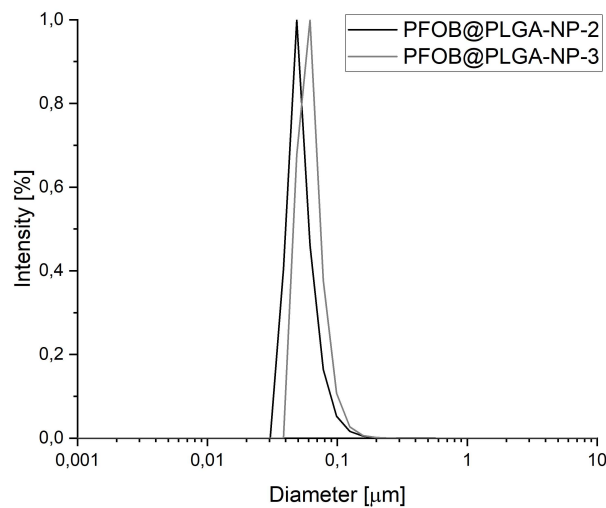


Fig. 2: Size distribution of PFOB@PLGA-NP-2 and PFOB@PLGA-NP-3.

### *PTX incorporation*

Paclitaxel incorporation efficiency (IE) was optimized by systematically varying emulsification speed and polymer molecular weight of the PLGA matrix. Different PTX concentrations were tested for optimal drug loading. The amount of encapsulated PTX increases with the initial PTX concentration, reaching a plateau around 0.1 mg per mg polymer, above which the polymer matrix appears to be saturated and no further PTX encapsulation occurs, even with higher initial amounts. Needle-shaped PTX crystals were observed in the suspensions alongside particles if oversaturation occurred (data not shown). A formulation with 0.1 mg PTX per mg polymer was selected as the optimal compromise between encapsulated PTX amount and encapsulation efficiency. These conditions were used for subsequent formulations (Table 2). Size distribution is in a similar range as unloaded particles (Fig. 7A,B).

## Results

*Table 2: PTX and PFOB content per mg particle, for a synthesis using 100 mg polymer, 90  $\mu$ l PFOB and 10 mg PTX. PFOB content expressed as PFOB per mg particle.*

| Sample             | Parameters                                                       | Diameter<br>[ $\mu$ m] | PTX<br>content<br>[mg/mg] /<br>IE [%] | PFOB<br>content<br>[ $\mu$ l/mg] / IE<br>[%] |
|--------------------|------------------------------------------------------------------|------------------------|---------------------------------------|----------------------------------------------|
| PTX@PFOB@PLGA-MP-1 | Emulsification:<br>24000 rpm, LA:GA:<br>75:25, $M_w$ : 76–115    | 2.8 $\pm$ 1.1          | 0.038 /<br>58                         | 0.458 / 51                                   |
| PTX@PFOB@PLGA-MP-2 | Emulsification:<br>8000 rpm, LA:GA:<br>75:25, $M_w$ : 76–115     | 10.2 $\pm$ 6.8         | 0.044 /<br>72                         | 0.574 / 64                                   |
| PTX@PFOB@PLGA-NP   | Emulsification:<br>ultrasonic tip, LA:GA:<br>75:25, $M_w$ : 4–15 | 0.300<br>PDI 0.278     | 0.037 /<br>66                         | 0.117 / 12                                   |

After optimization of synthesis parameters and purification procedure, no PTX crystals were observed in either microscopic or TEM images (Fig. 3). Similar PTX content and PTX IE were found for all samples. For PTX@PFOB@PLGA-MP-2 with a diameter of approximately 10  $\mu$ m the highest IE for PFOB as well as for PTX was obtained with 72%, whereas nano-sized PTX@PFOB@PLGA-NP had significantly lower PFOB loading and PFOB IE of around 12%. The shell thickness was found to be 19 nm in NPs, 0.5  $\mu$ m for MPs with a diameter of 2.8  $\mu$ m and 1.4  $\mu$ m in MPs with a diameter of approximately 10  $\mu$ m.

## Results

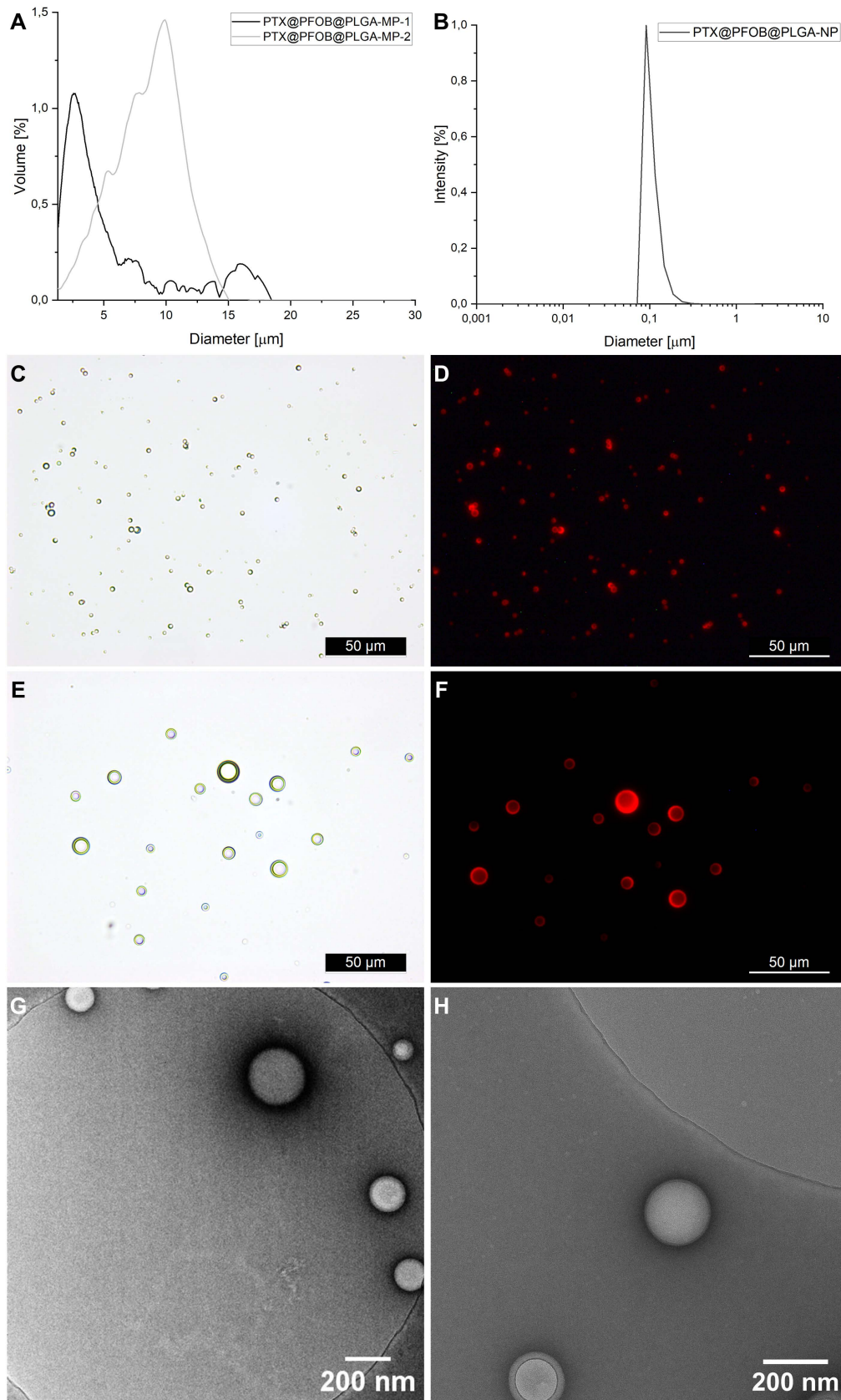
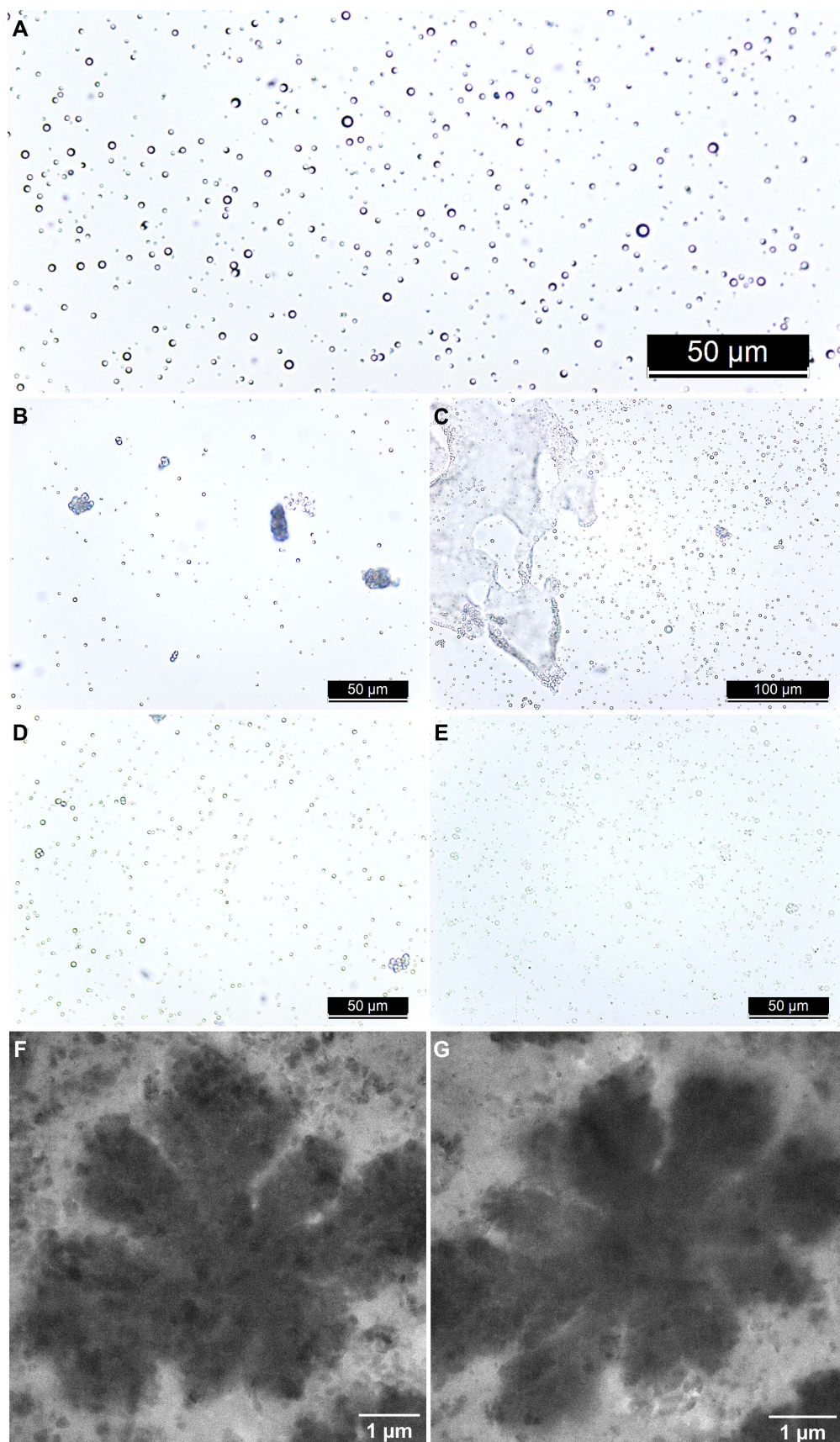


Fig. 3: Size distribution. A) PTX@PFOB@PLGA-MP-1 and PTX@PFOB@PLGA-MP-2 by Coulter Counter. B) PTX@PFOB@PLGA-NP by DLS. C) Optical microscope image of PTX@PFOB@PLGA-MP-1. D) Fluorescent microscope image of PTX@PFOB@PLGA-MP-1. E) Optical microscope image of PTX@PFOB@PLGA-MP-2. F) Fluorescent microscope image of PTX@PFOB@PLGA-MP-2. G), H) TEM images of PTX@PFOB@PLGA-NP-1.

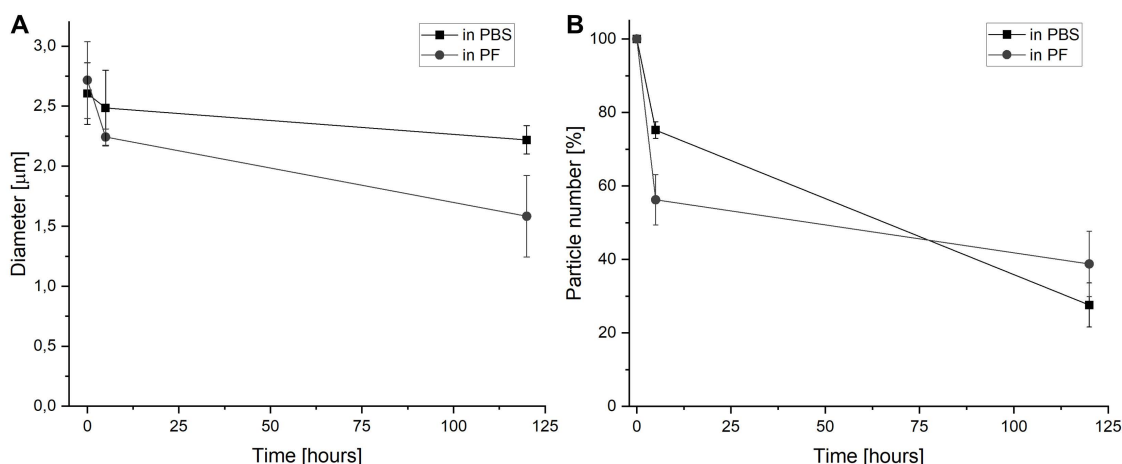
*In vitro degradability and release kinetic studies of particles*

Particle stability and degradation of PFOB@PLGA-MP-1 was monitored *in vitro* at 37 °C over 45 days. PF obtained from ascites patients was chosen as medium for close recreation of abdominal environment. PBS was used as control. Stability was inspected by microscope and Coulter Counter to track changes in particle number and morphology.



*Fig. 8: In vitro degradation monitoring of PFOB@PLGA-MP-1. A) Optical microscope image at the starting point. B) Optical microscope image after 14 days in PBS. C) Optical microscope image after 45 days in PBS. D) Optimal microscope image after 14 days in PF. E) Optimal microscope image after 45 days in PF. F), G) TEM images after 45 days in PF.*





*Fig. 9: In vitro stability over 120 hours at 37 °C in PBS and PF. A) Diameter in dependence of incubation time. B) Particle number in dependence of incubation time.*

At the starting point, distinct core-shell particles with a homogeneous distribution can be seen (Fig. 8A). After 14 days the particles in both PBS and in PF were prone to agglomeration but the original core-shell structure is maintained for particles. After 45 days in PBS, the polymeric shell of the particles appeared to form large polymeric aggregates found in the sample (Fig. 8C) while some remaining particles were intact. In contrast, the sample in PF appeared to develop minor conglomerates of three to four particles with almost conjoint polymer shells and fluorinated cores (Fig. 8E). Even more prominently, the polymer shells of the particles appear corroded with disordered yet spheric particle morphology in TEM images (Fig. 8F, G). Average particle diameters decreased more rapidly in PF compared to PBS, with a reduction from approximately 2.7 μm to 1.6 μm compared to 2.6 μm to 2.2 μm after 120 hours (Fig. 9A). Particle numbers also decreased more quickly in PF during the first 5 hours, dropping to 56% of the initial count, compared to 75% in PBS. However, after 120 hours, around 39% of the particles remain in PF, while only about 28% remain in PBS (Fig. 9B). This could possibly be explained by the disintegration of particles, resulting in a gradual shift of the overall size distribution toward smaller diameters and lower numbers.

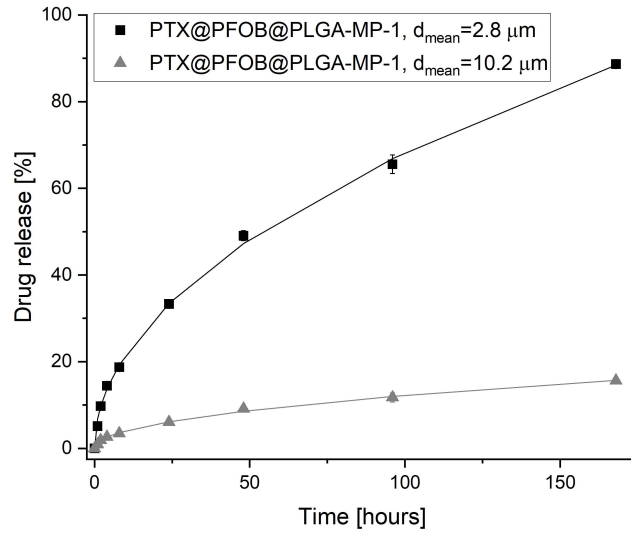


Fig. 10: Cumulative drug release of PTX@PFOB@PLGA-MP-1 (2.8 μm) and PTX@PFOB@PLGA-MP-2 (10.2 μm).

The cumulative release profiles of PTX from the particles in FBS were determined for two different sizes (Fig. 10). Particle dispersion of smaller average diameter (2.8 μm) showed at earlier times a more rapid release, with 33% of the encapsulated PTX released within the first 24 hours. After seven days, cumulative release from the smaller diameter particle dispersion reached 89%, whereas PTX release from PTX@PFOB@PLGA-MP-2 dispersion (10.2 μm diameter) was limited to 16%, respectively.

The kinetic of cumulative drug release can be described using a time exponential behavior[34]:

$$\frac{M_t}{M_\infty} = k * t^n \quad \text{Eq. 3}$$

where  $M_t$  and  $M_\infty$  is the absolute cumulative amount of drug released at time  $t$  and infinite time, respectively;  $k$  is a constant incorporating the diffusion coefficient of PTX within the drug carrier as well as the overall surface, and  $n$  is an exponent, which is indicative for the mechanism of drug release.

Both particle samples show a release kinetic in line with a *Fickian* transport of PTX ( $n = 0.48$ ,  $k = 0.013$  for  $d = 10.2 \mu\text{m}$  and  $n = 0.50$ ,  $k = 0.068$  for  $d = 2.8 \mu\text{m}$ ). The higher cumulative release of smaller particles compared to larger particles solely reflects the

higher surface area due to higher particle numbers of smaller particles for the same amount of PTX, which is subsumed in  $k$ .

### *Application of novel BaSSi sequence in vivo*

We investigated polymer particles filled with PFOB for image-guided i.p. drug delivery.  $^{19}\text{F}$ -MRI was employed to assess the biodistribution of polymer-shelled, PFOB-filled microcapsules following i.p. injection in a mouse as proof of concept of the novel sequence BASSI *in vivo*. Using PFOB@PLGA-MP-8 particles (Table 1), distinct  $^{19}\text{F}$  signal was detected in the abdominal region, as shown in overlay images combining anatomical  $^1\text{H}$  and functional  $^{19}\text{F}$ -MRI (Fig. 11A,B). After seven days, fluorine signal remained detectable, indicating slow excretion and sustained retention. While the particles largely remained in the same anatomical vicinity, a signal narrowing over neighboring voxels suggested gradual dispersion of particles over time. Histological analysis performed after seven days revealed particle accumulation on the outer surface of pancreas, liver and small intestine (Fig. 11E,F). Highest accumulation was found in the spleen and pancreas (Fig. 11D). Within the spleen and pancreas slices, individual particles were discernible, exhibiting clearly defined core-shell morphology (Fig. 11C,D).



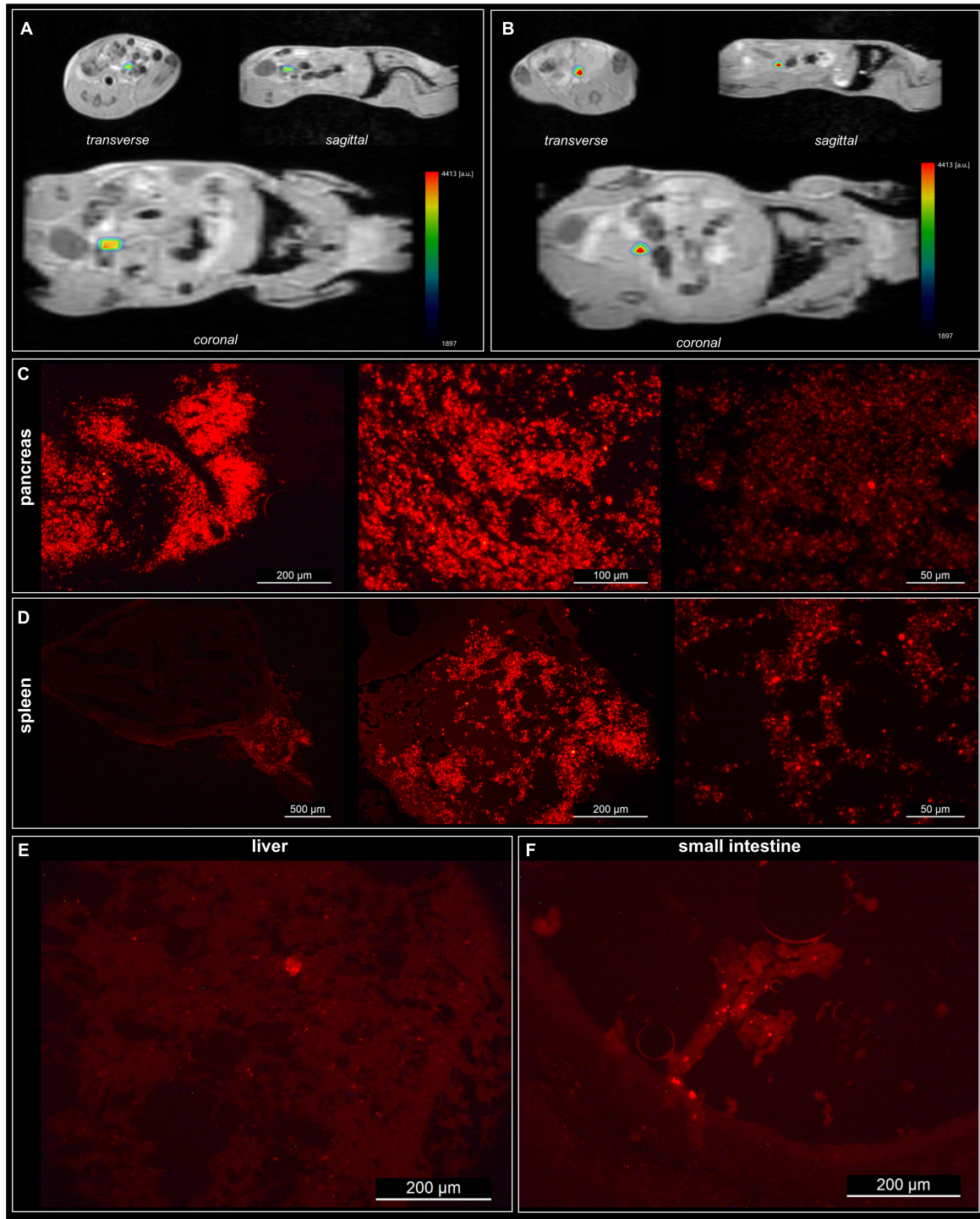


Fig. 11: I.p. injection of PFOB@PLGA-MP-8 in a mouse. A) Directly after injection. B) 7 days after injection. Fluorescence microscope images of histological tissue samples of C) pancreas, D) spleen, E) liver and F) small intestine.

### 3.1.5 Discussion

Here, we investigated PLGA capsules encapsulating PFOB and the drug PTX for potential application as i.p. DDS. The PFOB-core allows longitudinal *in vivo* particle tracking using  $^{19}\text{F}$ -MR imaging, which was demonstrated in a first proof of concept study in healthy mice. For synthesis of the particles, an emulsification process was used, where the organic DCM phase contained PLGA, PTX and liquid PFOB, while the aqueous phase contained a stabilizing surfactant. After emulsifying the organic phase, subsequent evaporation of DCM at ambient temperature induced gradual solvent removal, driving the system toward the binodal boundary due to the limited miscibility between PFOB and DCM. Phase separation within the droplets, led to formation of mobile, polymer-rich microdroplets that migrate to the oil-water interface, where they subsequently coalesce and form a polymer-shell around the perfluorocarbon core upon further solvent removal.[32] When PTX was added to the organic phase, the drug was incorporated into the polymer matrix as PTX is not soluble in PFOB.

The radius of the microcapsules and PFOB core can be theoretically calculated as described by *Böhmer et al.*,[35] showing that particle diameter depends mainly on the volume of the emulsion droplet and the amount of PFOB, while the shell thickness depends on the volume fraction of polymer and PFOB within the organic phase. Thus, the shell thickness should decrease linearly with increasing PFOB to polymer volume fraction. Experimentally, both shell thickness and particle diameter change only slightly with varying PFOB volume.

Increasing emulsification speed results in smaller emulsion droplets and consequently smaller particles with a more narrow size distribution and thinner polymer shell thickness. The theoretical ratio of PFOB to mg polymer influences shell thickness and overall diameter and is more sensitive to changes in nanoparticles compared to microparticles due to the different scaling of surface and volume ratio with size. However, factors beyond size such as surface properties or synthesis conditions also play a dominant role in PFOB encapsulation. Notably, pronounced differences in shell thickness across size scales may facilitate designing core-shell particles with optimized PFOB loading and structural integrity, with implications for biomedical and material science applications. The size distribution increases with increasing particle sizes resulting in broad distribution, which may hinder clinical translation. Achieving a more

uniform size distribution may be necessary, with microfluidic approaches presenting a promising solution to control particle uniformity.

Using higher molecular weight polymers were found to produce more stable structures resulting in larger particles. As expected, varying the lactic to glycolic acid ratio in PLGA from 65:35 to 50:50, polymer termination as well as initial PFOB volume during synthesis had minimal impact on particle size and PFOB incorporation, with differences falling within the standard deviation.

Encapsulating PTX within PLGA/PFOB theranostic carriers presents significant challenges due to the complexity of the four-component system and the emulsion-evaporation process. Main difficulties include retaining the imaging agent (PFOB) while loading the hydrophobic drug, prone to crystallization, within the polymer shell. *Boissenot et al.* demonstrated that PTX does not dissolve in PFOB but is encapsulated within the hydrophobic PLGA shell, consistent with prior studies on PLGA nanoparticles carrying hydrophobic drugs.[8,36,37] Our particles similarly feature a PFOB core surrounded by a PLGA shell, with PTX content attributed to the polymer matrix. PTX encapsulation did not impair particle morphology. Notably, significantly higher PTX loadings in the range of 4% were achieved, compared to *Boissenot's* 0.5% in nanoparticles. This may be due to our use of high molecular weight PLGA forming a more stable hydrophobic matrix capable of incorporating PTX without hydrophilic PEG. In contrast, *Boissenot* employed a PLGA-PEG copolymer (50:50 ratio, PLGA  $M_w=45,000$  g/mol with 10wt% PEG  $M_w=5,000$  g/mol), which may limit drug loading efficiency.[8]

As particle stability and degradation is strongly dependent on the nature of the incubation medium such as presence of proteins, we chose PF as medium, which contains electrolytes, proteins and various types of immune cells, such as macrophages, natural killer cells, or lymphocytes while using PBS as control.[38,39] Particles incubated in PF exhibited slow degradation over 45 days, reflecting physiological i.p. conditions, which was not observed in PBS. These findings suggest that degradation and also drug release properties can be tuned using different polymer shells. For example, using high molecular weight PLGA for thicker shells which may provide sustained release over weeks, while thinner shells composed from low molecular weight PLGA, yield more rapid degradation and drug release. Drug release kinetic studies show that cumulative release depends mainly on the overall surface

area of particles used. While both microparticle sizes release PTX by diffusion, overall release scales with the total surface area which is higher for smaller particles.[34] Normalizing the release kinetics to the particle number and, consequently, the total surface area results in release kinetics that are comparable across the two particle sizes.

To achieve prolonged i.p. retention and sustained release, a high molecular weight PLGA with an increased lactide content was selected ( $M_w=190,000\text{--}240,000$  g/mol, LA:GA (85:15)), forming microcapsules with a diameter of  $\sim 3\text{ }\mu\text{m}$ , thick shells, and superior monodispersity. To enhance imaging performance, we implemented an all-phase-encoded pseudo-spiral k-space trajectory (BaSSI), enabling broadband spectral acquisition ( $\sim 80$  ppm) without chemical shift artifacts. All imaging was conducted on a clinical 3.0 T MRI system equipped with a custom dual-tuned  $^1\text{H}/^{19}\text{F}$  RF coil, underscoring the clinical feasibility of BaSSI and its potential for advancing  $^{19}\text{F}$  MRI toward practical diagnostic applications.

Biodistribution was assessed *in vivo* using PFOB@PLGA-MP-8 particles, revealing a clear  $^{19}\text{F}$  signal located in one region of the abdomen following i.p. injection of 5.5 mg particles containing  $1.7\text{ }\mu\text{l}$  PFOB. After seven days, the fluorine signal remained detectable, indicating slow excretion and sustained retention in line with expectations after *in vitro* studies. The signal persisted in the form of a seemingly aggregated particle cluster in the abdomen. Considering a total dose of approximately  $1.7\text{ }\mu\text{l}$  ( $6.6\text{ }\mu\text{mol}$ ) PFOB was administered in 0.1 ml sodium chloride, signal dispersion should be taken into account following i.p. *in vivo* injection. This result appears to be in line with BaSSI's reported sensitivity of  $0.05\text{ }\mu\text{l}$  ( $0.2\text{ }\mu\text{mol}$ ) bulk non-diluted PFOB.[13]

Particle clearance or degradation with subsequent PFOB release appears to be strongly influenced by agglomeration and adhesion to tissue near the injection site, likely hindering lymphatic drainage from the peritoneal cavity. Literature indicates that lymphatic uptake is limited by the stomata size, with a  $\sim 3.6\text{ }\mu\text{m}$  cutoff in mice.[40] Despite the use of PLGA-based nano- and microparticles for i.p. delivery in the past, their *in vivo* distribution and clearance pathways remain poorly characterized, as most studies rely on histology or optical methods which are limited in temporal and spatial resolution as well as quantification.[41–46] Here, histological analysis revealed individual particles with clearly defined core-shell morphology in the spleen and pancreas, with additional capsule in multiple abdominal organs such as uterus, small

intestines or liver. These observations could be misinterpreted without complementary imaging data. While histology can provide insight into particle degradation and local tissue responses, it lacks spatial and quantitative information on biodistribution over time that imaging enables. Furthermore, observed particle aggregation in this study may result in high local drug concentrations which could lead to the strong inflammatory responses previously reported in preclinical and clinical studies involving polymer microparticles.[47,48]

Our findings demonstrate the importance of imaging for biodistribution studies, detecting particle aggregation after i.p. injection. This imageable approach may facilitate future studies, supporting the identification of key particle parameters for improved i.p. drug delivery and clearance.

### 3.1.6 Conclusion

Primary focus of this study was the formulation and use of PLGA-stabilized capsules containing PFOB to facilitate  $^{19}\text{F}$ -MR imaging and the drug PTX for sustained release application. Formulation process parameters were thoroughly investigated with respect to PTX and PFOB encapsulation efficiency as well as diameter and shell thickness. Particle stability was assessed *in vitro* using media that closely mimic human physiological conditions. Co-incorporation of chemotherapeutic drug PTX was facilitated in high efficiency. To enhance imaging sensitivity, we evaluated novel fluorine MRI sequence BaSSI with biodistribution studies *in vivo*. Particle fate and accumulation were monitored over seven days. Histological evaluation revealed major particle accumulation in the spleen and pancreas. This strategy advances the design of theranostic agents for improved i.p. drug delivery and imaging applications.

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### 3.2 Biodegradable radiopaque polymeric particles for CT image-guided intraperitoneal drug delivery

To address the challenge of rapid drug clearance in intraperitoneal chemotherapy, iodinated hydrophobic compounds were encapsulated in biodegradable poly(lactic acid) (PLA)-based particles via a solvent emulsion-evaporation method that enabled control over particle size. The iodinated oil, containing a 2,3,5-triiodobenzoate moiety, served dually as a CT contrast agent and solvent for the chemotherapeutic drug paclitaxel (PTX). Particles showed sustained release of PTX over seven days, with release levels depending on particle size. Following intraperitoneal administration in mice, dual-energy x-ray computed tomography (DECT) imaging provided quantitative tracking of particle biodistribution and clearance for up to 18 days. Imaging revealed particle aggregation near the injection site and approximately 50% clearance over the study period. The study highlights CT imaging as a promising tool for theranostic intraperitoneal drug delivery using radiopaque, PTX-loaded particles.

The concept of the following publication was developed by Prof. Dr. Holger Gröll and Xenia Kovalyk. The synthesis and formulation of the drug delivery systems were carried out by Xenia Kovalyk and Isabell Nies. Optimization of the physicochemical properties and *in vitro* drug release kinetics was performed by Xenia Kovalyk. Cell assays were conducted by Larissa-Marie Hamacher. Preclinical imaging experiments, including CT acquisition and *in vivo* evaluation, were conducted by Xenia Kovalyk, Sven Saniternik, Prof. Dr. Dr. Nils Große Hokamp and Dr. Markus Schütz. CT image analysis and iodine quantification were performed by Xenia Kovalyk with support from Felix Gremse. SEM measurements were conducted by Katharina Schwab. The manuscript was written by Xenia Kovalyk, with critical revisions and supervision by Prof. Dr. Holger Gröll, and scientific contributions from all co-authors.

This chapter is based on: Kovalyk X, Nies I, Saniternik S, Schütz MB, Gremse F, Schwab K, Schmidt AM, Hokamp NG, Gröll H. Biodegradable radiopaque polymeric particles for CT image-guided intraperitoneal drug delivery. Manuscript submitted for publication; 2025.

## 3.2.1 Abstract

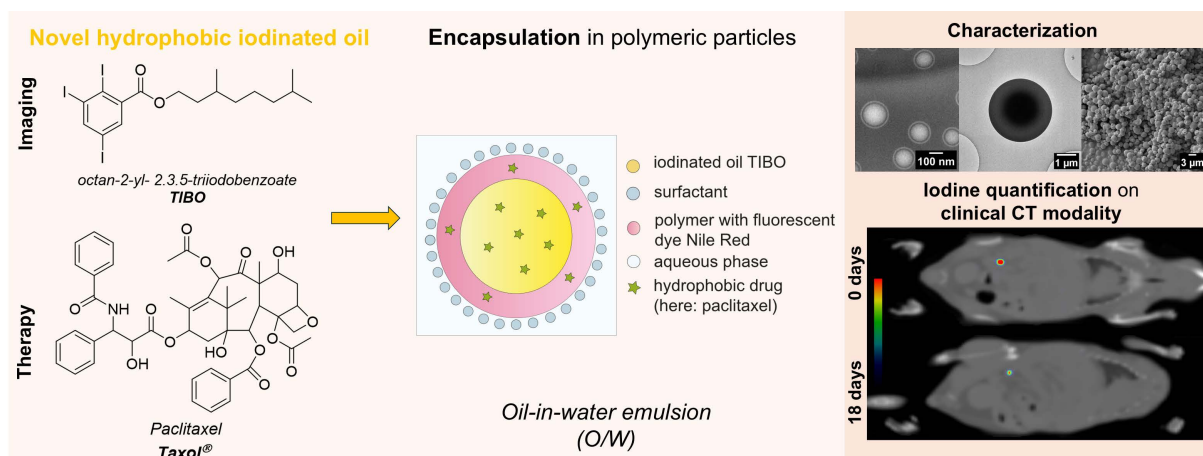


Fig. 1: Graphical abstract.

Iodinated hydrophobic compounds were encapsulated in biodegradable poly(lactic acid)-based polymer particles using a solvent emulsion-evaporation technique that provided control over particle size. The lipophilic iodinated oil containing the 2,3,5-triodobenzoate moiety served as radiopaque contrast agent for particle quantification using dual-energy x-ray CT (DECT) imaging and furthermore as solvent for the chemotherapeutic drug PTX. Release kinetics of PTX was studied in fetal bovine serum over seven days demonstrating sustainable drug release over the entire period of time. The cumulative release levels were mainly dependent on particle diameter. Intraperitoneal (i.p.) administration of a therapeutic dosage of radiopaque spheres in mice provided sufficient iodine contrast to image particle distribution and clearance over 18 days. Imaging showed particle aggregation *in vivo* with about 50% clearance over 18 days. These results show that DECT imaging is a viable option for a theranostic application in i.p. drug delivery using PTX-loaded, radiopaque capsules.

### 3.2.2 Introduction

Peritoneal carcinomatosis (PC) denotes the metastatic dissemination of malignant cells to the peritoneum, the serous membrane enveloping the abdominal organs.[1] The incidences of PC varies depending on the primary tumor. For instance, PC originating from pancreatic cancer accounts for 14% and from colorectal cancer it is estimated to be around 11%.[2,3] Treatment options at this advanced stage include systemic chemotherapy and cytoreductive surgery with optional hyperthermic intraperitoneal chemotherapy (HIPEC) mainly as palliative care.[4]

Any cancer cells remaining in the intraperitoneal space eventually cause recurrence due to their poor response to systemic chemotherapy. A possible solution for the above problems may be a post-operative treatment of any remaining cancer cells with i.p. drug delivery. The expected advantage is a more local exposure of intraperitoneal metastases to a high concentration of cytotoxic drugs over a prolonged time period using for example drug delivery systems (DDS) that provide a sustained release of cytotoxic drugs over days to several weeks.

Few earlier studies have demonstrated that accumulation and tumor targeting as well as tumor penetration of nano- and microparticles administered into the peritoneal cavity depend on size, surface charge and possibly on other material properties such as surface coatings.[5] Generally, i.p. administered DDS offer several advantages over systemically applied drugs leading to prolonged and more direct exposure. Size ranges have been studied from 50 nm to several microns mainly using *ex vivo* histological examination to study distribution within the peritoneal cavity as well as organ uptake and tumor accumulation.[6–8] However, the findings remain heterogeneous, precluding definite conclusions regarding the optimal particle diameter or surface charge. In this context, *in vivo* imaging such as magnetic resonance imaging, nuclear imaging or computed tomography (CT) could be used to track and quantify particle distribution relative to size and material properties. To this aim, the DDS has to be modified with a suitable contrast agent. This strategy, commonly referred to as “image-guided drug delivery” or “theranostics”, represents a powerful tool to elucidate the *in vivo* behavior of DDS. Imaging provides a non-invasive means to monitor biodistribution, accumulation at the target site, and drug release dynamics.

CT offers non-invasive three-dimensional (3D) anatomic images with excellent spatial resolution at relatively low cost and widespread availability. Therefore, it represents the

main modality for diagnosis and oncologic staging of cancer in general and for PC in particular.[9–11] Thus, CT imaging could also play a role for a new theranostic approach in PC management. As image contrast in conventional CT is based on differences in X-ray attenuation, images yield high contrast when there are significant differences between atomic weights (i.e., electron densities) within the imaged volume such as bone calcifications. Consequently, contrast variations between soft tissues with similar mass attenuation coefficients are low. To overcome this intrinsic low soft-tissue contrast, intravenous (i.v.) contrast agents are routinely administered. Currently, only iodine-based CT contrast agents are approved for i.v. administration providing high contrast enhancement due to the high ordinal number (high Z element). All contrast agents for i.v. injections are based on a 1,3,5-triodobenzene building block which is functionalized for improved hydrophilicity. Approved contrast agents contain an iodine concentration of 240–350 mg/ml but differ slightly in structure or overall charge. The disadvantage to these low molecular weight compounds (<2000 Da) is their quick elimination from circulation system and vascularized tissues, high free volume distributions, and rapid renal excretion.[12–14]

Lipiodol® is a lipophilic contrast agent derived from poppyseed oil with an iodine concentration of 480 mg/ml. Its lipophilic character allows for specialized applications such as hysterosalpingography and lymphography. Furthermore, this radiopaque oil is used for intraarterial injections during interventional liver procedures such as trans-arterial chemo-embolization (TACE). In addition, the growing interest in theranostic agents led to exploitation of its inherent properties for solubility and co-delivery of hydrophobic drugs alongside Lipiodol®.[15] The most common approach is to formulate Lipiodol® into emulsions or capsules stabilized by compounds such as amphiphilic lipids or polymers.[16,17] These formulations are particularly interesting as DDS for theranostic approaches as they can be detected using CT imaging while providing a reservoir for hydrophobic drugs dissolved in the oil core. However, as for any i.v. DDS application, small diameters are required making CT imaging difficult due to its limited sensitivity and the relatively low iodine payload of Lipiodol®. More recently, so-called DECT systems allow for a precise quantification of iodine, both *in* and *ex vivo*. [18–21] Here, the specific attenuation characteristics of low- and high-energy photons are detected separately and leveraged to allow for material decomposition and computation of material-specific maps.

In the past, *de Vries et al.* have developed a novel iodinated compound octan-2-yl-2,3,5-triiodobenzoate (TIBO) based on 2,3,5-triiodobenzoate with a particularly high iodine concentration (1120 mg/ml).[13] It was formulated into lipid- and polymer-stabilized emulsions providing almost three times higher CT contrast compared to Lipiodol®-based emulsions. Subsequently, these nanosized particles were tested *in vivo* as a CT blood pool contrast agent in mice. A contrast enhancement of 220 Hounsfield Units (HU) was measured directly after i.v. administration of 520 mg I/kg. The blood circulation half-life of a poly(butadiene)-*b*-poly(ethylene oxide) (PBD-PEO) stabilized emulsion was approximately 3 h without *in vivo* toxicity. As i.p. application of iodinated particles in a theranostic setting comes with less stringent requirement with respect to size, circulation times and clearance, radiopaque drug delivery systems could be a potential new application.

Here, we report the synthesis and characterization of poly(lactic acid)-stabilized capsules containing the iodinated oil TIBO using a facile solvent emulsion/evaporation process. This versatile technique allows co-incorporation of iodinated contrast agents and the hydrophobic drug PTX while controlling particle sizes and shell thickness. Drug release studies were performed in fetal bovine serum (FBS). In particular, we used a clinical DECT for all imaging studies, which allows to obtain iodine concentration maps in mg/ml instead of relative CT contrast measured in Hounsfield units based on the energy dependence of iodine absorption coefficients. As proof of concept, DECT imaging was used to follow particle distribution and subsequent degradation over 18 days in healthy mice after i.p. administration demonstrating a potential application in i.p. drug delivery.

### 3.2.3 Experimental section

#### *General/Materials*

All solvents were obtained from Sigma-Aldrich (St Louis, MO). Poly(lactic-co-glycolic acid) (PLGA) (Resomer<sup>®</sup> RG 756 S: LA:GA (75:25),  $M_w$ =76000–115000 g/mol, Resomer<sup>®</sup> RG 752 H: LA:GA (75:25),  $M_w$ =4,000–15,000 g/mol) was kindly provided by Evonik Operations GmbH (Essen, Germany). PLGA (LA:GA (75:25),  $M_w$ =66,000–107,000 g/mol) was purchased from Sigma-Aldrich (St Louis, MO). Poly(vinyl alcohol) (PVA) ( $M_w$ =67000 g/mol) was purchased from Sigma-Aldrich (St Louis, MO, USA). 9-(diethylamino)-5H-benzo[a]phenoxazin-5-one (Nile Red) and sodium cholate were purchased from Sigma-Aldrich (St Louis, MO, USA). All reagents were used as received from commercial sources without further purification. Water was purified using a Milli-Q system by Sartorius (Göttingen, Germany). A Hereaus Multifuge 3 SR Plus centrifuge (Hanau, Germany) was used for centrifugation. For emulsification, an IKA Ultra Turrax T25 (Staufen im Breisgau, Germany) and a microtip probe sonicator by GEX-600 Cole Palmer (Saint Neots, United Kingdom) were used.

#### *Synthesis*

##### *Octan-2-yl 2,3,5-triiodobenzoate (TIBO)*

The desired compound was synthesized in two steps according to the known procedure. The product was obtained as a light yellow viscous oil. NMR data matched the literature.[13]

##### *Single emulsion evaporation procedure for particle synthesis*

TIBO were encapsulated in PLGA capsules based on an adjusted protocol established by *Pisani et al.*[22] The polymer PLGA (100 mg) was dissolved under sonication in 4 ml of dichloromethane (DCM). For drug-loaded particles, paclitaxel (PTX) was added to the organic solution. The iodinated oil (60  $\mu$ l) and a Nile Red solution in acetone (0.08 mg/ml, 60  $\mu$ l) were added. The mixture was stirred for 10 minutes at room temperature (rt). The organic solution was poured into an ice-cooled beaker containing a 1.5% (w/v) sodium cholate aqueous solution (20 ml). The mixture was emulsified for 1 minute at 24000 rpm and 0 °C with an Ultraturrax-T18. For emulsification speeds below 8000 rpm PVA was chosen instead of sodium cholate. The reaction mixture was stirred for 3 hours at rt for the evaporation of the organic solvent. The suspension was filled up to 20 ml with double distilled water. 1% (w/v) PVA solution (20 ml) was added.

The mixture was centrifuged (2000 rpm, 4 °C, 20 minutes). After decanting the supernatant, the precipitated particles were redispersed in 15 ml of double distilled water. Washing was performed three times until HPLC results showed that PTX residue in the supernatant plateaued (below 5% of initially added PTX). Particles were stored at 4 °C.

For preparation of nano-sized particles, PVA was used as surfactant and an ultrasonic tip for emulsification. Similar synthesis was performed with Lipiodol® instead of TIBO as reference (SI). A double-emulsion procedure was performed for synthesis of iohexol-containing particles (SI).

## *Characterization*

### *Size distribution*

The hydrodynamic radius and the particles with diameters > 1.5 µm radius were measured with dynamic light scattering (DLS) and Coulter Counter, respectively. No further filtrations of the particles were performed throughout the entire experiment. In between measurements the samples were stored at rt.

The hydrodynamic radius and polydispersity of nano-sized particles was determined on a DLS/SLS goniometer system at a scattering angle of 90 degrees, 633 nm wavelength, 20 °C (ALV GmbH, Reutlingen, Germany) at rt. The samples were dispersed in purified water. The diffusion coefficient (D) was obtained from cumulant fits of the intensity correlation function using ALV software. Measurements were repeated 5 times and calculations were performed on the averaged correlation function of the 5 measurements.

Size distribution of micron-sized particles was determined using a Coulter Counter instrument (Multisizer 3, Beckmann Coulter, Brea, CA, USA) at rt. The mean diameter of particles was measured using a 50 µm aperture tube. For each measurement, 10 µl of the sample were mixed for with 20 ml of sodium chloride (0.9%) and analyzed in triplicate.

### *Microscopy*

TEM images were recorded on a side-entry electron microscope (Talos L120C, ThermoFisher, Waltham, USA). Samples for TEM were prepared by placing the emulsion (3 µl) on a 300-mesh carbon-coated copper grid and letting it dry at rt. Grids were previously hydrophilized with Alcian blue.



Scanning electron microscopy (SEM) images were taken with a Zeiss Neon 40 microscope (*Carl Zeiss AG*, Oberkochen, Germany) at 5 kV. For preparation, the dried capsules were attached to sample holders and coated with a thin layer of gold under vacuum before measurement.

All fluorescence and light microscopy images were recorded at rt on a Leica DM6000B instrument (*Leica*, Wetzlar, Germany). The samples were dispersed in purified water and deposited on a glass microscope slide covered with a glass coverslip. 63X objectives were used.

#### *High-performance liquid chromatography*

The drug loading efficiency was determined in triplicate by HPLC (Agilent 1100 series, *Agilent Technologies*, Diegem, Belgium). The mobile phase consisted of acetonitrile/0.1% trifluoroacetic acid (TFA) (v/v) in water (50:50 v/v). The reverse phase column was a Phenomenex Luna C18(2). The flow rate was set at 1.0 ml/min, and the detection wavelength was 227 nm. Particles were dissolved in acetonitrile and vigorously stirred to get a clear solution. Docetaxel was used as reference substance and added directly to the particle sample. The encapsulation efficiency was defined by the ratio of measured and initial weight of PTX encapsulated in particles.

$$\text{Incorporation efficiency [\%]} = \frac{\text{Weight of PTX in particles}}{\text{Initial weight of PTX}} \times 100 \quad \text{Eq. 1}$$

The drug loading was defined as the weight of incorporated drug divided by the polymer particle weight.

$$\text{Drug loading efficiency [\%]} = \frac{\text{Weight of PTX in particles}}{\text{Weight of particles}} \times 100 \quad \text{Eq. 2}$$

#### *In vitro drug release and anti-tumoral effect*

Chemotherapeutic-loaded particles (0.05 mg of PTX) were dispersed in 1.5 ml of FBS and incubated at 37 °C under shaking. At determined time intervals, the vials were centrifuged at 4500 rpm for 20 minutes. 0.75 ml of supernatant was taken and analyzed by HPLC as described above. Equal volume of FBS was replaced. Studies were carried out in triplicates.

CT26 cells were grown in DMEM culture medium supplemented with 10% FBS and 1% Pen Strep. The cells were maintained in an incubator supplied with 5% CO<sub>2</sub>/95% air humidified atmosphere at 37 °C. Cells were seeded in 96-well plates at the density of 7500 cells per well and incubated 24 h to allow cell attachment. The cells were then

incubated with PTX, PTX-loaded particles, and drug-free particles (polymer concentration equal to PTX-loaded particles) for 24 , 48 h and 72 h. At determined time points, the formulations were replaced with PBS containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) (5 mg/ml) and cells were then incubated for an additional 3 h. Dimethylsulfoxide (DMSO) was added to dissolve the formazan crystals. Absorbance was measured at 570 nm using an Epoch microplate reader (*Agilent Technologies*, Diegem, Belgium). The results were expressed as mean values of three measurements.

### *Phantom preparation and in vitro CT measurements*

Particles were measured for quantification of iodine content as a solid particle dispersion in water immobilized in gelatin gel. The dispersed microcapsules in water (1 ml) were mixed with 10 wt% gelatin gel (9 ml) and filled into 15 ml centrifuge tubes. The tubes were prefilled with pure gelatin (3 ml) to condense the sample volume in the remaining gel phase. After solidification of the gel at 4 °C, the tubes were filled with pure gelatine to the brim. These tubes were immobilized in a plastic tank which was filled with water. A detector-based DECT system (Philips 7500 Spectral CT, *Philips*, The Netherlands) has been used for scanning with a standard abdominal imaging protocol.[19–21] Images were acquired with 120 kVp and reconstructed with a field-of-view of 125 mm on a 512x512 cm<sup>2</sup> imaging matrix, rendering the in-plane resolution 0.244 mm. Images were computed using a dedicated image reconstruction algorithm providing quantitative iodine maps (unit: mg/ml) in addition to standard Hounsfield-Unit-based anatomical images; however, for the latter a 1024x1024 matrix was chosen (improving in-plane resolution to 0.122 mm). Images were evaluated with Imalytics Preclinical® (*Gremse-IT*, Germany) as established by *Gremse et al.*[23] Iodine content was defined as ratio of encapsulated iodine per mg particle.

### *In vivo CT measurement*

All animal experiments were approved by the State Agency for Consumer Protection and Nutrition of North-Rhine Westphalia (LAVE, Animal Ethical Proposal no.: 81-02.04.2020.A320) and were performed by authorized personnel. Healthy Balb/c mice (*Charles River*, USA) were i.p. injected with TIBO-containing particles. Dose was adjusted to 20 mg/kg bodyweight of PTX. Particles were dispersed in isotonic 0.9% NaCl solution. For injection, total injection volume was determined according to maximum allowed i.p. administration volume of 10 ml/kg bodyweight. The initial dose

was found to be  $91\pm 13\%$  of the injected dose (ID) directly after injection, indicating a loss in the syringe relative to the amount in the animal. Therefore, 100%ID at the initial time point in the animal was assumed for all subjects to standardize and enable comparative quantification of distribution and degradation. Images were acquired and reconstructed as described for *in vitro* measurements. After DECT scanning, mice were allowed to wake up. Subsequently, follow-up scans were conducted over 18 days. Images were evaluated with Imalytics Preclinical<sup>®</sup> (*Gremse-IT*, Germany).[23]

### 3.2.4 Results

The aim of this study was the synthesis of monodisperse polymer capsules containing the iodinated oil TIBO as well as the hydrophobic drug PTX to facilitate CT imaging. We expect size-dependent particle distribution in the peritoneum, potentially different release kinetics with different sizes, and higher iodine content for DECT imaging to exceed the lower detection limit. In addition, the chosen polymer can influence the efficiency of incorporation, degradation times, and biocompatibility.

#### *Particle synthesis and influence of parameter variation*

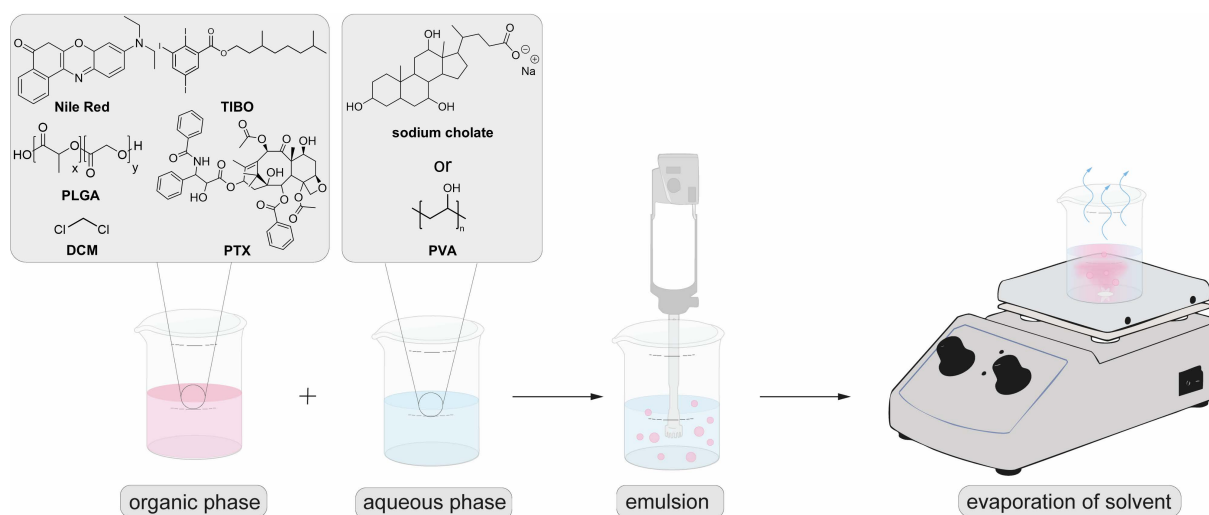


Fig. 2: Reaction scheme of solvent-emulsion process.

PLGA-shelled nano- and microcapsules containing the iodinated oil TIBO and the hydrophobic drug PTX were synthesized using a solvent-emulsion process (Fig. 2). Influence of process parameters on particle diameter, stability, and encapsulation efficiency were investigated. As first step, the organic DCM phase containing a given volume fraction of iodinated oil and PTX is emulsified. The emulsion is stabilized using either sodium cholate or PVA to prevent rapid coagulation. Due to the solution of DCM in the water-rich phase and subsequent evaporation, the polymer precipitates once it reaches its solubility limit and forms a polymeric shell surrounding the remaining oil. Nile Red incorporates mainly into the particle polymer shell and is to some extent dissolved in the oil, thus facilitating fluorescence microscopy. The diameter of the final capsules and the shell thickness depends on the volume of the emulsion droplet and the volume fraction of the compounds within the organic phase.[24]

In the first set of experiments, the volume fraction of iodinated oil was varied during synthesis using 60–90  $\mu\text{l}$  of TIBO in order to determine the effect on particle size, stability, and iodine loading (Table 1).

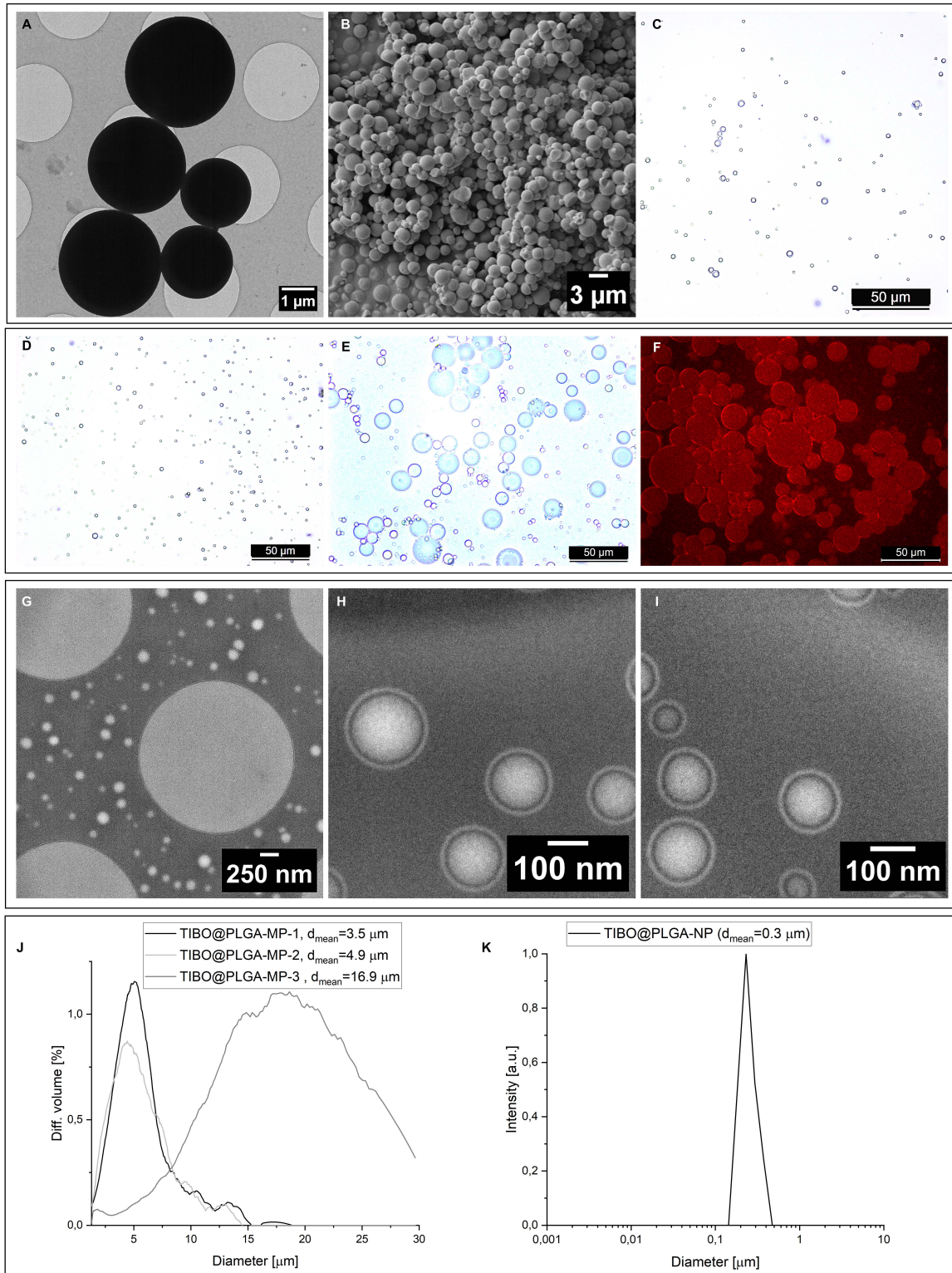
Secondly, to obtain particles within different size ranges, emulsification speed was varied from 24000 to 8000 rpm yielding particles with a mean diameter from 4.6  $\mu\text{m}$  to 16.9  $\mu\text{m}$ . For nanoparticles, an ultrasound microtip probe was used for emulsification, which allowed to obtain diameters of around 150 nm. Thus, the diameter of the initial emulsion largely determines the final particles diameter. However, with increasing diameter, polydispersity increases as well, indicating it may be more difficult to control the emulsification step and thus diameters of the dispersed organic phase using an emulsifier at low speed (Table 1). Particle diameter and morphology was further characterized using microscopy (Fig. 3). Third, experiments using PLGA with different molecular weights yielded particles with higher stability resulting in larger diameters for higher molecular weight.

A similar synthesis was performed with iodinated hydrophobic contrast CT agent Lipiodol® as reference. In general, particles encapsulating TIBO were more stable and displayed a more narrow size distribution compared to capsules containing Lipiodol® (PDI of 0.045 compared to 0.205, Table S1 in supplementary information (SI), Fig. S1).

*Table 1: Overview of particle diameters and respective reaction conditions. LA:GA ratio in all polymers corresponds to 75:25. For microparticle emulsification a dispersion tool was used, for nanoparticle emulsification an ultrasound microtip was utilized.*

| Sample         | Emulsification step    | Polymer molecular weight [g/mmol] | Volume fraction of TIBO [ $\mu\text{l}$ ] | Mean particle diameter [ $d_{\text{mean}}$ , $\mu\text{m}$ ] | Iodine content [mg/mg] |
|----------------|------------------------|-----------------------------------|-------------------------------------------|--------------------------------------------------------------|------------------------|
| TIBO@PLGA-MP-1 | 24000 rpm,             | 66–107                            | 60                                        | 3.5 $\pm$ 2.3                                                | 0.191                  |
| TIBO@PLGA-MP-2 | 1 min, 0 °C            |                                   | 90                                        | 4.9 $\pm$ 2.7                                                | 0.293                  |
| TIBO@PLGA-MP-3 | 8000 rpm,              | 76–115                            | 90                                        | 16.9 $\pm$ 7.9                                               | 0.246                  |
|                | 5 min, 0 °C            |                                   |                                           |                                                              |                        |
| TIBO@PLGA-NP   | 100% amplitude, 60 sec | 4–15                              | 90                                        | 0.264<br>PDI: 0.045                                          | 0.259                  |

## Results



**Fig. 3:** Images of TIBO@PLGA-MP-1 A) TEM. B) SEM. C): Light microscope. D) Light microscope image of Images of TIBO@PLGA-MP-2. E) Light microscope image of Images of TIBO@PLGA-MP-3. F) Fluorescence microscope image of TIBO@PLGA-MP-3. G), H), I) TEM images of TIBO@PLGA-NP. Size distribution obtained with (J) Coulter Counter for larger particles and (K) DLS for nanoparticles.

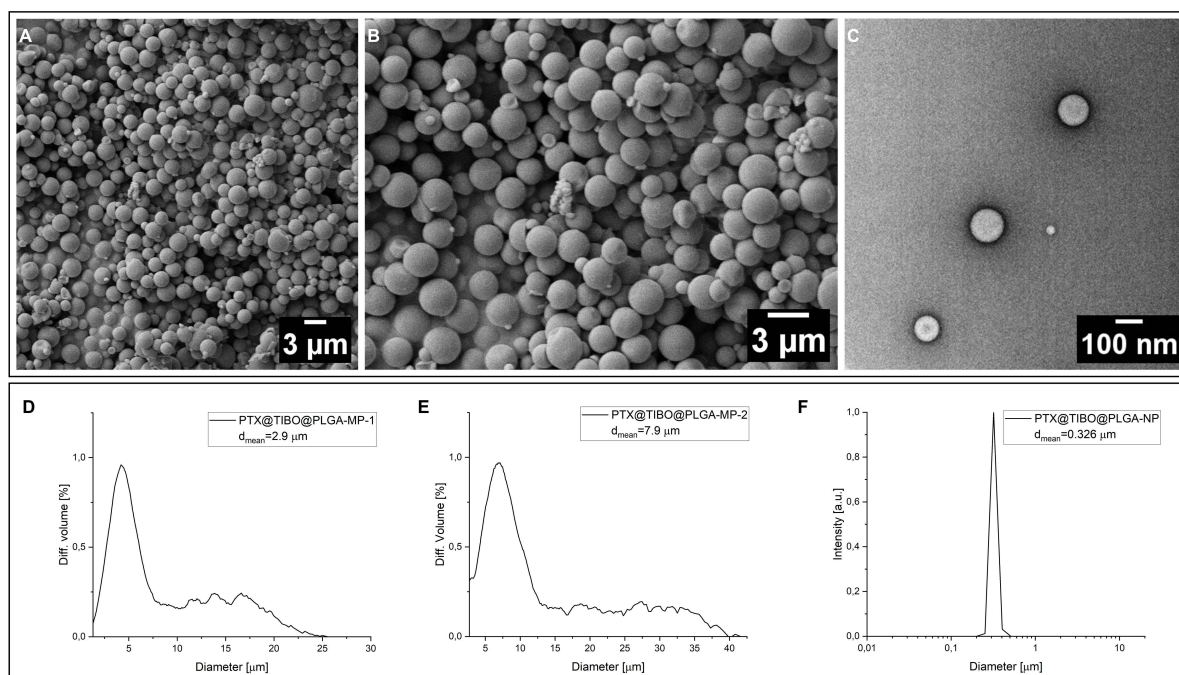


## Drug incorporation

Different PTX concentrations in DCM were tested for optimal drug loading of capsules using 90  $\mu$ l of TIBO for synthesis (Table 2). Exceeding a critical PTX concentration of 4.0 mg/ml in DCM during synthesis, particle morphology changed from core-shell to undesired acorn shapes (Fig. 4). Additionally, PTX crystallization could be observed (Fig. S2 in SI). Interestingly, nano-sized particles showed higher PTX incorporation efficiency and higher final drug loading compared to micron-sized particles.

*Table 2: Overview of particle diameters and respective reaction conditions. LA:GA ratio in all polymers corresponds to 75:25. PTX concentration in DCM during synthesis was fixed to 4.0 mg/ml. For microparticle emulsification a dispersion tool was used, for nanoparticle emulsification an ultrasound microtip was utilized. PTX@TIBO@PLGA-MP-1 emulsified at 24,000 rpm, PTX@TIBO@PLGA-MP-2 emulsified at 10,000 rpm.*

| Sample             | Polymer molecular weight [g/mmol] | Particle diameter [d <sub>mean</sub> , $\mu$ m] | PTX in particles [mg/mg] | Iodine content [mg/mg] |
|--------------------|-----------------------------------|-------------------------------------------------|--------------------------|------------------------|
| PTX@TIBO@PLGA-MP-1 | 66–107                            | 2.9 $\pm$ 2.2                                   | 0.052                    | 0.275                  |
| PTX@TIBO@PLGA-MP-2 | 76–115                            | 7.9 $\pm$ 3.0                                   | 0.052                    | 0.276                  |
| PTX@TIBO@PLGA-NP   | 76–115                            | 0.326                                           | 0.080                    | 0.265                  |
|                    |                                   | PDI: 0.044                                      |                          |                        |



**Fig. 4:** A), B) SEM images of PTX@TIBO-PLGA-MP. C) TEM image of PTX@TIBO@PLGA-NP. D) Size distribution of PTX@TIBO@PLGA-MP-1. Emulsified at 24,000 rpm. E) Size distribution of PTX@TIBO@PLGA-MP-2. Emulsified at 10,000 rpm. F) Size distribution of PTX@TIBO@PLGA-NP.

### *In vitro drug release and anti-tumoral effect*

The cumulative release profiles of PTX from the particles with three distinctly different mean diameters were determined in FBS. The nanoparticle dispersion PTX@TIBO@PLGA-NP (0.326  $\mu\text{m}$ ) exhibited a rapid initial release, with 38% of the encapsulated PTX released within the first 24 hours, approximately twice the amount released from PTX@TIBO@PLGA-MP-1 dispersion (2.9  $\mu\text{m}$ , 19%) and four times that from PTX@TIBO@PLGA-MP-2 dispersion (7.9  $\mu\text{m}$ , 9%) over the same period (Fig. 5). Notably, the cumulative release for nanoparticles was around 50% after seven days and remained lower for microparticles indicating sustained release over a prolonged time span.

The kinetic of cumulative drug release can be modeled using a time exponential behavior[25]:

$$\frac{M_t}{M_\infty} = k * t^n \quad \text{Eq. 3}$$

where  $M_t$  and  $M_\infty$  is the absolute cumulative amount of drug released at time  $t$  and infinite time, respectively;  $k$  is a constant incorporating the diffusion coefficient of PTX within the drug carrier as well as the overall surface, and  $n$  is an exponent, which is indicative for the mechanism of drug release.

The two larger particles show a release kinetic in line with a *Fickian* diffusion transport of PTX ( $n = 0.43$ ,  $k = 0.045$  for  $d = 2.9 \mu\text{m}$  and  $n = 0.53$ ,  $k = 0.0169$  for  $d = 7.9 \mu\text{m}$ ) while small nanoparticles show per particle a subdiffusive behavior with a slower release kinetic ( $n = 0.294$ ,  $k = 0.165$  for  $d = 0.326 \mu\text{m}$ ). The higher cumulative release of nanoparticles as well as the difference for the larger particles solely reflects the higher overall surface area of smaller particles for the same amount of PTX, which is subsumed in  $k$ . Multiplied by available surface area  $A$ , values for  $k' = kA$  are identical for the larger particles and about half for the nanoparticles, which is in line with hindered diffusion model for the latter.



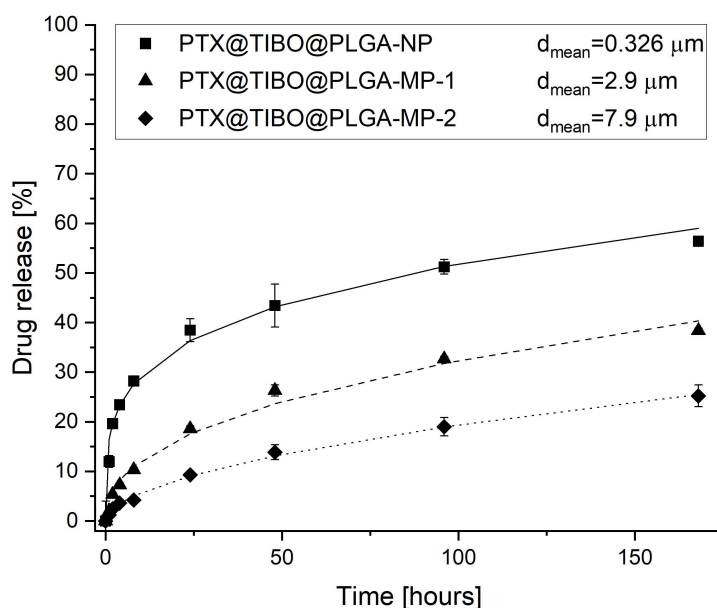


Fig. 5: Cumulative drug release of PTX@TIBO@PLGA-MP-1 (2.9  $\mu\text{m}$ ), PTX@TIBO@PLGA-MP-2 (7.9  $\mu\text{m}$ ) and PTX@TIBO@PLGA-NP (0.326  $\mu\text{m}$ ).

Cell viability studies were conducted for PTX-loaded TIBO-containing microparticles in comparison to free PTX (Fig. 6). Unloaded TIBO-containing particles served as control. After 24 hours, free drug exhibited superior cytotoxicity, with a half maximal inhibitory concentration ( $\text{IC}_{50}$ ) of 67.5  $\mu\text{g/ml}$  compared to 174.8  $\mu\text{g/ml}$  for the particulate formulation. However, after 48 hours PTX-loaded particles demonstrated similar cancer cell inhibition compared to free drug. By 72 hours, PTX-loaded particles achieved the lowest  $\text{IC}_{50}$  value of 3.6  $\mu\text{g/ml}$ , while the efficacy of free PTX declined, reflected in an increased  $\text{IC}_{50}$  of 67.9  $\mu\text{g/ml}$ . In addition, no cytotoxicity is observed with unloaded particles at all tested concentrations even after 72 h.

## Results

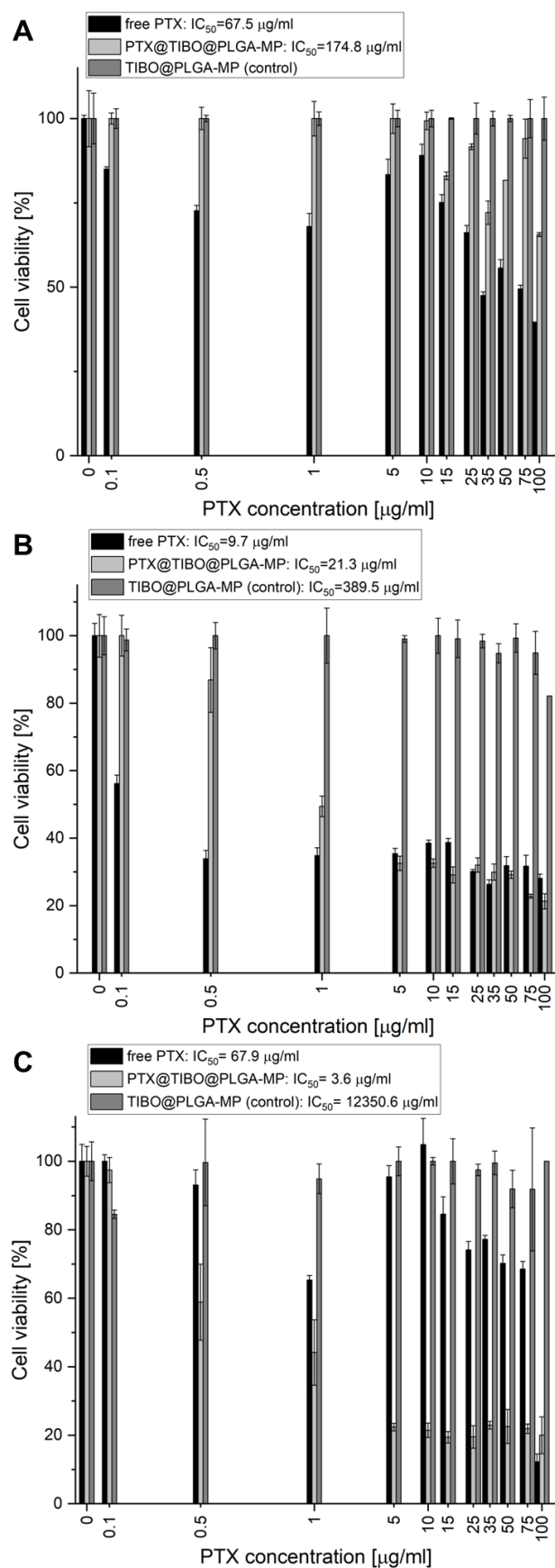


Fig. 6: Cell viability studies in CT26-WT cells of PTX@TIBO@PLGA-MP after A) 24 h, B) 48 h and C) 72 h incubation time.

## CT measurements

### Validation and in vitro phantom iodine quantification

DECT imaging was used to detect and quantify the DDS concentrations based on the incorporated iodinated oil using sodium iodide solutions of known concentrations as reference. Iodine maps were reconstructed and analyzed by placing volumes of interest (VOIs) into each phantom yielding concentration of iodine in mg per ml (Fig. 7).

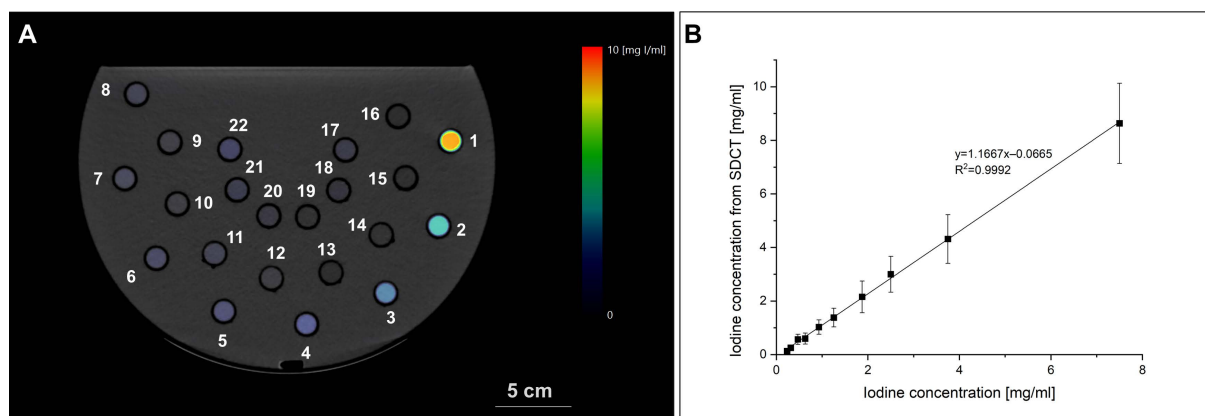


Fig. 7: A) Overlay of iodine map and CT image. Numbers 1 to 10 correspond to sodium iodide samples. Numbers 11 to 22 correspond to particulate samples. B) Calibration line for validation of iodine quantification with sodium iodide.

Subsequently, DECT was used to determine the iodine concentration of DDS dispersions with a known particle concentration. To prevent sedimentation and therefore concentration gradients, particle samples were measured while immobilized in gelatin. Based on DECT quantification, the amount of iodine per mg particle as well as the encapsulation efficiency of the compounds was calculated. The latter was found to vary between 60–80%, with no direct dependency on particle diameter or PTX incorporation (Table S2). Significantly higher iodine content (0.2–0.3 mg iodine per mg particle) was obtained with TIBO-containing capsules as oil phase compared to Lipiodol®-containing (0.1 mg iodine per mg particle) which is expected due to TIBO's higher iodine content. Encapsulation efficiency was also improved, ranging from 61–82% with TIBO versus 54–59% with Lipiodol® (Table S1 and S2, SI).

*In vivo biodistribution as a function of particle diameter in mice*

Feasibility to use DECT for imaging iodinated DDS after i.p. administration was tested *in vivo* in healthy mice. Mice (n=3 per group) were i.p. injected with TIBO@PLGA-MPs of three different diameters, i.e. 0.264  $\mu\text{m}$ , 4.9  $\mu\text{m}$  and 16.9  $\mu\text{m}$ , containing no PTX for ethical reasons. However, the injected dose was chosen to correspond to a therapeutical PTX dosage of 20 mg/kg bodyweight, corresponding to an iodine dose of approximately 50–75 mg/kg bodyweight. Afterwards, consecutive DECT scans were performed over a period of 18 days to quantify particle biodistribution. Animal weight measurements remained stable within the standard deviation throughout the study period, with no adverse events observed (Fig. S3, SI). The injected amount of DDS provided enough iodine payload for successful DECT in order to visualize particle distribution and concentration in the abdomen over time (Fig. 8A, B).

I.p. iodine concentration was quantified with DECT for three different DDS systems having diameters of 0.3  $\mu\text{m}$ , 4.9  $\mu\text{m}$ , and 16.9  $\mu\text{m}$ , expressed in%ID. Over time, the overall amount of iodine decreased possibly indicating particle degradation and clearance of the iodinated oil from the intraperitoneal cavity (Fig. 8). Notably, imaging reveals a large aggregate of particles which slightly relocates within the peritoneal cavity between the different imaging time points.

Smaller particles of 0.3  $\mu\text{m}$ , 4.9  $\mu\text{m}$  reached approximately 65%ID signal loss after eight days, in contrast to larger particles of 16.9  $\mu\text{m}$ , which retained 81%ID at the same time point. After 18 days, signal loss of approximately 50%ID was comparable for all particle sizes (Fig. 8C).

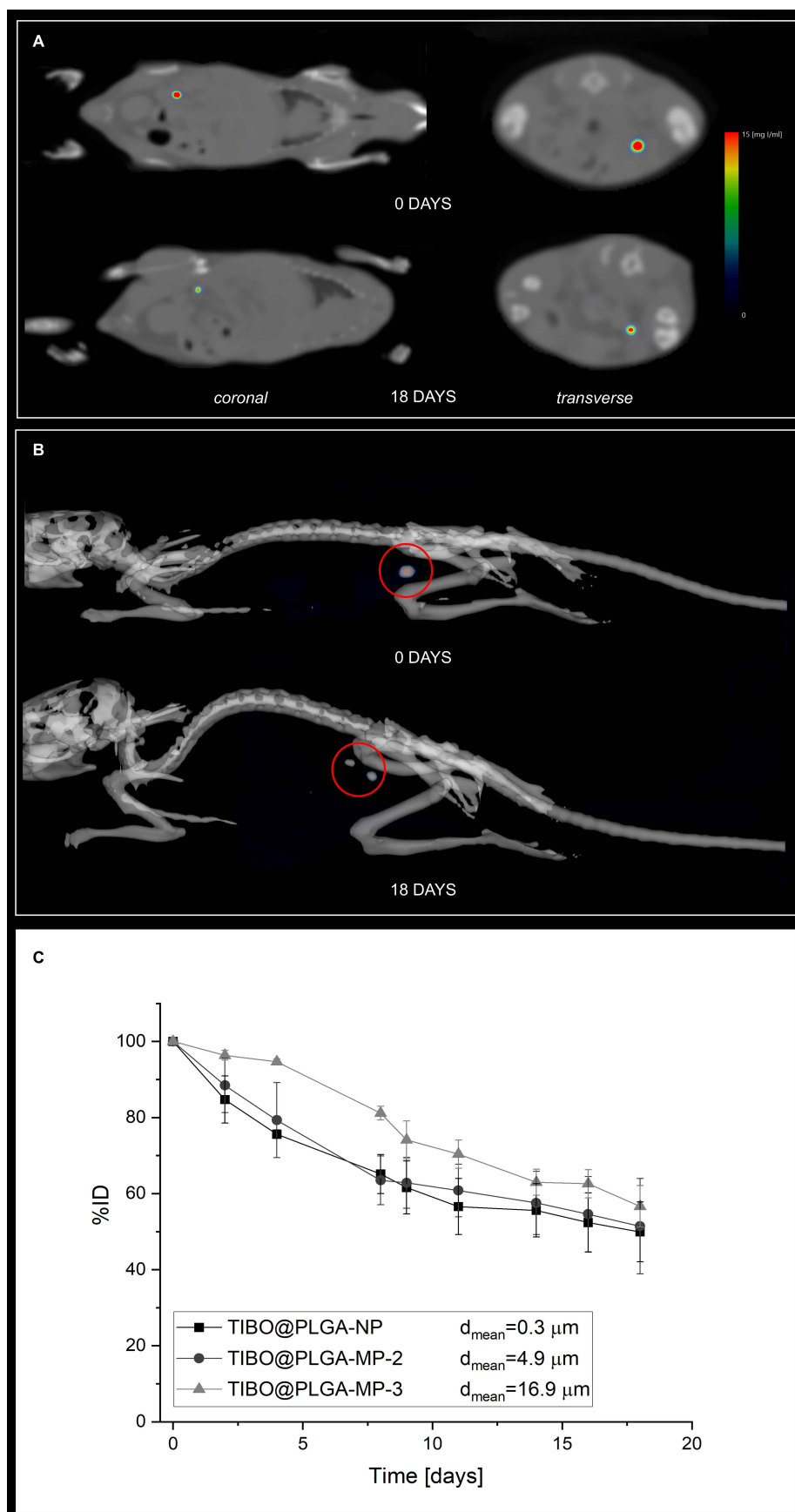
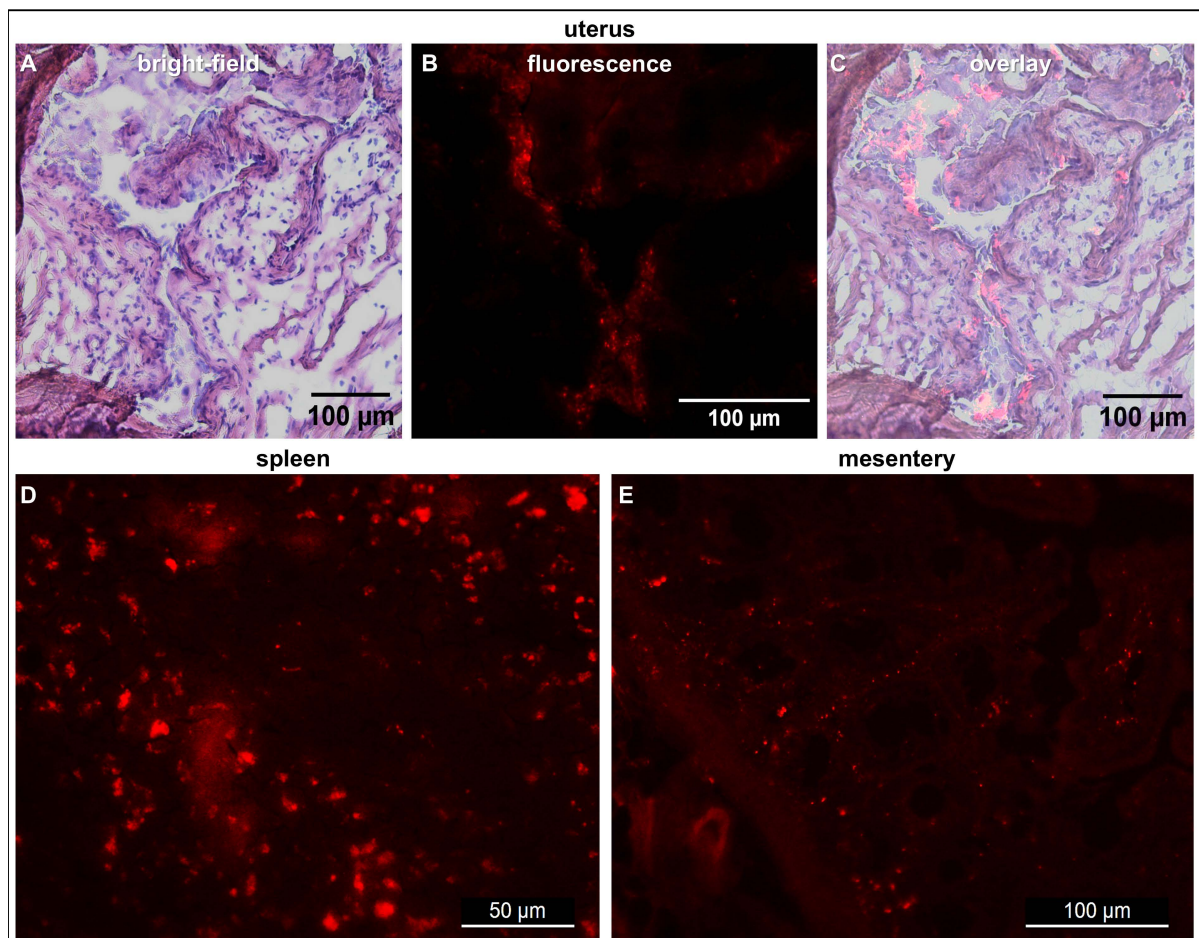


Fig. 8: A) Iodine map/CT overlay in vivo for one animal injected with TIBO@PLGA-NP on injection day and after 18 days. B) Representative visualization of iodine signal development over 18 days in one animal injected with TIBO@PLGA-NP. Iodine content marked in red circle. C) Iodine signal loss over study period of 18 days in dependence of injected particles.

Histological evaluation after 18 days showed for all three particle diameters to some extent a distribution across the entire peritoneum and additionally in the intestines, liver, uterus and ovaries (Fig. 9) as well as spleen and pancreas. The largest particles, which could be well visualized in the spleen, no longer exhibited a spherical morphology but appeared to be eroded, with a size range of 7–9  $\mu\text{m}$ . Structures identified in other organs, such as the uterus, liver, or mesentery, were even smaller measuring at only approximately 2–4  $\mu\text{m}$  (Fig. 9D, E).



*Fig. 9: A) Bright-field, B) fluorescent image and C) overlay of uterine slices of 4.9  $\mu\text{m}$  particles. Fluorescent images of 16.9  $\mu\text{m}$  particles in D) spleen and E) mesentery.*

### 3.2.5 Discussion

This work studied PLGA-shelled nano- and microcapsules filled with iodinated oil TIBO and the hydrophobic drug PTX for possible application in i.p. drug delivery.

A solvent-emulsion-evaporation technique was employed based on a process described by *Pisani et al.* for synthesis of polymer capsules.[22] This process allows for controlled particle synthesis with diameters ranging from 100 nm to 50  $\mu\text{m}$ . Here, particles with mean diameters ranging from 200 nm to 15  $\mu\text{m}$  were synthesized. Additionally, the volume fraction of oil and polymer in the organic phase provides control over the capsule shell thickness.[22,26,27] A fluorescent dye was incorporated to facilitate subsequent histological examination. TIBO not only provides higher iodine payloads for CT imaging but also yields more stable particles with a more narrow size distribution compared to Lipiodol<sup>®</sup>-based particles.

The radius of the microcapsules and iodinated oil can be theoretically calculated as described by *Böhmer et al.* showing that particle diameter depends on the volume of the emulsion droplets and the amount of oil, while shell thickness depends on the volume fraction of polymer and oil within the organic phase.[24] The results presented here show that emulsion droplet volume formed in the emulsification step is the most decisive parameter to control particle size. However, formation of large droplets using low mixing speeds also leads to an increasing polydispersity of emulsion droplets which translates into a broad size distribution for large particles. Here, other production methods such as microfluidic-based approaches provide higher control. Increasing the volume of iodinated oil during synthesis from 60  $\mu\text{l}$  to 90  $\mu\text{l}$  lead to an increase in particle diameter from 3.55  $\mu\text{m}$  to 4.86  $\mu\text{m}$ . Further increase of the TIBO volume fraction led to unstable particles yielding oil droplets rather than capsules. Polymer choice seemed to impact particle stability with higher molecular weight leading to more stable particles.

PTX loading was possible in high yields of 0.052–0.080 mg PTX per mg particle. The upper limit of PTX incorporation in the final formulation is at around 16.0 mg for 90  $\mu\text{l}$  TIBO, exceeding this concentration led to disordered particle morphology and PTX crystallization. Compared to solid PLGA-based particles, PTX incorporation was found to be ten-fold higher, achieving drug loadings of 5–8%, substantially exceeding the 0.4–0.7% typically reported for similar PTX-containing PLGA particles in the literature,

where drug loading is defined as ratio of PTX weight in particles and total particle weight.[28–30] This highlights TIBO's favorable dissolution properties for PTX.

For theranostic CT applications, PTX has been incorporated into nanoparticles containing Lipiodol® such as porphyrin nanoemulsions or radiopaque nanoemulsions for co-delivery of PTX and siRNA delivery, PTX loading in these systems was below 4%.[17] *Hwa Oh et al.* co-delivered PTX and siRNA in radiopaque nanoemulsions with a drug loading of around 1–2%, as described in supplementary materials.[31] An alternative method to achieve high PTX concentration per particle with a 2,3,5-triiodobenzoic acid-based prodrug strategy allowed for 10% PTX loading, enabling CT-guided chemotherapy.[32]

Release kinetic of PTX follows an exponential behavior which is mainly diffusion-driven for larger particles. For nanoparticles, a hindered diffusion was found, indicating a slower release per particle.[25] However, normalized to the same PTX concentration, cumulative release increases with decreasing particle diameter simply due to the higher surface area of smaller particles.[33] Thus, using the same particle systems with different diameters allows to tune the cumulative release obtained over time towards the need in i.p. drug delivery. At later time points, particle erosion and degradation of the polymer matrix especially in the biological environment provided by the i.p. fluid, may affect the release kinetic as well. As has been shown for solid PLGA-based particles, swelling and degradation of the polymer matrix leads to different release kinetics than expected for purely diffusion-driven release.[34] PTX release kinetic for our capsules-based system is expected to also depend on degradation of the polymer shell. Parameters such as shell thickness, molecular weight and the ratio of PLA to PLGA provide additional opportunities to tune the release properties, but were not subject of the study.

Results of our cell viability studies are in line with the release kinetic, showing an increasing toxicity over time. These results reveal that toxicity due to slow-release of PTX increases with longer incubation times, consistent with observations in related systems.[35–40] This characteristic is advantageous for our intended application of i.p. drug delivery, which relies on prolonged residence time and sustained drug release. These results reveal that toxicity from slow-release increases with longer incubation times, consistent with observations in related systems.[35–40] This characteristic is



advantageous for our intended application of i.p. drug delivery, which relies on prolonged residence time and sustained drug release.

With respect to DECT imaging, iodine signal at concentrations as low as 0.2 mg/ml iodine was detected and quantified in phantom studies using a clinical DECT modality. The phantom measurements indicate a slight overestimation of iodine concentrations measured by CT compared to expected values. This overestimation has also been reported in numerous previous studies.[19–21] Additionally, starting at iodine concentrations of approximately 2 mg/ml, the standard deviation is increasing, likely due to artifacts caused by photon starvation. As the iodinated capsules provide high iodine concentrations up to 0.275 mg iodine per mg particle, a theranostic approach at therapeutic relevant PTX concentrations is possible. As a proof of concept, Balb/c mice were injected i.p. with radiopaque particles of three different sizes in a therapeutical dosage of PTX. DECT was able to quantify iodine concentrations *in vivo* over 18 days, showing a signal loss of approximately 40–50% during this time span. Surprisingly, DECT imaging revealed that the majority of signal was very localized indicating an agglomeration of particles in the peritoneal cavity. Signal loss occurred more rapidly in smaller particles, compared to larger particles with diameters of 16.9  $\mu\text{m}$ , consistent with literature showing increased peritoneal retention with larger particle sizes.[41–44] However, signal loss for the 0.3  $\mu\text{m}$  and 4.9  $\mu\text{m}$  particles was comparable within the error, demonstrating that clearance of particles or degradation with subsequent clearance of TIBO is largely independent of the individual particle size. Rather, particle agglomeration and sticking to tissue close to the injection site seems to prevent clearance also for the nanoparticles from the peritoneal cavity by the lymphatic system. Entry into the lymphatic system and subsequent clearance from the peritoneum is mainly governed by the stomatal diameter, which has approximately a 3.6  $\mu\text{m}$  cut-off in mice.[5] Aluri *et al.* experienced similar unexpected behavior finding that decrease in particle size and faster drug release in nanocrystals did not lead to higher *in vivo* exposure of the drug after i.p. injection compared to microcrystals with slower release rates.[45] To date, the distribution and clearance pathways of PLGA-based nano- and microparticles after i.p. injection remain poorly understood, as studies have largely relied on histological analysis or optical methods.[46–51] These methods are limited to endpoint data or lack quantitative accuracy. Interestingly, our histological examinations show particles uptake in all abdominal organs which could lead to misleading interpretation without the additional information provided by imaging. Also, our

observation of particle agglomeration and therefore exposure of tissue to a high local drug concentration could explain the observation of strong inflammatory reactions in clinical studies using polymer microparticles for i.p. drug delivery.[52]

In most other studies radiopaque DDS were administered i.v. which imposes entirely different requirements such as diameters in the nanometer range, stability in blood, and long circulation times. For example, DECT has been successfully employed to distinguish gold NPs localized in tumors from liposomal iodine circulating in the vasculature, enabling simultaneous visualization of multiple contrast agents *in vivo*. [53,54] *Clark et al.* found therein a detection limit of 2.3 mg per ml iodine with a custom-built dual-source micro-CT system.[54] *Badea et al.* evaluated *in vivo* discrimination of iodine and gadolinium liposomal contrast agents using dual-energy and photon-counting spectral micro-CT. Both methods detected and quantified concentrations as low as 2.5 mg/ml. Imaging in sarcoma-bearing mice provided biodistribution data, demonstrating spectral CT's promise for multiplexed tumor imaging.[55] Nevertheless, most studies focus on micro-CT tailored for animal models, whereas this work conducted scanning on a clinical CT system designed for human use.[32] In addition, most recent CT systems overcome the need for a scintillating detector (used in all prior CT systems) through use of a semiconductor detector (so-called photon-counting CT). These systems are thought to improve both material separation capabilities and spatial resolution; both parameters of utmost importance for *in vivo* tracking and quantification of DDS.

DECT imaging allows real-time, quantitative, longitudinal particle tracking, providing novel insight into biodistribution and clearance dynamics *in vivo*. Past work in this field typically relies on conventional micro-CT and non-quantitative HU, limiting distinction between background tissue and drug carriers.[56,57] In contrast, this approach enables i.p. injection and longitudinal biodistribution tracking over several days using DECT-derived iodine maps, allowing precise anatomical localization and *in vivo* quantification of the administered DDS. This method can facilitate future systematic studies on i.p. distribution and clearance, helping to identify the most suitable particle parameters for optimized drug delivery.

### 3.2.6 Conclusion

Here, synthesis and formulation of PLGA-shelled nano- and microcapsules filled with the lipophilic, radiopaque oil TIBO is demonstrated. TIBO serves as a solvent for high PTX loading and simultaneously provides a high iodine payload for CT-based quantification. In particular, a clinical DECT system was used to obtain iodine concentration maps which allow to quantify biodistribution and particle degradation after i.p. administration over time. *In vitro* drug release of PTX in FBS together with cell viability assays reveal slow release of PTX with strongest cell viability inhibition after 72 hours. The release profile is considered favorable for future treatment of intraperitoneal metastasis. Notably, the high iodine payload in particles allowed to inject volumes according to therapeutic dosage of PTX into animals while still generating sufficient CT contrast for particle visualization. Signal loss is similar for particles in the nano- and micrometer range largely due to formation of large aggregates upon injection, which dominates the overall clearance rather than the individual particle size. At this point, it is unclear if signal loss is due to degradation of particles and subsequent clearance of TIBO, or related to redistribution and clearance of individual particles released from the aggregate.

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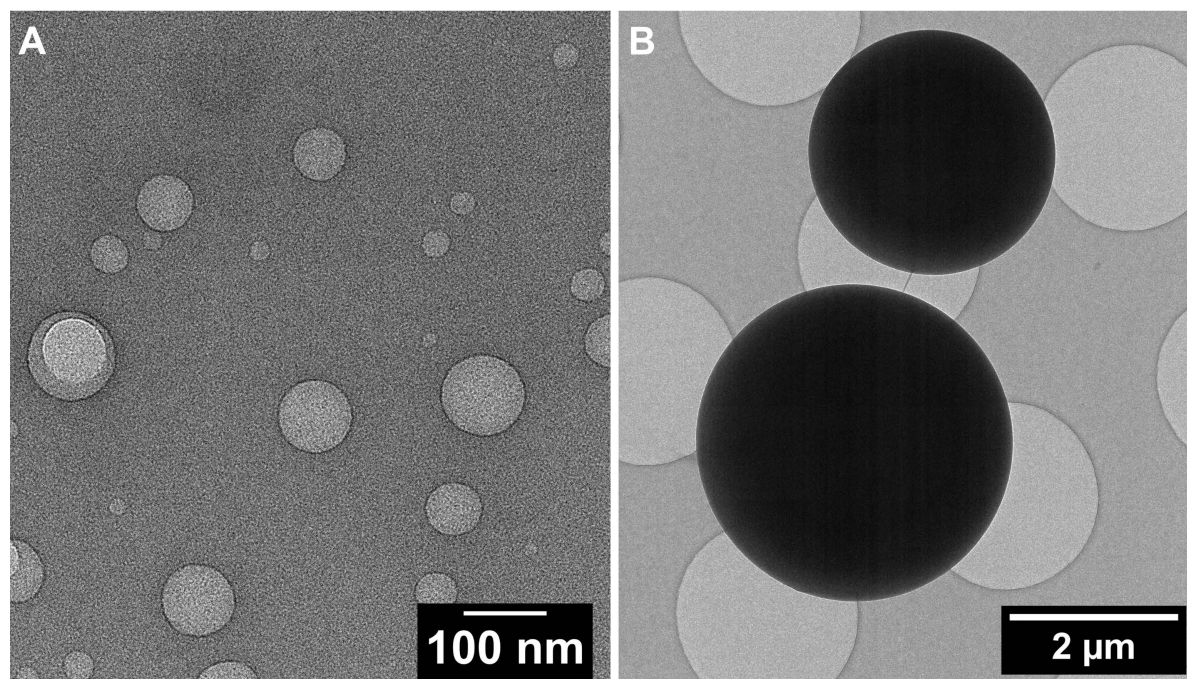


### 3.2.8 Supporting information

Following the protocol described in experimental section for particle synthesis with TIBO, particles with cores consisting of Lipiodol® were synthesized as reference.

*Table S1: Overview of obtained particles and respective reaction conditions.*

| Sample           | Emulsification step                         | Initial volume of iodinated species [ $\mu$ l] | Mean particle diameter [ $\mu$ m] | Iodine content [mg/mg] / IE [%] |
|------------------|---------------------------------------------|------------------------------------------------|-----------------------------------|---------------------------------|
| Lipiodol@PLGA-NP | Ultrasound microtip: 100% amplitude, 60 sec | 90                                             | 0.136<br>PDI: 0.205               | 0.095 / 53.7                    |
| Lipiodol@PLGA-MP | Dispersion tool: 24000 rpm, 1 min, 0 °C     | 60                                             | 3.3 $\pm$ 1.7                     | 0.070 / 59.1                    |



*Fig. S1: TEM images of A) Lipiodol@PLGA-NP and B) Lipiodol@PLGA-MP.*

## Results

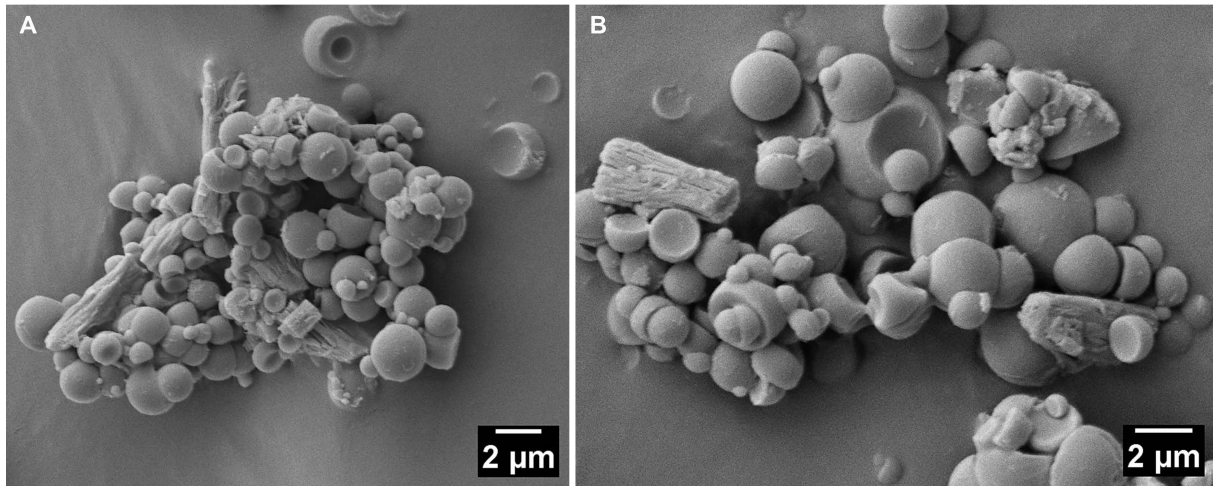


Fig. S2: TEM images of particles oversaturated with PTX: crystallization and acorn shapes are found.

Table S2: Obtained iodine concentrations from spectral CT measurements.

| Sample             | Iodine content per mg particle [mg/mg] | IE [%] |
|--------------------|----------------------------------------|--------|
| TIBO@PLGA-MP-1     | 0.191                                  | 60.8   |
| TIBO@PLGA-MP-2     | 0.293                                  | 81.8   |
| TIBO@PLGA-MP-3     | 0.246                                  | 68.1   |
| TIBO@PLGA-NP       | 0.259                                  | 71.6   |
| PTX@TIBO@PLGA-MP-1 | 0.275                                  | 80.9   |
| PTX@TIBO@PLGA-MP-2 | 0.276                                  | 76.3   |
| PTX@TIBO@PLGA-NP   | 0.265                                  | 70.6   |

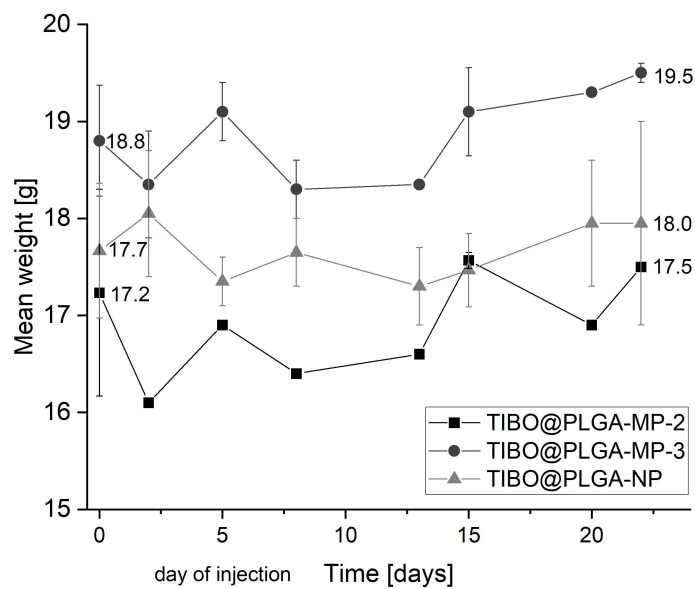


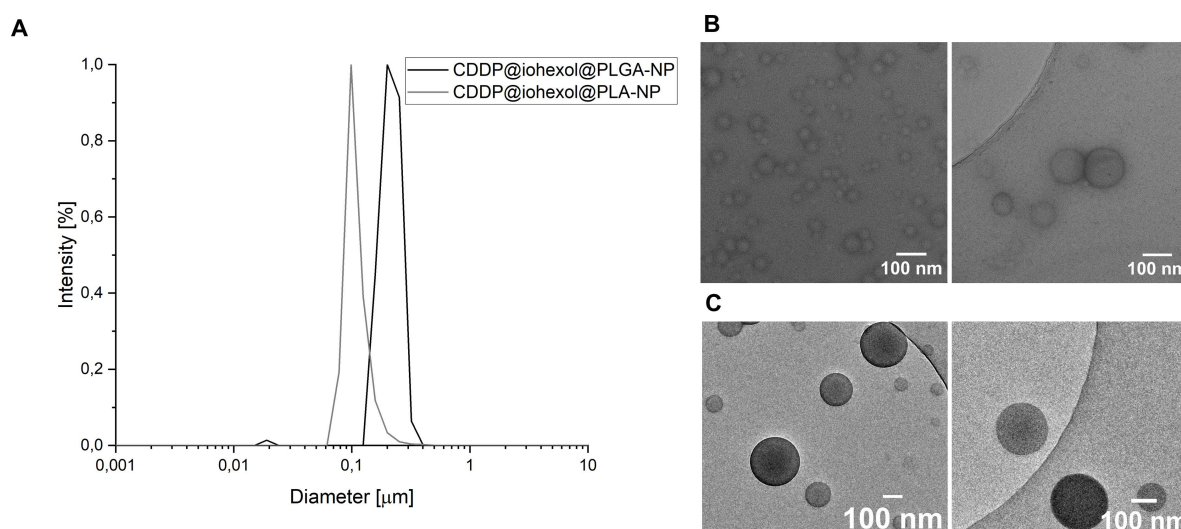
Fig. S3: Animal weight over the study period.

### Double emulsion evaporation procedure for particle synthesis

Particles loaded with iohexol were prepared by a water-oil-water (w/o/w) emulsion solvent evaporation method. Briefly, commercially available iohexol (0.8 ml) is emulsified in 4 ml of dichloromethane containing 100 mg of PLGA using an ultrasonic tip for 60 s. This w/o emulsion was added to 20 ml of PVA 1% (w/v) containing 6.6 ml of iohexol and emulsified again using an ultrasonic tip for 60 s. The obtained w/o/w emulsion was added dropwise to a mixture of iohexol:PVA (1:2, 40 ml) and stirred by a magnetic stirrer overnight at rt until complete evaporation of solvent. The particles were collected and purified by washing three times by centrifugation with purified water (2500 rpm, 4 °C, 5 minutes). The obtained particles were redispersed in water and stirred at 4 °C.

*Table S3: Double emulsion synthesis approach for hydrophilic cores loaded with CDDP and iohexol. Overview of results.*

| Sample               | Polymer                           | Iodine content [mg/mg] | Particle diameter [ $d_{\text{mean}}$ , $\mu\text{m}$ ] | CDDP in particles [mg/mg] |
|----------------------|-----------------------------------|------------------------|---------------------------------------------------------|---------------------------|
| CDDP@iohexol@PLGA-NP | LA:GA: 50:50, $M_w$ : 7–17 g/mmol | 0.246                  | 0.202<br>PDI:0.081                                      | 0.002                     |
| CDDP@iohexol@PLA-NP  | $M_w$ : 10–18 g/mol               | 0.271                  | 0.187<br>PDI: 0.013                                     | 0.002                     |



*Fig. S4: A) Size distribution by DLS of CDDP@iohexol@PLGA-NP and CDDP@iohexol@PLA-NP. B) TEM images of CDDP@iohexol@PLGA-NP. C) TEM images of CDDP@iohexol@PLA-NP.*

### 3.3 Gelatin and collagen-mimetic nanoparticles for intraperitoneal cisplatin delivery: from synthesis to in-vivo SPECT imaging of biodistribution

This study presents gelatin-poly(glutamic acid) (Gel-PGA) and collagen-like peptide-poly(glutamic acid) (CLP-PGA) nanoparticles as intraperitoneal delivery systems for *cis*-diamminedichloroplatinum (CDDP), commonly known as cisplatin. The CLP is produced recombinantly, offering animal-free, biocompatible formulation. NPs (~150 nm) were synthesized via *in situ* polymerization and showed sustained drug release. CLP-PGA NPs enabled higher CDDP loading and stability than Gel-PGA NPs. Single-photon emission computer tomography (SPECT/CT) imaging of radiolabeled NPs in mice revealed uptake in liver, spleen, pancreas, and ovaries, supporting their potential for targeted peritoneal carcinomatosis therapy.

The concept of the following publication was developed by Prof. Dr. Holger Gröll and Xenia Kovalyk. The synthesis and formulation of the drug delivery systems were carried out by Xenia Kovalyk. Optimization of physicochemical properties and *in vitro* drug release kinetics was performed by Xenia Kovalyk. Cell assays were conducted by Larissa-Marie Hamacher. Preparation of samples for ICP measurements were conducted by Ben Lasse Mink. ICP was measured by Sven Thoröe-Boveleth. Preclinical imaging experiments, including acquisition and *in vivo* evaluation, were conducted by Xenia Kovalyk, Sven Saniternik, and Larissa-Marie Hamacher. Contributions to formulation development were provided by Dr. Thomas Endres and Dr. Maria Montero-Mirabet. TEM imaging was conducted by Patricia Bach. The manuscript was written by Xenia Kovalyk, with critical revisions and supervision by Prof. Dr. Holger Gröll and scientific input from all co-authors.

This chapter is based on: Kovalyk X, Saniternik S, Hamacher LM, Mink BL, Thoröe-Boveleth S, Bach P, Endres T, Montero-Mirabet M, Schmidt AM, Gröll H. Gelatin and collagen-mimetic nanoparticles for intraperitoneal cisplatin delivery: from synthesis to in-vivo SPECT imaging of biodistribution. Manuscript under review; 2025.

## 3.3.1 Abstract

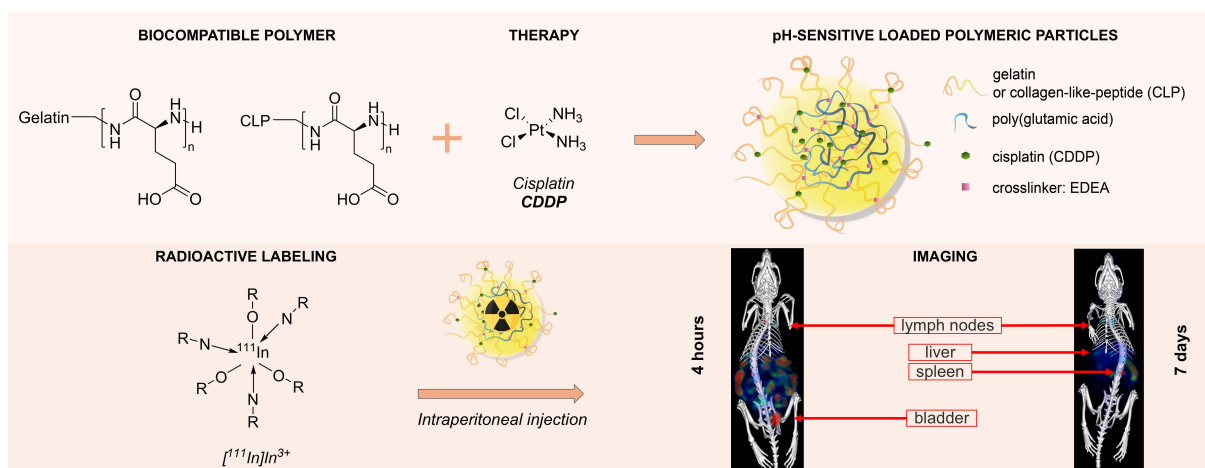


Fig. 1: Graphical abstract.

Peritoneal carcinomatosis is a metastatic condition with poor prognosis, commonly associated with abdominal cancers. Current treatments comprise cytoreductive surgery with systemic chemotherapy or may include more invasive options such as hyperthermic intraperitoneal chemotherapy, and pressurized intraperitoneal aerosol chemotherapy. However, currently, all treatment options are considered palliative as remaining cancer cells cause recurrence. This study explores new intraperitoneal (i.p.) drug delivery systems (DDS) based on gelatin-poly(glutamic acid) (Gel-PGA-NPs) or collagen-like-peptide-poly(glutamic acid) nanoparticles (CLP-PGA-NPs) loaded with CDDP for localized treatment of peritoneal carcinomatosis. These NPs, synthesized via *in situ* polymerization, offer prolonged drug release and continuous biodegradation. The used CLP is produced by a recombinant manufacturing method avoiding any animal-derived material, which has advantages in regard to biocompatibility and reproducibility. Characterization by dynamic light scattering and transmission electron microscopy showed NPs averaging 150 nm in size. CDDP loading in CLP-PGA NPs facilitated high drug loading and stability compared to Gel-PGA NPs. Single-photon emission computer tomography (SPECT/CT) imaging of radiolabeled NPs in healthy mice revealed primary uptake in the spleen and liver, and further accumulation in the pancreas, ovaries and adherence to abdominal tissues highlighting the potential use for treatment of intraperitoneal metastases.

### 3.3.2 Introduction

Peritoneal carcinomatosis (PC) is a complex disease characterized by the metastatic spread of cancer cells to the peritoneum, the serous membrane lining the abdominal cavity and enveloping the abdominal organs. This condition is often diagnosed in patients with advanced-stage malignancies, particularly cancers originating in the gastrointestinal tract, such as ovarian, colorectal, pancreatic, and gastric cancers among others.

The incidence of PC is estimated to be up to 14% for pancreatic cancer, approximately 11% for colorectal cancer, around 14–50% for gastric cancer, and as high as 70% for ovarian cancer.[1–4] The presence of PC often reflects poor prognosis, indicating a widespread disease mostly without curative approaches. Treatment options at this advanced stage comprise cytoreductive surgery often followed by hyperthermic intraperitoneal chemotherapy (HIPEC) or pressurized intraperitoneal aerosol chemotherapy (PIPAC) alongside with further systemic chemotherapy. However, treatment aims at life extension and reduction of symptoms but is considered as palliative care due to recurrence originating from remaining cancer cells.[5] One of the major challenges in treating PC is the poor perfusion of surface tumors, which are often poorly connected to the main vascular system. As a result, these tumors are less responsive to conventional intravenous (i.v.) chemotherapy. I.p. drug delivery has been proposed as a promising strategy to improve localized drug exposure to peritoneal metastases. I.p. administration of DDS offers the advantage of longer residence time and thus provides an extended and more direct exposure to drugs compared to i.v. administration.

Post-operative treatment of remaining cancer cells with i.p. drug delivery is currently explored in first clinical trials and is subject of research in a preclinical setting.[6–11] As of today, no formulation has been specifically approved for i.p. delivery.[12] Earlier research has shown that tumor-targeting as well as tumor penetration, but also clearance from the intraperitoneal space of drug-loaded NPs and microparticles (MPs) after i.p. administration depend on size and possibly on other material properties such as surface charge and coatings.[13] Particle-based DDS have been studied with sizes between 50 nm to several microns in small animal models of PC. Tumor uptake was mainly assessed using *ex vivo* histological examination.[14–16] The findings are diverse and do not allow to conclude on the most suited diameter or surface properties. The choice of drug primarily depends on the type of primary tumor, with *cis*-

diaminodichloroplatinum (CDDP), doxorubicin (DOX), and paclitaxel (PTX) being commonly used in clinical applications of i.p. chemotherapy.[17–19] Particularly, i.p. administration of platinum-based chemotherapeutics such as CDDP showed promising results in patients with gynecological cancers or in combination with mitomycin C for colorectal cancers.[20,21] However, due to their molecular size (smaller than 2 nm), these chemotherapeutic drugs are rapidly eliminated from the peritoneal cavity via blood vessels.[22] The use of sustained-release DDS with large diameters could help maintain adequate drug exposure to residual tumor cells within the peritoneal cavity.[23–25]

CDDP is one of the most potent drugs for treatment of several abdominal or gynecological tumors and is used for HIPEC treatments as well.[21] One of the disadvantages of CDDP is its poor solubility in water as well as organic solvents such as dimethylformamide (DMF) or dimethylsulfoxide (DMSO) making synthesis of DDS with sufficient CDDP payload challenging. However, the use of DMSO during synthesis is strongly discouraged as it inserts into the complex as a ligand.[26] The strategy to simply entrap CDDP in polymeric NPs suffers from low encapsulation efficiency and yields systems with poor release profiles.[27,28] A better option is the chemical complexation of CDDP by amphiphilic block copolymers that contain carboxylic groups, leading to micellar structures with polymer-metal complexes which may improve the drug release profile and increase the drug load.[29,30]

These typically core-shell-structured polymeric micelles can be fabricated via self-assembly procedures. However, they require a block copolymer precursor, which entails time-intensive and complex synthesis procedures. In past studies, advantage was taken of the simultaneous polymerization and self-assembly between the components for development of a more facile procedure using macromolecules as template.[31] This approach is based on polymerization of monomers in a biopolymer-monomer pair reaction system consisting of biopolymer and polymerizable monomer. Particularly, gelatin was studied as template and acrylic acid (AA) as monomer for polymerization of AA in the gelatin aqueous solution. Stable core-shell NPs with multiple functional groups were obtained.[31] In subsequent studies by *Ding et al.*, incorporation of CDDP into these gelatin-poly (acrylic acid) (Gel-PAA) NPs through the interaction between platinum of CDDP and carboxylic groups in the NPs was investigated.[32–34] The NPs, which contain many carboxyl groups from both gelatin and PAA, enable high CDDP loading with an encapsulation efficiency up to 70%. In

addition, a slow-release drug profile with a small initial burst of only 13–28% after 8 hours is found.[32] These particles showed promising efficacy after i.v. and i.p. injection in a subcutaneous model of hepatocellular carcinoma.[32,34]

Here, we present a novel DDS based on Gel-PGA-NPs loaded with CDDP. Poly(glutamic acid) (PGA) has been extensively studied in drug delivery and cosmetic applications and is considered a biocompatible, safe and non-toxic building block.[35,36] In contrast, possible toxicity may arise from PAA and its monomer AA, especially when considering the need for an external initiator for the polymerization such as potassium persulfate.[37–41] Next to animal-derived gelatin, we use a new type of collagen-like-peptide (CLP) marketed under the brand Vecollan<sup>®</sup>, which is obtained via advanced recombinant protein production. Vecollan<sup>®</sup> contains no animal- or human-derived ingredients and has precisely defined chemical properties translating into more defined synthesis procedures and particles with narrower size distribution.[42–44]

In this work, we present the synthesis, characterization and release profiles in fetal bovine serum (FBS) of CDDP-loaded Gel-PGA NPs in comparison to CDDP-loaded CLP-poly(glutamic acid) NPs (CLP-PGA-NPs). Moreover, we present the first results of the *in vivo* biodistribution of radiolabeled CLP-PGA-NPs after injection into the i.p. cavity using single-photon emission computed tomography (SPECT/CT) imaging. Longitudinal imaging is crucial to gain an understanding of the relationship between the physicochemical properties of particle-based carriers and their biological fate following i.p. administration.



### 3.3.3 Experimental section

#### *General/Materials*

All solvents were obtained from Sigma-Aldrich (St Louis, MO, USA). Type-B gelatin with 100–115 mmol of carboxylic acid per 100 g of protein and an isoelectric point (pI) of 4.7–5.2 were purchased from Sigma-Aldrich (St Louis, MO) with an average molecular weight of 20000–25000 g/mol (50–120 gel strength). Gelatin was refined once by dissolving it in distilled water followed by precipitating with acetone and drying at room temperature (rt). Vecollan<sup>®</sup>, a recombinantly manufactured CLP, was kindly provided by Evonik Operations GmbH (Essen, Germany). CDDP was purchased from TCI Chemicals (Tokyo, Japan). N-tert-butoxycarbonyl-L-glutamic acid gamma-benzyl ester (Boc-(L)Glu(OBzl)-OH) and propanephosphonic acid anhydride (T3P<sup>®</sup>) were purchased from Sigma-Aldrich. All reagents were used as received from commercial sources without further purification. Water was purified using a Milli-Q system by Sartorius. A Hereaus Multifuge 3 SR Plus and Eppendorf Centrifuge 5425 (Wesseling, Germany) centrifuges were used for centrifugation. [<sup>111</sup>In]InCl<sub>3</sub> was acquired from Curium (Petten, Netherlands) as a solution in 0.02 M hydrochloric acid (HCl) (usually 185 MBq in approx. 500 µl).

### *Synthesis of Gel-PGA and CLP-PGA NPs*

#### *Preparation of (S)-glutamic acid 5-benzyl ester-N-carboxyanhydride*

(S)-glutamic acid 5-benzyl ester N-carboxyanhydride ((S)Glu(OBzl)-NCA) was synthesized in a single step following a procedure adapted from *Laconde et al.*[45] Under an inert atmosphere, Boc-(L)Glu(OBzl)-OH (25 g) was dissolved in ethyl acetate (150 ml). A solution of T3P® (1.2 eq) in ethyl acetate was added dropwise followed by pyridine (1 eq.). The mixture was stirred at rt for 2 h. Subsequently, ice-cold water (100 ml) was added. The organic layer was separated, washed twice with a cold saturated sodium chloride (NaCl) solution (2 x 50 ml), and then dried over MgSO<sub>4</sub>. After filtration, the solvent was removed under reduced pressure obtaining a colorless solid. Diethylether (100 ml) was added to the colorless residue. Recrystallization from diethylether followed by filtration yielded (S)Glu(OBzl)-NCA as colorless crystals which were stored under argon atmosphere at 4°C. Yield: 71%. NMR data matched the literature.[46]

**<sup>1</sup>H-NMR** (300 MHz, CDCl<sub>3</sub>): δ 7.35 (m, 5H), 6.80 (s, 1H), 5.13 (s, 2H), 4.38 (t, 1H), 2.58 (t, 2H), 2.32–2.11 (m, 2H).

**<sup>13</sup>C-NMR** (75 MHz, CDCl<sub>3</sub>): δ 172.47, 169.58, 152.25, 135.37, 128.81, 128.66, 128.43, 67.17, 56.96, 29.77, 26.94.

#### *Preparation of Gel-PGA and CLP-PGA NPs*

Synthesis was based on a procedure described by *Ding et al.*[33] Purified gelatin (0.35 g) or CLP (0.35 g) was dissolved in 50 ml of purified water. (S)Glu(OBzl)-NCA (0.1 g for gelatin-based and 0.085 g for CLP-based NPs) was dissolved in 4 ml DMF and cooled in an ice bath. The organic solution was slowly added over a dropping funnel. The polymerization of (S)Glu(OBzl)-NCA monomer was initiated by the amino groups of gelatin obtaining poly((S)-5-benzylglutamate) (PBLG). As opalescence appeared in the reaction mixture, which was a signal for NP formation, the reaction was allowed to proceed for another 15 minutes at rt. The resultant suspension was then filtered to remove any larger aggregates and dialyzed against water for 24 h using a dialysis membrane bag (12000 Dalton molecular weight cutoff) to remove residual monomers. Deprotection of carboxy groups of PBLG was conducted according to an adjusted procedure by *Conejos-Sanchez et al.*[46] In a round bottom flask, to benzyl-protected Gel-PBLG NPs in aqueous solution, 2 equiv. of NaOH per carboxylic group of the PBLG block was added in 2 ml of purified water. The mixture was stirred

overnight for 16 hours. The deprotected NPs were again dialyzed against distilled water for 24 h. Subsequently, the crosslinking reaction of the NPs was conducted by adding the desired amount of 2,2'-(ethylenedioxy)-bis(ethylamine) (EDEA) as crosslinker in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) at rt for 8 h. The crosslinker to carboxy group ratio amounted to 1:2. The EDC to crosslinker ratio was 0.5:1. The cross-linked product was again dialyzed against distilled water for 24 h.

#### *Preparation of CDDP-loaded Gel-PGA and CLP-PGA NPs*

CDDP was added to an aqueous suspension of CLP-PGA NPs in a ratio of 2:1 of CDDP to carboxy groups in NPs. The resultant mixture was shaken at 400 rpm at 37 °C for two days. The unbound CDDP was removed by dialysis against distilled water using a 12000 Dalton molecular weight cutoff dialysis membrane for two days.

#### *Radiolabeling of CLP-PGA NPs*

For labeling experiments, 10 mg of the CLP-based NPs were lyophilized and redispersed in 0.25 ml of 1 M sodium acetate buffer in a 1.5 ml reaction vial. 0.25 ml of [ $^{111}\text{In}$ ]InCl<sub>3</sub> (100 MBq) was added to the reaction vial resulting in a solution with pH~5. The labeling mixture was incubated at 37 °C for 120 minutes. To verify the stability of the NP complex, 5  $\mu\text{l}$  of the competing chelating agent diethylenetriaminepentaacetic acid (DTPA; 10 mM) was added and incubated for 10 minutes at 37 °C. The  $^{111}\text{In}$ -labeled NPs were separated from unbound [ $^{111}\text{In}$ ]In<sup>3+</sup> or DTPA-complexed radionuclides using a PD MiniTrap G-25 Sephadex column (*GE Healthcare*, Uppsala, Sweden) eluted with physiological saline solution. The radiochemical purity and yield were determined by thin layer chromatography (radio-TLC) on silica plates. Mobile phase consisted of 0.1 M citrate buffer. The exposed plates were analyzed on a FLA-7000 phosphor imager (*FujiFilm*, Tokyo, Japan) using the AIDA software. Radiochemical purity and yield were determined.

#### *Characterization*

The hydrodynamic diameter, polydispersity of nano-sized particles and surface potential was determined using a Zetasizer Nano ZS (*Malvern Panalytical Ltd.*, Malvern, UK). No further filtrations of the particles were performed throughout the entire experiment. In between measurements the samples were stored at 4 °C. TEM images were recorded on electron microscopes (Talos L120C, *ThermoFisher*, Waltham, USA and Leo 912, *Zeiss*, Oberkochen, Germany). Samples were prepared

by placing the emulsion (3  $\mu$ l) on a 300-mesh carbon-coated copper grid and letting it dry at rt. NMR spectra were recorded on a Bruker Avance II spectrometer operating at 300 MHz using deuterated solvents. For measuring the CDDP content, 1 ml of rhodium solution (1 mg/l) was added to the samples as an internal standard and then destroyed upon heating in fuming nitric acid ( $\geq 99\%$ ). After filtration with a 0.2  $\mu$ l syringe filter, the Pt concentration in the solution was determined by inductively coupled plasma mass spectrometry (ICP-MS, 8900-QQQ, *Agilent*, Waldbronn, Germany). Measurements were carried out under following conditions: A MicroMist quartz glass Meinhard nebulizer was used. The RF power was set to 1550 W, the plasma flow was adjusted to 15 l/min and the nebulizer flow was set to 1.15 l/min. A 2-mm quartz glass injector was employed.

### *In vitro drug release and in vitro toxicity studies*

NPs were dispersed at a concentration of 0.1 mg CDDP/ml in 1.5 ml of FBS (pH 7.4) and incubated at 37 °C under shaking. At determined time intervals, the vials were centrifuged at 10000 rpm for 20 minutes. 0.5 ml of supernatant was taken and analyzed by ICP-MS as described in section 0. Equal volume of FBS was replaced. Studies were carried out in duplicate.

Colorectal cancer (CT26) cells were grown in DMEM supplemented with 10% FBS and 1X Penicillin/Streptomycin. The cells were cultured in flasks at 37 °C in an atmosphere of 95% air and 5% CO<sub>2</sub>, in an incubator. Cells were plated in 96-well plates at the density of 2000 viable cells per well in 0.2 ml of complete DMEM medium and incubated 24 h. The total cell counts, and viability were counted by the trypan blue dye exclusion method. The cells were incubated with CDDP, CDDP-loaded NPs and drug-free control NPs (in the same amount of weight as drug-loaded NPs for comparison) for 48 and 72 h. After treatment, the formulations were replaced with 40  $\mu$ l of a 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) in PBS and cells were then incubated for an additional 3 h. The resulting formazan crystals were solubilized by the addition of 150  $\mu$ l DMSO. Absorbance was measured at 570 nm using an Epoch microplate reader (*Agilent Technologies*, Diegem, Belgium). The results were expressed as mean values with standard deviation of three measurements.

### *In vivo biodistribution*

All animal experiments were approved by the State Agency for Consumer Protection and Nutrition of North-Rhine Westphalia (LAVE, Animal Ethical Proposal no.: 81-02.04.2020.A320) and were performed by authorized personnel. Healthy Balb/c mice (8 weeks old, 19–22 g, *Charles River*, USA) were i.p. injected with  $^{111}\text{In}$ -labeled CLP-based NPs ( $23.7 \pm 0.6$  MBq in 0.2 ml per animal of a 20 mg/ml NP dispersion in isotonic 0.9% NaCl solution) and imaged using SPECT/CT. On injection day, the animals were imaged sequentially for four hours with scans performed every 30 minutes. Subsequent imaging occurred daily for seven days, after which the animals were sacrificed. Following pharmacokinetic studies, biodistribution was determined by dissecting the animals, weighing, and isolating the respective tissues (blood, thymus, urine, head, femur muscle, tumor, small intestines, colon, stomach, stomach content, femur bone, skin, kidneys, liver, pancreas, spleen, heart, lung, tail, bladder, ovaries, mesenteries, peritoneum, brain). Tissue radioactivity was quantified using gamma-counting. All presented data are decay-corrected. The particle distribution was studied using autoradiography, on 10  $\mu\text{m}$  slices of cryogenically embedded samples. The relative distribution was normalized to the tissue exhibiting the highest signal per unit area. Therefore, a circular region of interest (ROI) with an area of 1.2 mm<sup>2</sup> was selected for quantification.

### *Longitudinal SPECT/CT*

After i.p. injection of the radiolabeled particles, one SPECT image per hour was acquired over four hours total (scan parameters: 25 s/projection, 12 projections, 100.0 mm axial FOV; nanoSPECT®, *Mediso*, Budapest, Hungary). From day one to seven, one scan per day was acquired using the same settings. After the last scan, mice were sacrificed for autoradiographic and biodistribution analysis. Images were evaluated with Imalytics Preclinical® (*Gremse-IT*, Germany). All presented data are decay-corrected.

## 3.3.4 Results

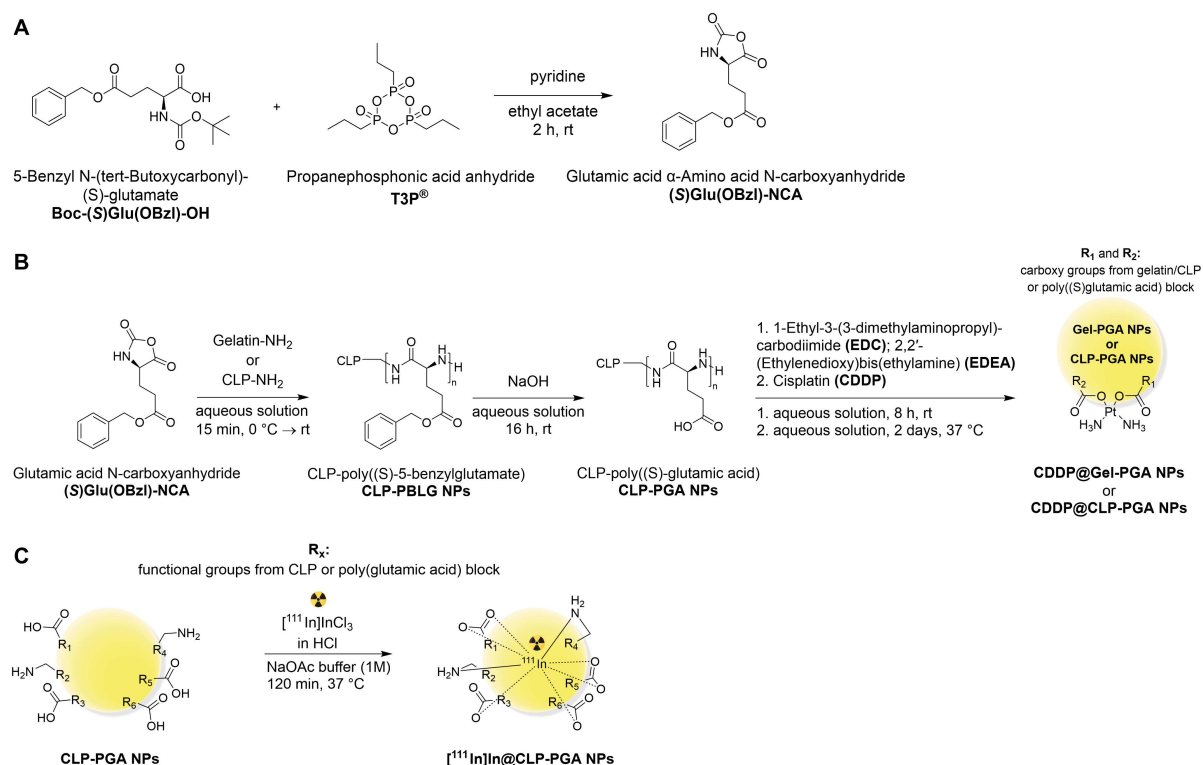
*Gelatin or CLP Particle synthesis and influence of parameter variation*

Fig. 2: A) Reaction scheme for synthesis of (S)Glu(OBzl)-NCA. B) Schematic representation of the synthesis process for CDDP-loaded NPs. C) Reaction scheme for NP radiolabeling.

Gelatin or CLP-poly(glutamic acid)-based NPs are synthesized via an *in situ* polymerization of glutamic acid anhydride initiated by the amino-groups present in gelatin and CLP, respectively. Thus, gelatin or CLP serve simultaneously as a macromolecular polymerization template and initiator. First, (S)Glu(OBzl)-NCA was obtained in a mild reaction procedure based on a protocol by *Laconde et al.*[45] To gently deprotect the acid function of PGA, a modified approach of *Conejos-Sanchez et al.* was applied.[46] Crosslinking of the resulting particles is carried out using EDEA in presence of EDC in order to activate the carboxy group. Finally, the particles are reacted with CDDP to form metal-polymer complexes.

*Preparation of (S)Glu(OBzl)-NCA*

Tert-butyloxycarbonyl (Boc)- and benzoyl-protected (S)-glutamic acid was reacted with T3P® in the presence of pyridine at rt (Fig. 2A). After purification by recrystallization, colorless crystals were collected in a yield of 71%. NMR data match literature values. The corresponding spectra are attached in the supplementary information (SI, Fig. S1).

*Preparation and characterization of CDDP-loaded Gel-PGA or CLP-PGA NPs*

Polymerization of (S)Glu(OBzl)-NCA was started *in situ* by the amino groups of gelatin or CLP leading to NP formation (Fig. 2B). The initial gelatin or CLP solution was transparent and became opalescent upon the slow addition of BLG-NCA, indicating the formation of NPs. The ratio of gelatin to anhydride, the amount of the solvents as well as the reaction time affect particle size, with longer reaction times leading to larger particles. Cooling of the reaction mixture during synthesis is beneficial to obtain narrow size distributions. Additionally, anhydride purity is crucial for achieving small diameters and a narrow distribution as minimal impurities lead to particle aggregation. Reaction conditions and obtained particle sizes are summarized in Table S1 (SI). For all further experiments, gelatin-based NPs, were synthesized according to the optimized procedure, using 350 mg gelatin in 50 ml water and 100 mg anhydride in 4 ml DMF. For CLP-based NPs the amount of anhydride was adjusted to 85 mg in 4 ml DMF. Subsequently, the acid moieties in the PGA block were deprotected by addition of sodium hydroxide. IR spectra of protected particles display a distinctive IR peak at around  $700\text{ cm}^{-1}$  associated with OBzl-group (out-of-plane bending vibrations of C-H bonds in aromatic rings), which is significantly reduced in the deprotected product (Fig. S2 in SI).[48,49] Next, NPs were crosslinked using EDC/EDEA to increase stability following a protocol described by *Ding et al.*[33] After crosslinking, particles exhibited a more narrow size distribution in DLS with well-defined boundaries visible in TEM images (Fig. 4C, F). Furthermore, CLP-based particles have smaller hydrodynamic diameters at each reaction step compared to the gelatin-based NPs (Table 1). Finally, CDDP was loaded into deprotected gelatin and CLP particles using a ratio of 2:1 of CDDP to carboxy groups, free CDDP was removed by dialysis. Notably, CLP-based NPs exhibited a significantly higher platinum (Pt) content, with 76  $\mu\text{g}$  of Pt per mg of NPs, compared to only 25  $\mu\text{g}$  of Pt per mg of NPs in the gelatin-based formulation. In addition, this approach allows for the incorporation of other platinum-based chemotherapeutics, such as [(1*R*,2*R*)-cyclohexane-1,2-diamine](ethanedioato-*O*,*O'*)platinum(II) (oxPt) (see Table S2, SI). Surface charge, size distribution and morphology were assessed for each particle system and reaction step using electrophoretic and dynamic light scattering, and TEM, respectively.

## Results

Table 1: Characteristics of gelatin- and CLP-based NPs after each reaction step.

| Reaction step     | Hydrodynamic Diameter [nm] | PDI   | Surface charge [mV] | Hydrodynamic Diameter [nm] | PDI   | Surface charge [mV] |
|-------------------|----------------------------|-------|---------------------|----------------------------|-------|---------------------|
|                   | Gelatin-based NPs          |       |                     | CLP-based NPs              |       |                     |
| Polymerization    | 258                        | 0.253 | -14.1               | 296                        | 0.251 | 9.6                 |
| Deprotection      | 281                        | 0.168 | -24.1               | 256                        | 0.122 | -18.4               |
| Crosslinking      | 241                        | 0.177 | -19.5               | 223                        | 0.137 | -10.4               |
| CDDP complexation | 191                        | 0.219 | -0.8                | 155                        | 0.125 | 21.7                |
| Pt content        | 0.025 mg Pt/mg NPs         |       |                     | 0.076 mg Pt/mg NPs         |       |                     |

For each reaction step, a significant change of the surface charge is observed, in line with earlier results for similar systems (Fig. 3A).[32] As the surface charge is dependent on the dissociation and protonation of carboxy and amino groups, we studied the effect of pH value on surface charge by adding sodium hydroxide (0.1 M) or hydrochloric acid (0.1 M) to a dispersion of polymerized Gel-PBLG NPs. Surface potential measurements reveal a linear dependence of the surface charge on the pH of the solution (Fig. 3C). Accordingly, the surface charge after each reaction step changes with the pH obtained or used for each reaction step (Fig. 3B).

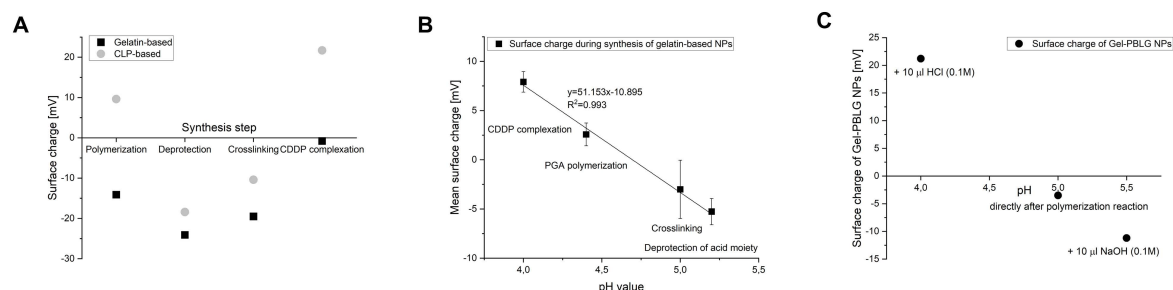
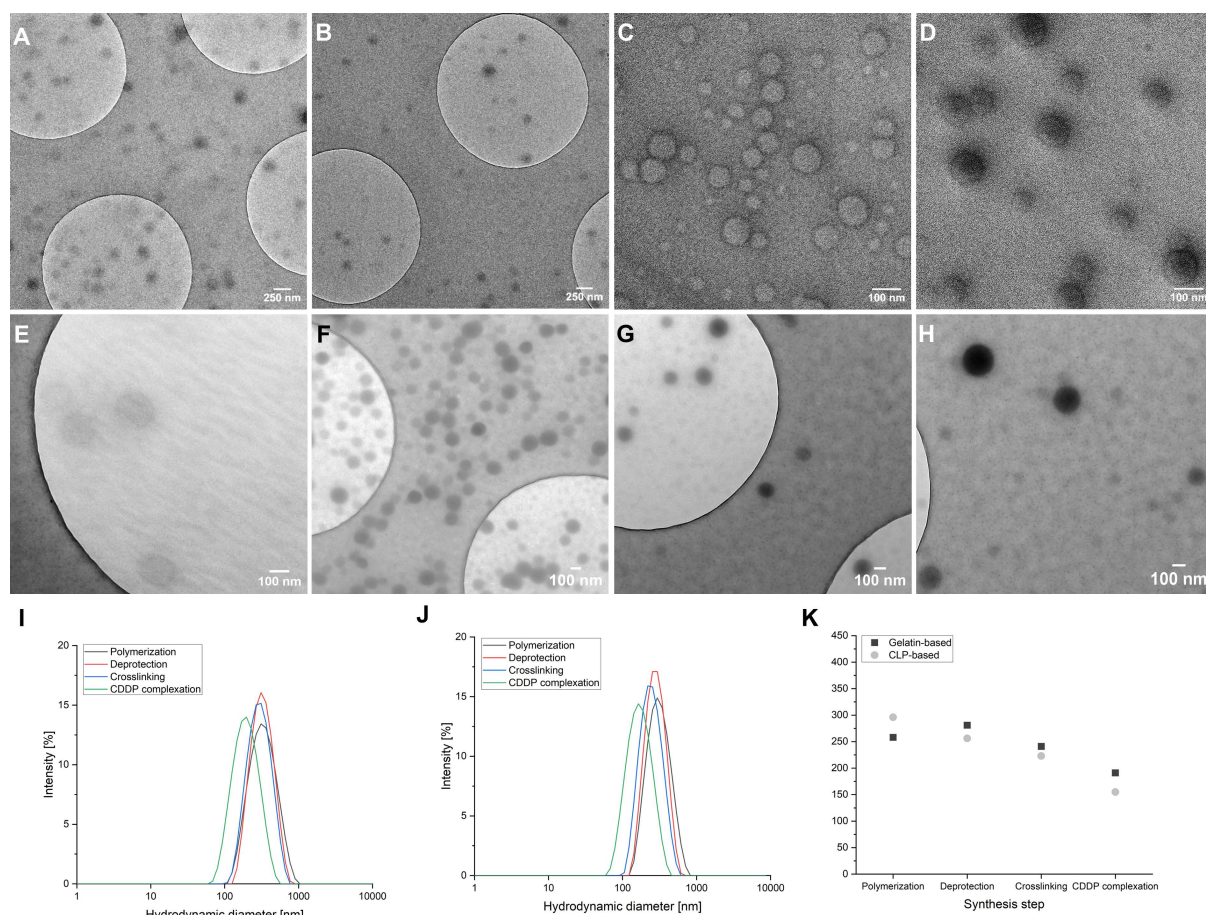


Fig. 3: Investigation on surface charge. A) Surface charge in gelatin- and CLP-based NPs depending on reaction step. B) Overview of surface charge in gelatin-based NPs as a function of pH according to reaction step ( $n=2$ ). C) Dependency of surface charge on pH value of Gel-PBLG-NP dispersion.

TEM images show that deprotection does not affect particle structure and the next crosslinking step leads to formation of smaller particles with a well-defined spherical morphology (Fig. 4). After CDDP loading, NPs appear darker with a clear edge delineation due to a higher electron density (Fig. 4D). CLP-based NPs appear smaller in diameter compared to gelatin-based NPs (Fig. 4G, H).

Overall, CLP-based NPs exhibit a narrower size distribution and smaller hydrodynamic diameters in comparison to gelatin-based NPs (Fig. 4K). CDDP complexation leads to a further reduction in particle diameters without formation of aggregates.





**Fig. 4:** TEM images of gelatin-based NPs after A) polymerization, B) deprotection, C) crosslinking D) CDDP-loading. TEM images of CLP-based NPs after E) polymerization, F) deprotection and crosslinking. TEM images of CLP-based NPs after CDDP-loading: G) overview image, H) magnified to visualize single particles. I) Hydrodynamic diameters of gelatin-based NPs in dependence of synthesis step. J) Hydrodynamic diameters of CLP-based NPs in dependence of synthesis step. K) Comparison of hydrodynamic diameters in gelatin- and CLP-based NPs in dependence of reaction step.

### *In vitro drug release and anti-tumoral effect*

Cell viability assays with CT26 cells were performed for 48 h and 72 h incubation with free CDDP, CDDP-loaded particles and non-loaded (empty) control particles (Fig. 5A, B). The cytotoxicity of CDDP-loaded NPs is lower than that of free CDDP at the tested concentrations, which can be contributed to the slower CDDP release from the spheres. Notably, no significant effect on cell viability was observed with the empty NPs. Drug release studies conducted in FBS over a seven-day period revealed an initial burst release of 37% within the first eight hours. The release profile gradually plateaued, reaching a maximum of 52% by day seven. (Fig. 5C).

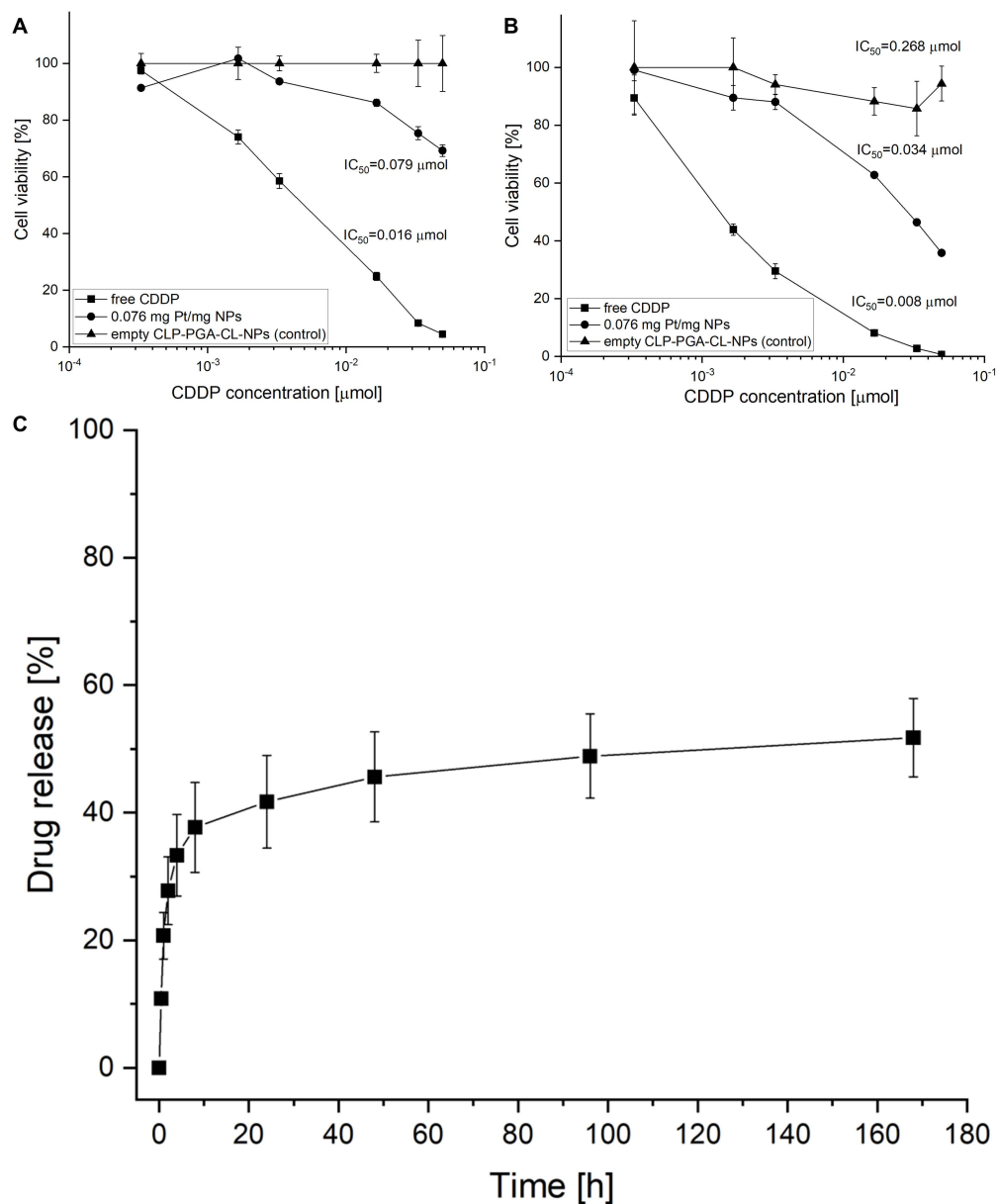


Fig. 5: Cell viability assays of CT-26 colorectal cancer cells ( $n=3$ ) after A) 48 h and B) 72 h incubation time of CLP-based NPs. C) Drug release of platinum in FBS over seven days.

## *Radiolabeling and in vivo biodistribution studies*

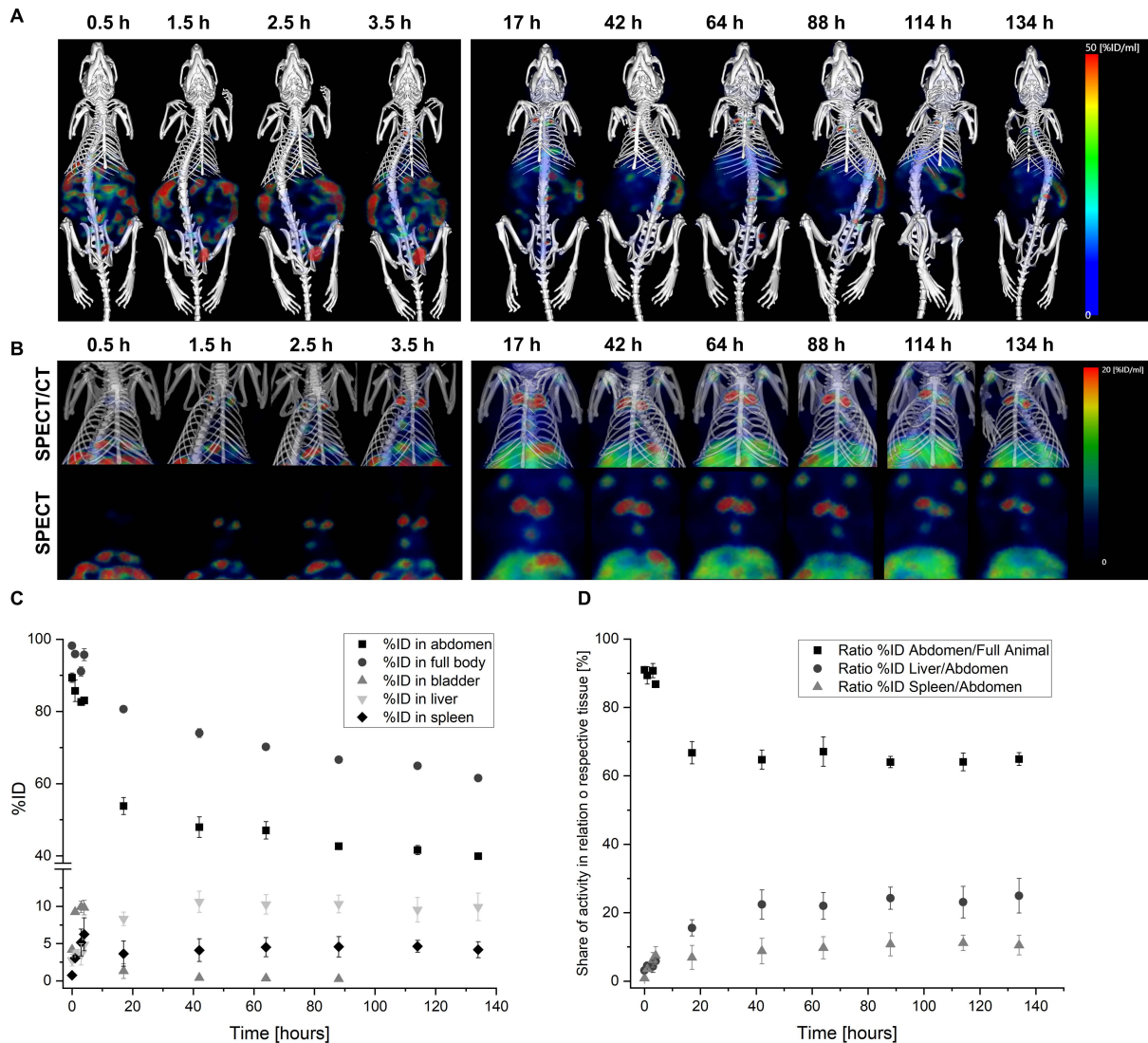
### *Radiolabeling*

The radionuclide indium-111, in its trivalent metal cation form ( $[^{111}\text{In}]\text{In}^{3+}$ ) frequently employed in SPECT imaging, was used to label crosslinked, but unloaded CLP-PGA NPs (Fig. 2C). Unbound  $[^{111}\text{In}]\text{In}^{3+}$  was removed via size exclusion chromatography (SEC), resulting in a specific activity of 5 MBq of  $[^{111}\text{In}]\text{In}^{3+}$  per mg of NPs and a radiochemical purity of 90% (Fig. S3 in SI). The surface charge increases from  $-10.4$  mV to  $-9.4$  mV, suggesting  $[^{111}\text{In}]\text{In}^{3+}$  complexation by carboxylic acid groups, while the hydrodynamic diameter increased from 223 nm to 287 nm (Table S3 in SI). Notably, introduction of commonly employed functional chelator 2,2',2'',2'''-(1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrayl)tetraacetic acid (DOTA) did not increase radiochemical yield nor improve stability of labeled NPs (data not shown).

### *In vivo biodistribution*

The biodistribution of  $^{111}\text{In}$ -labeled CLP-based NPs was assessed in healthy mice ( $n=2$ ) using longitudinal SPECT/CT imaging (Fig. 6). Directly after i.p. injection, the scans reveal rapid signal distribution within the abdominal cavity, with notable adherence to the bowel tissue. Within the first four hours, particles rapidly redistribute within the peritoneal cavity with changing areas of accumulation, which are difficult to link to CT-based anatomical structures. About 10% of the injected activity (%ID) is cleared to the urine within the first 90 minutes. Furthermore, activity accumulation is observed at the hemisphere close to the diaphragm and above the liver. The next day, about 80%ID remains in the entire animal followed by continuous clearance to 62%ID on day seven (Fig. 6C). SPECT imaging reveals a redistribution of activity over several days within the abdominal cavity showing a gradual uptake inside the liver and spleen, which can be clearly recognized on day seven. Activity in the spleen reaches about 6%ID after 4 hours with a plateau value of 4%ID after 7 days. However, at early timepoints, due to the limited spatial resolution of SPECT imaging, it is impossible to distinguish between particle adherence to the outside of the spleen and uptake within the organ. Liver uptake proceeds exponentially for 42 hours with the highest value reaching 11%ID. At the following time points, activity accumulation remains consistent until reaching 10%ID on day seven (Fig. 6C). The activity found in the abdominal cavity compared to the entire animal remains from day two almost constant with a ratio of 65%. In contrast, the activity in the liver and spleen relative to the remaining abdominal

space increases during the first two days, stabilizing thereafter by day seven at a constant ratio of 25% and 11%, respectively (Fig. 6D). Furthermore, within the first four hours, activity is detected in the thymus region, which remains constant from day two to seven. In detail, activity is observed in the parasternal lymph nodes (LNs) indicating uptake and clearance via the lymphatic system. Additionally, the mediastinal LNs and also the cisterna chyli can be identified within SPECT images.



**Fig. 6:** SPECT/CT images of one mouse over the course of A) seven days (representative). B) SPECT images of the same mouse with window adjusted to activity levels observed in lymph nodes (20%ID/ml). For clarity, the same scan is displayed with and without overlay of the corresponding CT image to allow a better view of the lymph nodes. C) SPECT evaluation: %ID of full body, abdomen, bladder, liver and spleen (n=2). D) SPECT evaluation: Ratio of activity found in abdomen compared to whole body, liver to abdomen and spleen to abdomen (n=2).

Biodistribution studies performed after day seven are in line with SPECT data, with the highest uptake of activity in the spleen (121%ID/g), followed by the kidney with 34%ID/g. All abdominal organs are in the range of 30%ID/g, see Fig. 7 and Fig. S4 in

SI. The mesentery also demonstrated notable uptake with 24%ID/g, while the peritoneum of healthy mice showed only an uptake of 6%ID/g. For further localization of NPs, tissue specimens were examined with autoradiography and correlated with microscopy images (Fig. 8A). Here, autoradiography measurements show NP uptake inside liver and spleen, but also partly within the pancreas and ovaries (Fig. 8A, Table S4 in SI), while NP s seem to adhere to the outside of other abdominal organs such as the colon (Fig. 8B).

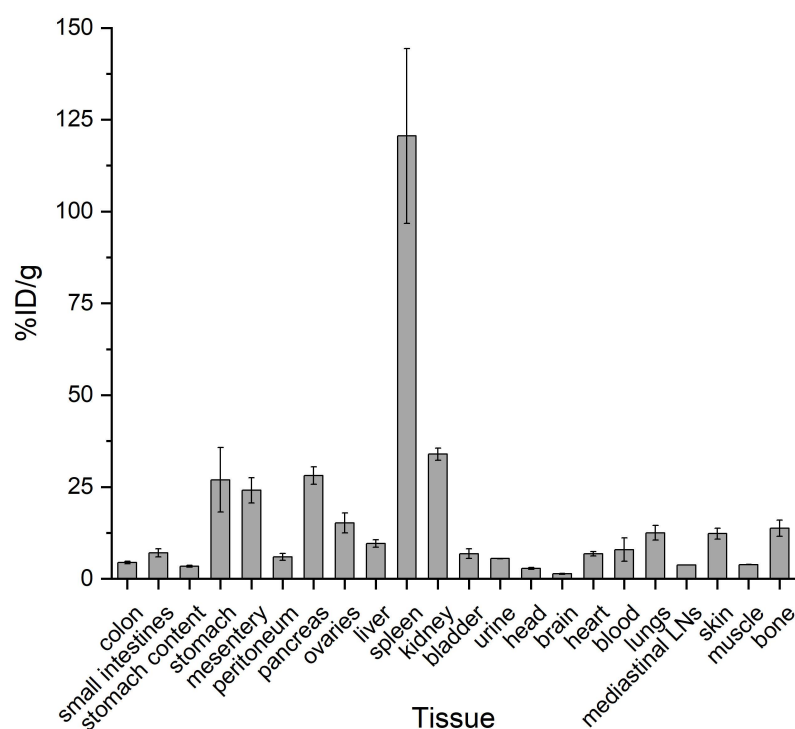


Fig. 7: Biodistribution of  $[^{111}\text{In}]\text{In@CLP-PGA-NPs}$  by gamma counter ( $n=2$ ).

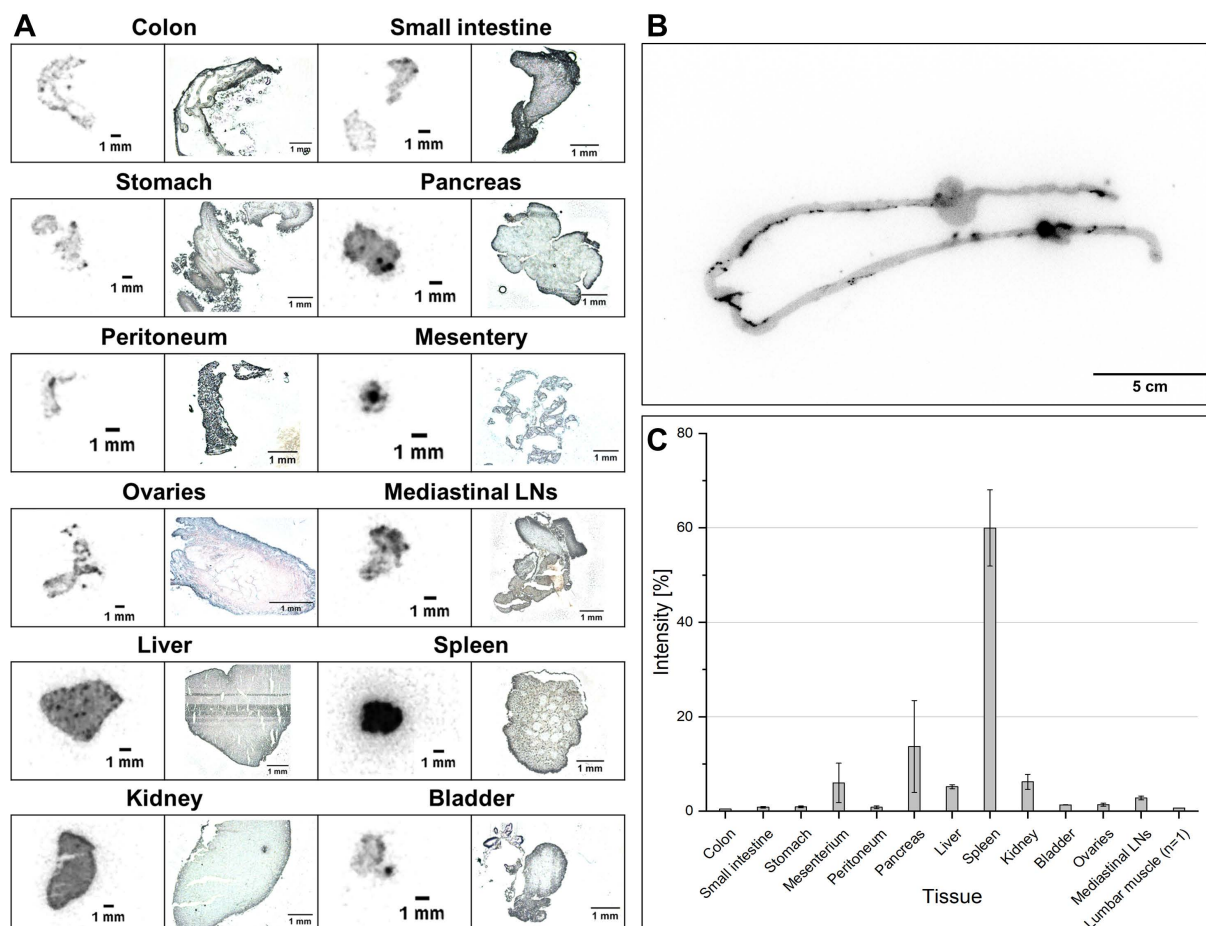


Fig. 8: A) Autoradiography and corresponding light microscope images of explanted tissues of one mouse (representative). Dark spots in autoradiography correspond to activity accumulation. B) Autoradiography image of whole colon. C) Activity accumulation in explanted tissues by autoradiography.



### 3.3.5 Discussion

Treatment of patients diagnosed with PC is currently limited to palliative care. Current treatment options comprise cytoreductive surgery often followed by HIPEC or PIPAC procedures and systemic chemotherapy. However, as peritoneal metastases seed in the peritoneum, are often poorly vascularized and not (yet) connected to the systemic vascular system, systemic chemotherapy typically fails to reach therapeutically relevant drug concentrations. Moreover, tumor cells further disseminate via the lymphatic system, which is another source of recurrence and systemic spread. I.p. drug delivery has been recognized as viable option for additional treatment as higher and more local drug exposure can be achieved compared to systemic chemotherapy. As clearance of i.p. delivered DDS occurs via the lymphatic system, reminiscent micrometastases in adjacent LNs may also receive a higher drug exposure. i.p. retention, distribution but also clearance pathways depend strongly on the DDS, with size and surface charge being the most important parameters, among others.[50–52] Platinum-based drugs such as CDDP or oxPt present the most important cytostatics for treatment of PC with colorectal and gynecological cancers as primary tumor. Consequently, development of adequate DDS providing a high payload of platinum in particles with adaptable surface charge and size is an active field of research.

In this study, we synthesized NPs with diameters from 155–296 nm based on gelatin or CLP and PGA, providing a high load of CDDP. Our synthesis route is similar to the procedure first proposed by *Zhang et al.* to synthesize gelatin-polyacrylic acid NPs, which were subsequently used for loading with CDDP.[31–34] In our case, AA was substituted for glutamic acid due to its favorable biocompatibility and biodegradation properties. The polymerization and ring opening reaction of benzoyl-protected glutamic acid is initiated *in situ* by amino groups present in gelatin or CLP. The *in situ* polymerization of BLG-NCA induced by gelatin or CLP led to NP formation in the diameter range of 100 to 200 nm. Particle diameter can be controlled by choosing different ratios of gelatin to anhydride, amount of solvents as well as reaction time. The NPs are formed by micellization, which in turn is induced by conjugation between PBLG and gelatin or CLP respectively, forming a core-shell structured particle with insoluble inter-polymer complexes of PBLG and Gel/CLP as the core and soluble Gel/CLP as the shell. Hydrophilic amino-, carboxy- and hydroxyl groups in gelatin or CLP ensure water solubility and electrostatic stabilization of the particles.

Deprotection of the PBLG carboxy groups is reflected by the reduction of signal intensity in the range of 700 to 750  $\text{cm}^{-1}$  in the IR spectra, typically associated with benzyl groups. Deprotection leads to formation of PGA increasing the electrostatic interactions and hydrogen bonds with gelatin or CLP reflected further in a decrease of particle diameter.

Next, particle stability was further ensured through a selective crosslinking using EDEA in the presence of EDC, which led to decrease of the particle diameter as well as PDI. Subsequent complexation with CDDP led to well-defined particles with visible boundaries in TEM images related to the high electron density of platinum. Following CDDP loading, the surface charge of both compounds increased from  $-19.5$  mV to  $-0.8$  mV, for gelatin-based NPs and from  $-10.4$  mV to  $21.7$  mV for CLP-based NPs. Notably, CLP-based NPs have with  $76$   $\mu\text{g}$  of CDDP per mg of NPs a significantly higher CDDP content, compared to just  $25$   $\mu\text{g}$  of CDDP per mg of NPs in the gelatin-based NPs. The higher CDDP loading in CLP-based NPs may be attributed to CLP's chemical structure with its higher abundance of free carboxy groups facilitating higher complexation of platinum. Furthermore, incorporation of other platinum-based drugs such as oxPt is possible with this approach.

Electrophoretic light scattering performed at different pH values showed that the surface potential of gelatin-based NPs is positive ( $7.9$  mV) at a pH about 4 after CDDP loading and negative ( $-5.3$  mV) upon an increase in pH above 5 indicates the surface charge switch property of NPs in response to the change of medium pH. Since type-B Gel utilized in this study has an pI between 4.8 and 5.0, we suggest the microscopic structure of Gel-PGA NPs consisting of a mainly gelatin-based shell with positively charged gelatin corresponding to ionized amino groups at  $\text{pH} < \text{pI}$  or negatively charged gelatin with ionized carboxylate groups at  $\text{pH} > \text{pI}$ , which ensures NP stability at physiological pH. The particle core presumably consists of the Gel-PGA interpolyelectrolyte complex.[32] The here employed CLP has a pI of 5.5 and shows similar pH-sensitive behavior exhibiting elevated surface charge switches between reaction steps. TEM images provide an insight into the particles' spherical morphology. Importantly, our synthesis route omits any external and potentially toxic initiator and uses only biocompatible and biodegradable building blocks. Furthermore, the employed CLP is produced by a recombinant protein manufacturing process, providing a vegan protein source with an end product having well-defined chemical properties such as a consistent amino acid sequence, monodisperse molecular weight,



pharmaceutical grade purity and production reproducibility. Animal-sourced materials can be linked to undesired immunological responses, pathogens, inconsistent quality and may require more stringent regulatory control. In a clinical setting, Vecollan<sup>®</sup>-based systems may be advantageous from a regulatory perspective.

As CLP-based NPs have a higher drug load and are more defined in structure and diameter all further studies proceeded with CLP-based particles (CLP-PGA-NPs). The toxicity of empty and CDDP loaded CLP-NPs was studied in cell viability assays compared to free drug. Empty NPs did not show any toxicity, indicating their biocompatibility. The IC<sub>50</sub> for free CDDP is in the lower molar range. The IC<sub>50</sub> for CDDP-loaded NPs decreased with incubation time indicating a slow but steady release. This was reinforced by drug release studies in FBS, which demonstrated an initial burst release of 37% within eight hours, stabilizing in a gradual release pattern reaching 52% after seven days. Most likely, the release yields not CDDP but the active metabolite *cis*-diammineaquachloroplatinum(II), which also forms after protein binding of CDDP.[53] The release profile is thus suitable for the intended application of i.p. drug delivery, where a sustained release profile is required.

As distribution within the peritoneal cavity, as well as retention and clearance pathways are essential for future optimization of the DDS, we employed consecutive SPECT/CT studies over one week after i.p. injection to follow the *in vivo* behavior of CLP-PGA-CL-NPs. To this aim, particles were radiolabeled using [<sup>111</sup>In]InCl<sub>3</sub>. With a half-life of 2.8 days, we could track their biodistribution and clearance over seven days.[54] The high density of carboxyl and amino groups present at the NP surface facilitated the formation of strong coordination bonds with [<sup>111</sup>In]In<sup>3+</sup> without the need to introduce additional chelators such as DOTA moieties, as previously reported in the literature.[55–57] DLS data revealed an increase of the mean hydrodynamic particle diameter and a slight broadening of the size distribution by 60 nm, most like due to some [<sup>111</sup>In]In<sup>3+</sup>-induced crosslinking of individual particles. Radiolabeling barely affected surface charge with an increase from –10.4 mV before labeling to –9.8 mV after labeling.

After i.p. injection, SPECT analysis revealed an initial renal clearance of about 10%ID to the bladder, of either [<sup>111</sup>In]In<sup>3+</sup> after initial release or small compounds after transchelation.[55] At later time points, no renal clearance was observed. Our SPECT measurements are in line with earlier observations regarding particle clearance from

the IP cavity.[59–62] Particles with diameters above 20 nm are primarily cleared via the lymphatic system. The predominant route is the parasternal lymphatic pathway, where particles enter through ventral, diaphragmatic stomata which empty into lymphatic vessels parallel to the internal thoracic artery and finally flow to the parasternal LNs and then mediastinal LNs, clearly recognizable on SPECT images four hours post-injection and all later timepoints. Finally, they enter into the right or thoracic lymphatic vessels with subsequent vascular recirculation.[60,61,63,64] Secondly, particles taken up via other diaphragmatic stomata flow through lymphatic vessels along the posterolateral aspect of the thoracic cavity, also draining into the mediastinal LNs. This pathway is therefore also known as the “thoracic cavity lymphatic” route. The third route involves visceral abdominal lymphatics that drain into the cisterna chyli and later into the thoracic ducts. Uptake in visceral LNs and the cisterna chyli can be identified on most SPECT images as well. Imaging indicates that particles are redistributed via the lymphatic system, recirculated, and ultimately taken up by the liver and spleen which are the main clearance organs of the mononuclear phagocyte system (MPS). SPECT imaging showed that by day seven, approximately 40%ID remained overall in the peritoneal cavity, with 14% having accumulated in the liver and spleen. Taking these two sites into consideration, a total activity of 26%ID remained in the abdominal cavity after seven days. As the limited spatial resolution of SPECT imaging does not allow to distinguish between NP uptake and adsorption to outer tissue boundaries, autoradiography images were acquired for tissue cross-sections. While liver and spleen sections show homogeneous uptake of radiolabeled particles, NPs are mainly sticking to the outer tissue layer of other abdominal organs such as colon, mesothelium, peritoneum, kidney, etc. The mesentery, which is one of the prime locations of intraperitoneal metastasis, showed notable uptake, accumulating approximately 24%ID/g. Interestingly, the pancreas and ovaries showed mixed results with NP accumulation on the organ’s surface as well as uptake within the organs.

In summary, SPECT imaging, autoradiography as well as biodistribution data demonstrate that NPs are cleared through known lymphatic pathways, which feed into the venous system. Subsequent recirculation, alongside phagocytosis leads to clearance by the MPS with uptake in liver and spleen. Previous studies showed that particle size and surface charge are the main factors determining clearance pathways, retention time within the intraperitoneal cavity, potential adhesion near injection site but also tumor uptake. With respect to size, contradictory requirements exist as larger

particles (i.e. diameter above 2–5  $\mu\text{m}$ ) show prolonged retention as the stomata have a size cut-off in that range, while small particles seem to show improved tumor uptake.[50,51,65,66] As our CLP-based NPs may be tuned in size from nano- to microparticles as well as in surface charge by possible surface modification with charged groups, they are an ideal platform to further study the most suitable properties for i.p. drug delivery. Furthermore, they can be loaded with other platinum-based drugs such as oxPt or carboplatin, next to CDDP addressing different tumor entities. SPECT imaging, combined with autoradiography, is a perfect tool to track the pharmacokinetics of particle clearance as well as their spatial redistribution in the intraperitoneal cavity.

### 3.3.6 Conclusion

In this study, CDDP was loaded into Gel-PGA or CLP-PGA NPs through the interaction between the platinum of CDDP and the carboxyl groups of the NPs with high drug loading and stability. The obtained NPs have a spherical structure with an average diameter of about 150 nm as measured by DLS and TEM. Subsequently, longitudinal SPECT/CT imaging using radiolabeled NPs was used to assess kinetic and biodistribution in healthy mice. After i.p. injection, particles are cleared via the lymphatic system into the venous system. Subsequently, particles are recirculated with final uptake in liver and spleen. After seven days, a significant amount is still present in other abdominal organs such as pancreas and ovaries, in addition to particles adhering to the surface of abdominal tissues. The NPs demonstrated extended release characteristics, suggesting their potential for sustained drug release. Overall, the results support the use of CLP-PGA NPs as a promising drug carrier for passive distribution and controlled release, with potential for localized treatments in i.p. drug delivery.

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## 3.3.8 Supporting information

## Synthesis

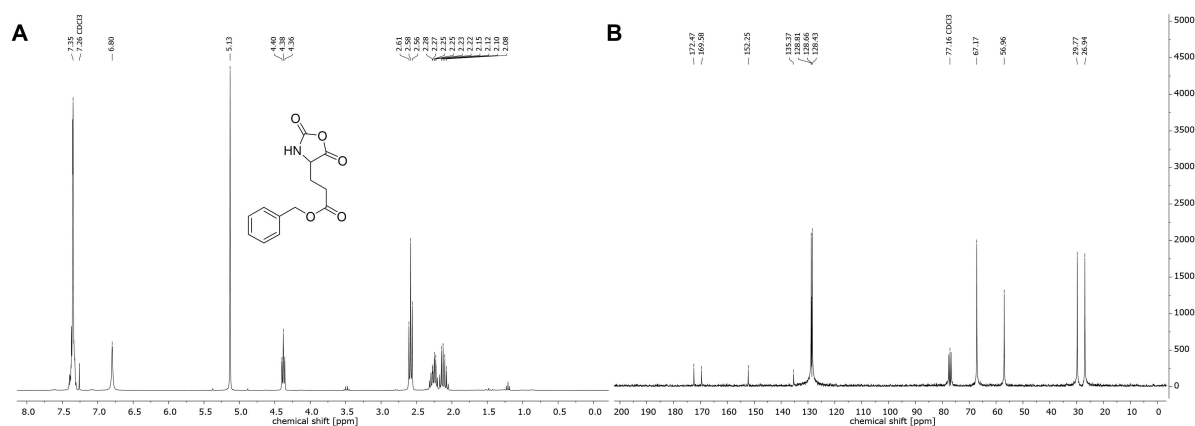


Fig. S1: NMR spectra of (S)Glu(OBzl)-NCA. A) <sup>1</sup>H-NMR spectrum. B) <sup>13</sup>C-NMR spectrum.

Table S1: Overview of results after polymerization step. The solvent amount was varied. Reaction was conducted at 0 °C for 15 minutes with 350 mg of gelatin and 100 mg (S)Glu(OBzl)-NCA.

| Sample        | Water  | DMF   | Hydrodynamic Diameter | PDI   | Surface potential |
|---------------|--------|-------|-----------------------|-------|-------------------|
| Gel-PBLG-NP-1 | 50 ml  | 10 ml | 122 nm                | 0.246 | 1.4 mV            |
| Gel-PBLG-NP-2 |        | 4 ml  | 178 nm                | 0.190 | 1.6 mV            |
| Gel-PBLG-NP-3 | 100 ml | 10 ml | 135 nm                | 0.386 | 1.7 mV            |
| Gel-PBLG-NP-4 | 25 ml  | 4 ml  | 233 nm                | 0.237 | 2.4 mV            |

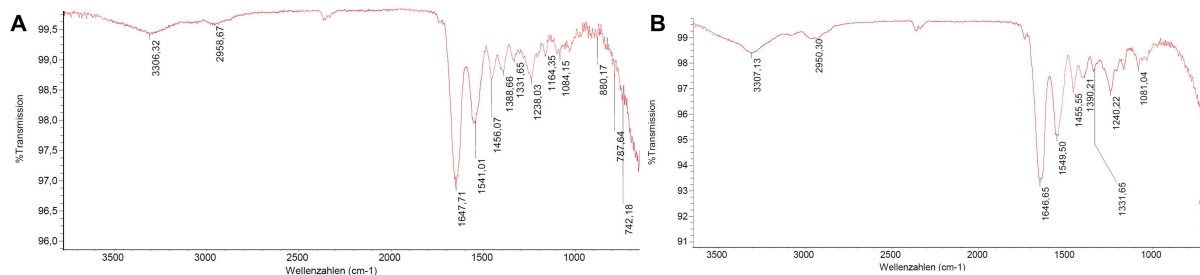


Fig. S2: IR spectra of A) benzoyl-protected gelatin-based particles and B) deprotected particles.

Table S2: Overview of results after oxPt loading.

| Sample          | Hydrodynamic Diameter | Surface potential | Pt content         |
|-----------------|-----------------------|-------------------|--------------------|
| oxPt@CLP-PGA-NP | 210 nm                | -2.6 mV           | 0.041 mg Pt/mg NPs |

### Radiolabeling and biodistribution

TLC analysis indicates no free  $[^{111}\text{In}]\text{In}^{3+}$  but the presence of approximately 10% of another  $^{111}\text{In}$ -containing compound, which could not be separated by SEC purification. For these first experiments, we chose to proceed with a radiochemical purity of 90% for *in vivo* studies. (Table S3).  $^{111}\text{In}$ -labeling led to an increase in particle radius by 60 nm and a slightly broader size distribution (Table S3).

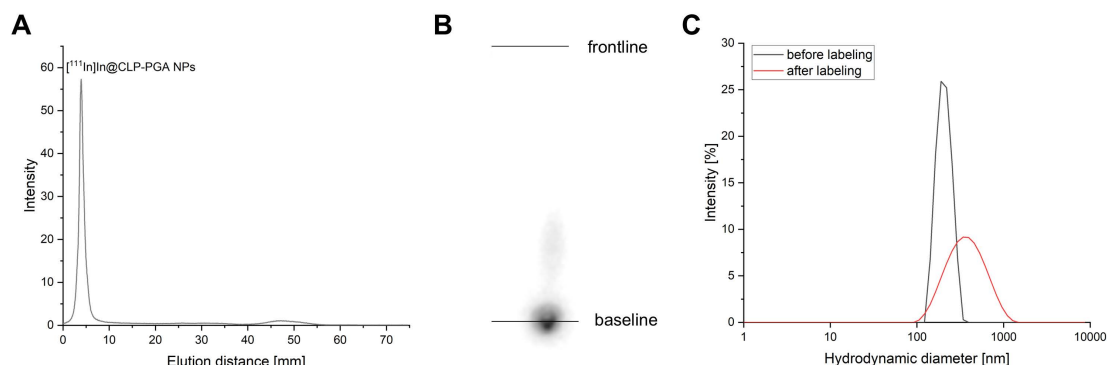


Fig. S3: A) Thin-layer chromatogram of  $[^{111}\text{In}]\text{In@CLP-PGA NPs}$ . B) Autoradiography image of TLC of  $[^{111}\text{In}]\text{In@CLP-PGA NPs}$ . C) DLS spectra of CLP-based NPs before and after labelling with  $[^{111}\text{In}]\text{InCl}_3$ .

Table S3: Overview of CLP-PGA NPs before and after labelling with  $[^{111}\text{In}]\text{InCl}_3$ .

| Sample                                   | Hydrodynamic diameter [nm] | Surface charge [mV] | Radiochemical purity [%] | Specific activity |
|------------------------------------------|----------------------------|---------------------|--------------------------|-------------------|
| CLP-PGA NPs                              | 223<br>(PDI: 0.137)        | -10.4               |                          | -                 |
| $[^{111}\text{In}]\text{In@CLP-PGA NPs}$ | 287<br>(PDI: 0.186)        | -9.8                | 90                       | 5 MBq/mg NPs      |

## Results

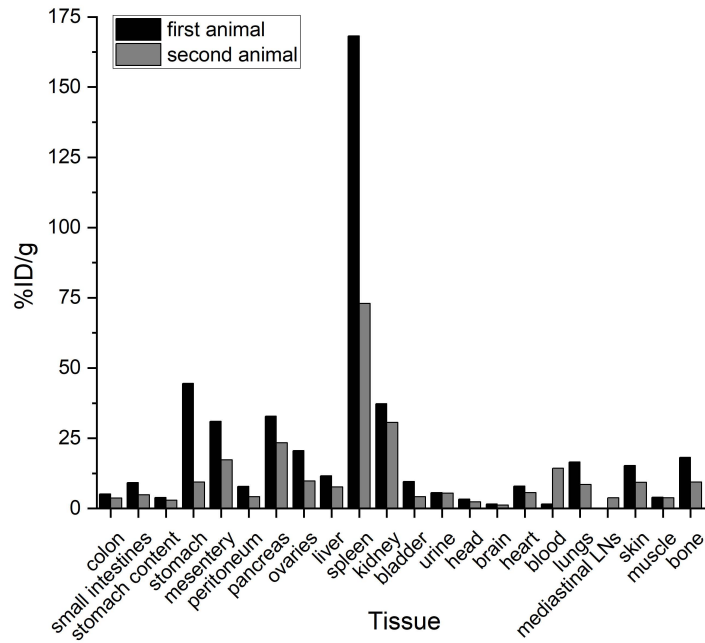


Fig. S4: Biodistribution of both mice individually as determined by gamma counter.

Table S4: Activity accumulation in explanted tissues by autoradiography.

| Tissue          | Intensity [%]<br>(first animal) | Intensity [%]<br>(second animal) | Mean [%]     |
|-----------------|---------------------------------|----------------------------------|--------------|
| Colon           | 0.471                           | 0.459                            | 0.465±0.006  |
| Small intestine | 1.010                           | 0.662                            | 0.836±0.174  |
| Ovaries         | 1.046                           | 1.704                            | 1.375±0.329  |
| Mesenterium     | 10.181                          | 1.811                            | 5.996±4.185  |
| Peritoneum      | 0.536                           | 1.122                            | 0.829±0.293  |
| Kidney          | 4.631                           | 7.800                            | 6.216±1.584  |
| Liver           | 4.765                           | 5.604                            | 5.185±0.419  |
| Bladder         | 1.363                           | 1.314                            | 1.339±0.024  |
| Thymus          | 3.206                           | 2.399                            | 2.803±0.404  |
| Stomach         | 0.732                           | 1.101                            | 0.916±0.184  |
| Spleen          | 68.063                          | 51.891                           | 59.977±8.086 |
| Pancreas        | 3.994                           | 23.437                           | 13.716±9.722 |
| Lumbar muscle   | -                               | 0.695                            | 0.695        |

## 4 General discussion

Currently, management of peritoneal carcinomatosis (PC) presents a major clinical challenge with limited treatment options. Treatment comprises cytoreductive surgery complemented by intravenous and intraperitoneal (i.p.) chemotherapy. The latter is often applied during HIPEC or PIPAC interventions. As in most cases residual cancer cells eventually cause recurrence, above treatment is considered palliative care.[1] For any future and hopefully curative treatment, the problem of remaining cancer cells has to be addressed, raising interest in DDS for i.p. application. The DDS should provide a sustained release of drugs over time leading to a high local concentration at tumor sites. Thus, their clearance from the peritoneal space should be slow without causing any additional toxic effects. Preferentially, the DDS should accumulate within the lesions. Research performed so far points at a few key aspects such as diameter and surface charge as influence on residence time in peritoneum, tumor accumulation and clearance pathways. Particles above a diameter of approximately 2–5  $\mu\text{m}$  are retained in the peritoneum much longer than smaller particles.[2–4] The prolonged retention is related to the cut-off size of stomata forming the entry into the lymphatic system. Smaller particles are cleared via the lymphatic system, drained into the venous circulation and then recirculated.[4–6] Eventually, these particles are mainly cleared via the ERS with uptake in liver and spleen. While larger particle diameters prolong retention in the peritoneal space, smaller particles show favorable uptake in tumor tissue.[7] As of now, there is no consensus on the most suitable particle diameter balancing uptake in tumor tissue and peritoneal retention. Besides particle diameter, the surface charge plays a key role. Research shows that particles with a positive surface charge have preferential electrostatic interaction with the negatively charged membrane covering the abdominal space. This interaction leads to prolonged retention, but can also reduce distribution across the peritoneum.[8,9] Further complication presents agglomeration of positively charged particles *in vivo* induced by interaction with proteins within the peritoneal fluid.[10,11] Surface modifications may reduce agglomeration and enhance stability, but can also lead to faster clearance.[9,12] Overall, the interplay between size and surface charge is not well understood and has been only studied using particular systems such as liposomes or polymers, silica or polymeric particles.[13] On top, more important than the particular fate of the DDS is the pharmacokinetic of the formulation. Release kinetic has to be seen in the context of the drug carrier biodistribution. For example, long retention of

DDS that rapidly release their drug payload will not provide a substantially increased benefit over i.p. administered chemotherapy of the free drug. Vice versa, slow release of a drug from rapidly cleared DDS will only lead to increased drug levels in clearing organs such as liver and spleen.

To date, most research employs bioluminescence or endpoint histological analysis to investigate the fate of DDS *in vivo*, looking at uptake in tumor and other organs at distinct timepoints, offering limited temporal and spatial resolution.[3,9,14–18] As quantification of the entire particle distribution is lacking, evaluation of how DDS modifications such as size, surface charge or PEGylation affect their *in vivo* fate is difficult. Some studies simply rely on quantification of drug levels achieved in tumors compared to healthy tissues, which is obviously the most relevant information linked to therapeutic efficacy.[19] However, this approach doesn't allow to link drug uptake to the PKPD and drug release kinetics of the DDS.

A comprehensive understanding of carrier fate after i.p. injection is essential to design drug carriers tailored to specific therapeutic needs. Here, imaging-based approaches come into play. Imaging, such as magnetic resonance imaging (MRI), computer tomography (CT) or nuclear imaging not only allow to assess tumor payload but can also track particle distribution *in vivo*. A prerequisite is the modification of the DDS with contrast agents, such as fluorine-19 ( $^{19}\text{F}$ )-based compounds for MRI, iodine for CT or radionuclides for positron emission tomography (PET) or single-photon emission computer tomography (SPECT) imaging.

In this thesis, different DDS are developed and studied *in vitro* as well as *in vivo* using multimodal imaging approaches.

**Chapter 3.1** presents synthesis and characterization of capsules comprising a poly(lactic-co-glycolic acid) (PLGA) shell and a liquid perfluorooctylbromide (PFOB) core for image-guided i.p. drug delivery. PTX was encapsulated within the polymer shell using a versatile solvent emulsion-evaporation technique, demonstrating sustained release kinetics. Systematic variation of synthesis parameters optimized imaging contrast, drug loading, and degradation profiles. Using  $^{19}\text{F}$ -MRI, capsule biodistribution and degradation were longitudinally monitored in a healthy mouse over seven days. From  $^{19}\text{F}$ -imaging perspective, a new acquisition scheme termed BaSSI with spectral encoding was used for  $^{19}\text{F}$ -MR imaging, providing superior sensitivity compared to conventional  $^{19}\text{F}$ -MRI sequences. *In vivo*  $^{19}\text{F}$ -MRI revealed that the

majority of capsules remained in the vicinity of the injection site with gradual degradation over one week with their typical core-shell structure largely intact as confirmed by histological analysis. *Post mortem* histological analysis confirmed additional particle presence at peritoneal tumor-relevant sites including the pancreas, ovaries and small intestines, highlighting potential for targeted therapy in PC.

This study showed that  $^{19}\text{F}$ -MRI can be successfully used to detect  $^{19}\text{F}$ -containing DDS *in vivo* providing a hot-spot image overlaid on an anatomical MR image. In particular, the scans were performed on an older generation clinical MR scanner at 3 Tesla. Notably, most  $^{19}\text{F}$ -MRI studies so far were performed at higher field strength (i.e. > 7 Tesla). As SNR scales with field strength, shorter acquisition times can be achieved at higher field strengths compared to our study. However,  $^{19}\text{F}$ -MR imaging serves primarily as a preclinical tool for DDS development in order to understand better particle distribution and may not be needed for clinical translation as such. While PFOB is the component of choice when it comes to *in vivo* use as MR contrast agent, its chemical properties make it less suited as a solvent for hydrophobic drugs such as PTX. After formation of the emulsion droplets of the organic phase, which contains PFOB, PTX and PLGA, PTX accumulates solely in the PLGA shell, interfering at higher concentrations with particle formation. PTX release studies performed with two different particle diameters over seven days shows exponential release kinetic in accordance with a *Fickian* diffusion-driven PTX release from microparticles. Overall, achievable drug loading per mg polymer is limited, which requires injection of a considerably high number of capsules in order to reach a therapeutically relevant PTX dose. An option to increase PTX loading per particle and therefore reduce the number of particles is the use of an alternative oil core to PFOB, which acts as a solvent for PTX. Thus, during capsule formation, PTX is not only incorporated into the PLGA shell but also remains dissolved in the oil reservoir.

In **chapter 3.2**, a similar system of PLGA-shelled capsules was investigated but using the iodinated oil octan-2-yl 2,3,5-triodobenzoate (TIBO) instead of PFOB. As TIBO contains the 2,3,5-triodobenzoate moiety it provides a high iodine content per mg particle rendering them radiopaque with the possibility to quantify particle distribution and degradation using CT imaging. In particular, a clinical CT scanner was used equipped with an energy resolving dual-energy detector (DECT), which allows to perform material delineation based on the unique energy-dependent absorption

coefficients of iodine in comparison to absorption caused by endogenous atoms such as calcium.

By adjusting the parameters of the formulation method, control over particle characteristics was achieved. Most importantly, emulsification speed yielding emulsion droplets of different sizes provided control over final particle diameter. Furthermore, volume ratio of PTX, TIBO and polymer dissolved in the organic phase provided control over particle size and shell thickness. As TIBO serves as a solvent for PTX, these particles incorporate up to two times more PTX per mg polymer compared to capsules with PFOB depending on the diameter of the particles. For PTX-loaded particles, sustained drug release was evaluated over seven days. Release kinetic of PTX follows similar behavior as in PFOB-loaded particles which is mainly *Fickian* diffusion-driven for larger particles. For nanoparticles, a hindered diffusion was found, indicating a slower release per particle. However, normalized to the same PTX concentration, cumulative release increases with decreasing particle diameter simply due to the higher surface area of smaller particles. PTX release proceeds slower from TIBO-based particle dispersion than from PFOB-based dispersion, likely due to higher particle numbers required for equal drug loading and a two-step release mechanism. In the TIBO system, PTX must first diffuse from the core into the shell before being released, whereas in the PFOB system, release occurs directly from the shell, resulting in faster kinetics. When taking higher particle number into account, the release kinetics are comparable between the two formulations.

For proof of concept, an *in vivo* study was performed in healthy mice using a clinical DECT to follow particle distribution over seven days. After i.p. administration of the particles at a dose required for a potential therapeutic study, sufficiently high iodine concentrations were achieved to provide 3D hot-spot iodine concentration maps over 18 days. Comparable to results obtained with  $^{19}\text{F}$ -imaging, this study showed particle agglomeration in the vicinity of the injection side. Notably, the iodine concentration decreased to about 60% over the study period depending on particle diameter. The signal is mainly localized in a few spots around the injection site, indicating agglomeration of particles *in vivo*. Reduction of iodine concentration over time can either be related to distribution and possible clearance of particles or to decomposition of particles with subsequent clearance of TIBO. Histological analysis after 18 days revealed particle distribution in the intestines, liver, uterus, ovaries as well as spleen and pancreas. This was seen across all particle sizes, though larger particles allowed

clearer visualization. Notable differences in particle morphology were observed between fluorine- and iodine-containing formulations. It should be noted that PFOB-based experiments were terminated after seven days, while animals injected with TIBO-based particles were euthanized after 18 days. Particles with fluorinated cores largely preserved their core-shell structure. Whereas iodine-filled counterparts revealed deformed and non-spherical structures in the spleen ( $\sim 7\text{--}9\text{ }\mu\text{m}$ ), while smaller structures ( $\sim 2\text{--}4\text{ }\mu\text{m}$ ) were observed in other organs. The longer timeframe may explain the greater degradation observed for the iodine-containing particles.

While the TIBO-filled capsules allow to incorporate significant amounts of PTX, incorporation of hydrophilic drugs such as CDDP yielded unfavorable results. Here, CDDP is accumulating only to a small degree inside the polymer matrix, while the majority of the drug is found in the water-rich outer phase after the evaporation of the organic phase. The emulsion process was therefore extended to a double-emulsion process, with water-based CDDP-containing emulsion droplets dispersed in an oil-phase within the polymer capsule. This approach was investigated as well but yielded only low incorporation efficiency with 25 to 40 times lower drug loading compared to PTX. Therefore, capsules are less suited for delivery of hydrophilic drugs requiring a different DDS with an alternative loading mechanism.

Another approach is to load CDDP into particles via complexation. CDDP is a planar platinum(II) complex with two amine and two chloride ligands where the latter are leaving groups due to their weaker interaction with platinum. Using drug carriers with a high number of carboxy groups allows to incorporate a high amount of platinum via complex formation. *In vivo*, hydrolysis supported by a degradation of the drug carrier leads to a slow release of the active metabolites *cis*-diammineaquachloroplatinum(II) and *cis*-diamminediaquaplatinum(II), which subsequently bind to the DNA and interfere with mitosis.

In **chapter 3.3**, biopolymer-based particles were investigated that allow loading of CDDP by complex formation. The biopolymer is based on a co-polymer formed by either gelatin-poly(glutamic acid) (Gel-PGA) or collagen-like peptide-poly(glutamic acid) (CLP-PGA) respectively. Though similar in their composition, gelatin and CLP differ in their production method. Gelatin is derived from animal sources while CLP can be produced by a (vegan) recombinant protein production method which improves biocompatibility and batch-to-batch reproducibility. Loading of CDDP was achieved by



complexation, where CLP-PGA-NPs demonstrated superior drug loading and stability over Gel-PGA-NPs yielding well defined particles with diameters around 150 nm and a drug loading of 0.076 mg Pt per mg particles. Radiolabeling with [ $^{111}\text{In}$ ]InCl<sub>3</sub> could be performed without the need to introduce additional macrocyclic chelators as the highly abundant carboxy and amine groups provided highly stable labeling.

SPECT/CT imaging in healthy mice was used to monitor *in vivo* biodistribution of radiolabeled CLP-PGA-NPs over seven days. Imaging revealed fairly rapid clearance from the peritoneal space via the lymphatic system and subsequent uptake in the spleen and liver, with additional accumulation in the pancreas, ovaries, and along peritoneal surfaces. Interestingly, after seven days, around 26% of injected activity still remain in the peritoneal space excluding abdominal organs liver and spleen. Furthermore, significant accumulation in common metastatic sites such as the pancreas or ovaries were found. The observed biodistribution also highlighted possible application for targeting the lymphatic system, which is relevant in disease spread, based on surface charge and size. As the release kinetic of CDDP shows steady release over seven days, this DDS system is particularly interesting for future therapeutic application. In particular, the CLP-PGA particles can be potentially produced in different sizes ranging from nano- to microparticles. The surface potential can be tuned by conjugation with additional negatively or positively charged groups to investigate how particle diameter and surface charge influence particle distribution in a PC model in mice.

In this thesis, a range of DDS based on biocompatible and biodegradable materials capable of incorporating both hydrophilic and hydrophobic drugs were developed, making them adaptable for diverse cancer types. These systems are tunable in size and surface charge, allowing tailored design. Using healthy mice for a first proof of concept, particles were i.p. administered using different imaging modalities to follow their *in vivo* behavior. For PLGA-based systems, hot-spot images obtained with  $^{19}\text{F}$ -MRI as well as with DECT reveal that particles stay in the vicinity of their injection sites, most likely as larger aggregates. Both studies show consistently that after seven days, about 60–80% of the originally injected amount of particles remain detectable within distinct spots. Although these results seem logical as the outer PLGA-shell is identical, it is reassuring that both imaging techniques provide similar quantification results. Furthermore, the particle diameter for the PLGA-based capsules has little effect on distribution within the abdominal cavity or clearance. Most likely, particles form

aggregates which stick to the abdominal membrane upon injection, make the diameter of the individual particles less important. In both studies, histological examination showed particles widely dispersed across the abdominal organs. As histology doesn't allow any quantification and only examines small samples, any judgment of particle distribution is impossible without additional quantitative tomographic imaging information as provided in these cases by  $^{19}\text{F}$ -MRI or iodine-DECT. From a therapeutic aspect, the TIBO-containing particles are more interesting as higher amount of PTX can be delivered. In contrast, PFOB-containing particles, though less efficient in PTX incorporation, can offer improved detectability due to the high sensitivity of  $^{19}\text{F}$ -MRI (0.05  $\mu\text{l}$  PFOB detectable with  $^{19}\text{F}$ -MRI and 200  $\mu\text{l}$  sodium iodide detectable with DECT in our study).[20] However, this imaging advantage comes with the trade-off of longer acquisition times and more complex experimental setups. Incorporation of PTX in PFOB-based capsules could be improved by adding an additional oil to the organic phase which acts as a solvent for PTX. This concept has already been shown in the past and could be further explored for  $^{19}\text{F}$ -MRI applications.[21] The more important aspect is the possible agglomeration of PLGA particles upon injection.

To date, the particle distribution and clearance of PLGA-based nano- and microparticles after i.p. injection are poorly understood. Past investigations have largely relied on histological analysis or optical methods.[14–18] While *Tsai et al.* labeled PTX for imaging by autoradiography, the fate of the PLGA carrier itself has not been determined.[19] *Amoozgar et al.* coated PLGA-based particles with polydopamine and PEG to enhance drug retention and extend the release profile. However, *in vivo* studies did not include uncoated particles for comparison.[22] During one phase I clinical study with poly(lactide)-based PTX-containing microspheres, adverse effects such as inflammatory responses and bowel obstruction were observed leading to an early termination of the trial.[23] Animal studies have been shown to produce similar results depending on particle size and polymer molecular weight.[24] Agglomeration of particles as observed with  $^{19}\text{F}$ -MR and DECT imaging studies could lead to extremely high local drug concentrations causing above inflammatory response of healthy tissue. Thus, clinical translation approaches would significantly benefit from introduction of quantitative imaging for closer investigation of carrier fate and evaluation of safety.

For delivery of platinum-based drugs, the biopolymer-based system composed of CLP-PLA seems most promising. CDDP could be loaded with high efficiency additionally

providing a release kinetic which is suited for therapeutic application. As expected for particles with a diameter around 150 nm, SPECT imaging revealed clearance via the lymphatic system. However, after seven days, a considerable number of particles (26%) is still present and distributed within the abdominal cavity. Autoradiography shows adherence of the particles to the outer surface of abdominal organs and the mesothelium. This system in combination with highly sensitive SPECT imaging offers future opportunities to investigate the dependence of particle distribution, clearance and tumor uptake as a function of particle size and surface modification.

In future studies, these DDS should be evaluated in relevant tumor models to assess their therapeutic efficacy against cancer. For this purpose, the use of imageable tumor models is essential to enable continuous, longitudinal monitoring of tumor response, moving beyond reliance on endpoint analyses alone that is as of today still the most common method for preclinical evaluation of treatment success.

Optimizing DDS performance requires multidisciplinary evaluation to ensure safety and biocompatibility. Yet most research remains chemistry-focused, using generic drugs and non-specific models, which limits clinical relevance. Standardization and alignment with clinical needs are crucial for better translation. This thesis introduces a versatile platform to tailored i.p. therapy for PC. Combination of real-time longitudinal imaging with therapeutic, biocompatible, and biodegradable drug carriers is proposed for development of tailored strategies specific to individual tumor types. This approach shows potential to improve treatment outcomes in the clinical problem of PC.

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## 5 Acknowledgements

First and foremost, I would like to express my sincere gratitude to Prof. Dr. Gröll for making this work possible. Thank you for your supervision and for giving me the opportunity to be the first to explore and expand this exciting field of research in your lab. I truly appreciated being able to drop by at any time and rely on your support. I hope this work serves as a first step toward realizing the vision behind it. Holger, since you of course are my “Doktorvater,” you’ve also “raised” me in a way. And these are the values I’ve especially taken away from our time together and want to highlight: autonomy, confidence, teamwork, curiosity about life, art, music, and literature, the ability to dive into completely new fields, overcoming the fear of the unknown, doing things even when you don’t feel fully ready, focusing on the big picture instead of getting lost in details, being a leader who sincerely cares for his coworkers on a profound and personal level, not taking failures too seriously, perseverance, intrinsic interest in research and science on every level, frustration tolerance, physical chemistry with all its many equations and calculations (though I’m still not a fan), and truly immersing yourself deeply into literature research and your own topic. Coming back to a conversation after a particularly rough patch when you said, “Yes, I still believe in this project”: I do, too!

I would also like to express my sincere gratitude to Prof. Dr. Neumaier for kindly agreeing to serve as the second examiner of this thesis. I greatly appreciate having your broad expertise in this field to thoroughly evaluate this work. During my master’s studies, I had the chance to complete a research project in your group that introduced me to radiochemistry, sparked my interest in the field, and helped shape my scientific path. Thank you as well for the support of your PhD students, who were always extremely helpful and knowledgeable. My thanks go to Jan Bertram, Dmitrii Antuganov and Michael Gemki for the insightful discussions during various hurdles along this long journey.

Thank you to Prof. Dr. Annette Schmidt for being my second supervisor all these years having seen the whole journey. I appreciate the many fruitful discussions and the reviews. Your guidance and access to lab resources greatly contributed to my work. Moritz Raphael, Linus Kuckling, Katharina Schwab, Patricia Bach: thank you all so much for providing help and performing various analyses, which would not have been possible without your support!

I would like to express my gratitude to Prof. Dr. Mathias Wickleder for kindly agreeing to serve as chair of my thesis committee. During my master's studies, I had the opportunity to complete two research projects in your group, which intensified my interest in radio- and nanochemistry. This experience has influenced my academic path and research focus.

I would also like to sincerely thank Prof. Dr. David Maintz for his support and for welcoming our group into the radiology department. This has opened up many valuable opportunities for research, both for our group and for me personally. I am also grateful to the entire radiology team for their helpfulness and support. A special thank you to Prof. Dr. Nils Große Hokamp for taking time out of your day to conduct experiments with me even after office hours are over. I think we're finally there!

Thank you very much to Evonik Foundation not only for the financial support without which none of this would be possible but also for the valuable insights into your work, the expert meetings, the scholarship events, and the opportunity to connect with other scholarship holders. I am truly grateful for the chance to meet so many inspiring people along the way, some of whom I now have the pleasure of calling friends. These experiences greatly enriched my journey as a PhD student. Special thanks to Thomas Endres and Maria Montero-Mirabet for their practical advice and for providing samples of various products which significantly advanced my research.

A very special and heartfelt thank you goes to Dr. Markus Schütz, who introduced me to this group and topic. Thank you for always having a listening ear, good advice, and plenty of time for me. Even after moving on to new territories, you always made time for all of us and offered invaluable guidance!

Thanks to Dr. Muhammed Yildirim and Dr. Johannes Lindemeyer for introducing me to MRI and supporting me during the experiments. Muhammed, our communication (sometimes even across continents) gave me hope, especially after long 16 hour days at the MRI with no results, only to find out there was a hardware error afterward.

Dr. Sin Yui Yeo: you lead by example, and we love to follow you! Thank you for teaching and helping me, for inspiring me to continuously improve myself, and for showing that research always remains hard work but is worth it. Also, thank you for inviting us to work alongside you in your office with all the free drinks. Let us not underestimate the positive impact that it had on this thesis!

Dr. Felix Gremse, thank you for generously dedicating your time to assist me, even with minor issues that were certainly straightforward to you. Your support with imaging and quantification data has been and continues to be invaluable.

Sven Saniternik: from the beginning until the end, we've been through it all. We studied chemistry together, wrote our bachelor theses side by side, and joined this group one after the other. We pushed and helped each other when needed, sharing countless inside jokes and memories like our own language. You are the best colleague anyone could ask for (I'm sure everyone in our group would agree). Now it's your turn to finish your PhD project, and as you know, I will always be by your side for this!

Jannika Jönsson and Larissa Hamacher: your help with cell assays and animal experiments, your shoulders to cry on and your support have helped me immensely, thank you so much! Gernot Welters, you have grown from a medical student completing an internship in our lab to a close friend, which I truly appreciate. You have of course also been a (very strong) shoulder to lean on. On numerous occasions, especially during animal studies, you were my last beacon of hope and ultimate savior. Thank you for being there!

I am also grateful to the entire Grüll research group. Despite our many different projects, all of you offered advice, practical support, and encouragement. Because of your wide-ranging expertise, I was able to learn so much from every discipline, and that's truly what makes our interdisciplinary group so unique to me. Special thanks, of course, go to everyone who assisted with the animal studies. I hope one day we can revisit "our" castle together!

All my students: thank you for giving me the opportunity to grow with you and improve myself as a teacher. Thank you for sharing the same interest and passion for this topic as I do and for showing up with such amazing results.

Of course, I must thank my friends. All the laughter and sometimes just a comforting conversation made this journey so much easier.

A very special thank you to (almost Dr.) Jan Bertram. Your help, understanding, support, expertise, our walks, your sweet treats, and your jokes helped me get through even the most stressful phases without losing sight of the light at the end of the tunnel. You inspire me. I look forward to growing with you personally and professionally forever. How lucky am I to be able to share every part of my life with you!



My deepest gratitude goes to my family. Thank you to my brother Viktor Kovalyk for always being there. To my parents: your constant support and guidance have made all the difference. You are my greatest role models. “Danke“ and „спасибо“!