Paclitaxel-Loaded Polymersomes for Enhanced Intraperitoneal Chemotherapy

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Abstract

Peritoneal carcinomatosis is present in more than 60% of gastric cancer, 40% of ovarian cancer, and 35% of colon cancer patients. It is the second most common cause of cancer-related mortality, with a median survival of 1 to 3 months. Cytoreductive surgery combined with intraperitoneal chemotherapy is the current clinical treatment, but achieving curative drug accumulation and penetration in peritoneal carcinomatosis lesions remains an unresolved challenge. Here, we used flexible and pH-sensitive polymersomes for payload delivery to peritoneal gastric (MKN-45P) and colon (CT26) carcinoma in mice. Polymersomes were loaded with paclitaxel and in vitro drug release was studied as a function of pH and time. Paclitaxel-loaded polymersomes remained stable in aqueous solution at neutral pH for up to 4 months. In cell viability assay on cultured cancer cell lines (MKN-45P, SKOV3, CT26), paclitaxel-loaded polymersomes were more toxic than free drug or albumin-bound paclitaxel (Abraxane). Intraperitoneally administered fluorescent polymersomes accumulated in malignant lesions, and immunofluorescence revealed an intense signal inside tumors with no detectable signal in control organs. A dual targeting of tumors was observed: direct (circulation-independent) penetration, and systemic, blood vessel–associated accumulation. Finally, we evaluated preclinical antitumor efficacy of paclitaxel-polymersomes in the treatment of MKN-45P disseminated gastric carcinoma using a total dose of 7 mg/kg. Experimental therapy with paclitaxel-polymersomes improved the therapeutic index of drug over free paclitaxel and Abraxane, as evaluated by intraperitoneal tumor burden and number of metastatic nodules. Our findings underline the potential utility of the polymersome platform for delivery of drugs and imaging agents to peritoneal carcinomatosis lesions.

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Introduction

Peritoneal carcinomatosis, dissemination of cancerous tissues in the peritoneal cavity, is a common manifestation in digestive tract and advanced gynecologic cancers, such as gastric, colorectal, ovarian, and appendiceal cancer. Up to 60% of patients with gastric cancer show disease progression and die of peritoneal carcinomatosis, with a median survival of 1 to 3 months (1). Forty percent of colorectal cancers lead to peritoneal carcinomatosis (2), and the mortality from ovarian cancer peritoneal carcinomatosis is about 35% (3). Systemic chemotherapy is less active against peritoneal metastasis than intraperitoneal chemotherapy (IPC), presumably because early-stage peritoneal tumor nodules are poorly vascularized and drug delivery and penetration into advanced tumors is inefficient. Compared with intravenous administration, the intraperitoneal route increases the drug concentration within the abdominal cavity; therefore, the local effects of the drug are increased (1–3). Despite proven significant clinical benefit of the intraperitoneal paclitaxel chemotherapy in the optimally debulked epithelial ovarian carcinoma patients (4), the therapy is not widely used, mostly due to the complexity of the regimen and drug side effects (5). An ideal intraperitoneal chemotherapeutic agent should be able to directly target and show high cytotoxic activity toward peritoneal tumors, persist in the peritoneal cavity for extended periods, and also result in some degree of systemic exposure (5). When IPC is administered under mild hyperthermia, the effects of chemotherapy are extended by vasodilation and damage of tissue barriers, increasing the penetration of anticancer drugs (1–3). Yet, even in the case of hyperthermic IPC, drug penetration is limited to 3 to 5 mm and malignant nodules as small as 3 mm need to be excised prior to IPC to achieve adequate therapeutic efficacy (3).

Paclitaxel is a potent cytotoxic drug used to treat ovarian, breast, lung, and pancreatic cancer, among others. Because of its low solubility in aqueous solutions, paclitaxel is administered with Cremophor EL as solvent, which is responsible for much of the clinical toxicity (6). Abraxane is an alternative formulation, in which paclitaxel is bound to albumin, forming water-soluble...
Polymersomes for Peritoneal Carcinomatosis Treatment

Materials and Methods

Materials

CHCl₃, MeOH, isopropanol, dimethylformamide (DMF), Rhodamine B octadecyl ester, doxorubicin, paclitaxel, and MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were purchased from Sigma-Aldrich. Phosphate buffer saline (PBS) was purchased from Lonza.

MKN-45P human gastric cancer cells were isolated from parental MKN-45P cells and authenticated as described previously (30). MKN-45P-luc cells were made by infecting MKN-45P cells with lentivirus (Biogenova). SKOV-3 and CT26 cell lines were purchased from ATCC (SKOV-3 ATCC HTB-77, CT26 ATCC CLR-2638). Cell lines were used without further authentication. The cells were cultivated in DMEM with 100 IU/mL of penicillin, streptomycin, and 10% of heat-inactivated FBS (GE Healthcare).

Athymic nude mice were purchased from Harlan Laboratories and Balb/c mice were purchased from Charles River Laboratories. Animal experimentation procedures were approved by the Estonian Ministry of Agriculture, Committee of Animal Experimentation, project #42.

POEGMA–PDPA copolymer synthesis

The 4-(2-bromoisobutyryl ethyl)morpholine initiator (MEBr) was prepared according to a previously published procedure (31). The protected maleimide initiator (Mal-Br) was prepared according to a previously published procedure (32). The Rhodamine 6G-based initiator (Rho-Br) was prepared according to a previously published procedure (30). Atom-transfer radical-polymerization (ATRP) synthesis and purification of POEGMA-PDPA copolymer synthesis was carried out as described previously (19). The resulting copolymer composition was determined by 1H nuclear magnetic resonance in CDCl₃ and the polydispersity was determined by size exclusion chromatography in acid water (0.25 vol% trifluoroacetic acid). The deprotection of Mal-P(OEG10MA)20-PDPA₉₀ was carried out according to a previously described procedure (19).

Reaction of Mal-P(OEG10MA)₂₀-PDPA₉₀ with cysteine-terminated 5(6)-carboxyfluorescein (Cys-FAM)

The deprotected Mal-P(OEG₁₀MA)₂₀-PDPA₉₀ (20 mg, 0.7 μmol) was dissolved in 1 mL of CHCl₃:MeOH 2:1, and 2 equivalents of Cys-FAM dissolved in 1 mL of nitrogen-purged DMF were added to the solution. The reaction mixture was stirred at 300 rpm overnight at room temperature. The CHCl₃ and MeOH were evaporated, and the rest of the solution was dialyzed against water using a dialysis cassette (Thermo Fisher Scientific) with a molecular weight cutoff of 10 kDa to remove the excess of Cys-FAM. The resulting suspension was freeze-dried and a yellow powder was obtained.

Polymersomes formation and paclitaxel, Rhodamine B octadecyl ester, and doxorubicin encapsulation

For the formation of labeled polymersomes, FAM-P(OEG₁₀MA)₂₀-PDPA₉₀ was mixed with the nonlabeled copolymer in a ratio 2:8. For the labeling of polymersomes with Rhodamine, the Rho–POEGMA–PDPA was mixed with the nonlabeled copolymer at a ratio of 1:9. For the formation of polymersomes and encapsulation of paclitaxel or doxorubicin, 20 mg of copolymer was dissolved in 4 mL of CHCl₃:MeOH 2:1, and 2 equivalents of Cys-FAM dissolved in 1 mL of nitrogen-purged DMF were added to the solution. The mixture was stirred at 300 rpm overnight at room temperature. The CHCl₃ and MeOH were evaporated, and the rest of the solution was dialyzed against water using a dialysis cassette (Thermo Fisher Scientific) with a molecular weight cutoff of 10 kDa to remove the excess of Cys-FAM. The resulting suspension was freeze-dried and a yellow powder was obtained.

Polymersomes for Peritoneal Carcinomatosis Treatment

Polymersomes can encapsulate water-insoluble compounds in the hydrophobic membrane and water-soluble compounds within the vesicular lumen (12–18). The surface of polymersomes can be functionalized with affinity ligands and dyes for targeted delivery and for tracking (19). The high molecular weight of block copolymers enables the formation of highly entangled membranes (20). This results in increased resilience with elastomer-like mechanical properties, which allows polymersomes to undergo up to 100% surface deformation before rupturing (21–23). These unique mechanical properties enable polymersomes to translocate across pores up to an order of magnitude smaller than their diameter (17).

Here we use polymersomes assembled from diblock copolymer poly(oligoethylene glycol methylacrylate)-poly(2-(diisopropylamino)ethyl methacrylate; P[OEG₁₀MA)₂₀-PDPA₉₀ or POEGMA–PDPA. POEGMA is a polyethylene oxide–based hydrophobic block that protects the particles from the immune system, extending the in vivo half-life (24), whereas the PDPA block makes the polymersomes pH-sensitive (Supplementary Fig. S1). PDPA is not soluble in water at neutral pH, thus allowing the formation of the polymersomes. However, under mildly acidic conditions (below pH 6.5), the amine group of the PDPA becomes protonated, resulting in fast disassembly of the polymersomes (25). Upon endocytosis and trafficking within acidic early endosomes, the rapid polymersome destabilization triggers an osmotic shock with consequent endosomal membrane lysis and cytosolic cargo release (14, 26, 27).

PDPA–based polymersomes have been used for intracellular delivery of DNA (14), antibodies (15), antibiotics (28), anticancer drugs (12, 13), and peptides (29). Particularly, for cancer cells, an efficient cytoplasmic delivery improves the drug efficacy (12). More recently, it has been demonstrated that polymersome-mediated intracellular delivery enables drugs to act considerably faster than when administered alone (13).

In the current study, we evaluated intraperitoneal administration of pH-sensitive polymersomes as a treatment for peritoneal cancer lesions in mice. We demonstrate that polymersomes allow efficient intracellular payload delivery in vitro and in vivo, specific homing to peritoneal tumors, and increase the therapeutic activity of paclitaxel at a very low drug concentration.
added. The solution containing the polymer and the dye or drug was dried under vacuum for the formation of the polymer film. The film was hydrated with 2 mL of PBS (pH 7.4) and stirred at 300 rpm for 2 weeks. After that, the suspension was sonicated for 30 minutes at room temperature. The polymer-some samples were purified by centrifugation. The suspension was spun down at 500 g for 20 minutes at room temperature, and the resulting supernatant was spun down at 20,000 g for 20 minutes at room temperature. The pellet was resuspended in 2 mL of PBS (pH 7.4).

Dynamic light scattering (DLS; Zetasizer Nano ZS, Malvern Instruments) was used to assess the polydispersity and average size of polymersome preparations. Z-potential measurements were conducted to determine the surface charge of the vesicles, using the Zetasizer Nano ZS, Malvern Instruments. Transmission electron microscopy (TEM) was used to assess the size, surface topology, and morphology of assembled vesicles. Polymersomes in PBS were deposited onto copper grids at a concentration of 1 mg/mL, and stained using 0.75% phosphotungstic acid (pH 7). Images were acquired using a transmission electron microscope Tecnai 10, (Philips).

The encapsulated paclitaxel was quantified by ultra-performance liquid chromatography (UPLC equipment from Waters), using free paclitaxel dissolved in MeOH to make the standard curve. Fifty microliters of paclitaxel-polymersomes were mixed with 30 µL of MeOH and 2 µL of HCl (1 mol/L). Five microliters of this mixture were run using water/Acetonitrile as eluent and Acquity Ultraperformance UPLC BEH C18 1.7 μmol/L 2.1 x 50 mm column.

**Paclitaxel release studies and stability test**

For paclitaxel release studies, paclitaxel-polymersomes were incubated at 37°C, and after different time points, the sample was centrifuged at 20,000 × g. The supernatant was then used for quantification of paclitaxel by UPLC, as described above. For the short-term stability test of polymersomes and paclitaxel-polymersomes, the vesicles were incubated at 37°C over 24 hours in PBS, or in PBS containing 4.5 mg of BSA (GE Healthcare), and the stability was studied by monitoring size changes by DLS. For the release test of paclitaxel-polymersomes incubated with cells, 10,000 MKN-45P cells or 5,000 CT26 cells were incubated in 96-well plate with DMEM containing FBS, and the stability was studied by monitoring size changes by DLS. For the release test of paclitaxel-polymersomes incubated with cells, 10,000 MKN-45P cells or 5,000 CT26 cells were incubated in 96-well plate with DMEM containing FBS for 24 hours. Paclitaxel-polymersomes were added to the cells at a final concentration of 10 μmol/L and after 24 hours the culture medium was centrifuged at 20,000 × g. The supernatant was mixed with methanol and HCl and analyzed with UPLC for the detection of paclitaxel.

**In vitro cytotoxicity assay**

MKN-45P, CT26, or SKOV-3 cells were plated in a 96-well plate (10,000 MKN-45P or SKOV-3 cells for the 6- and 24-hour time point; 5,000 MKN-45P cells for the 48-hour time point; 5,000 CT26 cells for the 6- and 24-hour time point; and 2,500 CT26 cells or the 48-hour time point) and incubated for 24 hours at 37°C in 5% CO₂ atmosphere. Polymersomes, paclitaxel-polymersomes, Abraxane, or free paclitaxel samples were then added to the wells at a final concentration of 10, 1, 0.5, or 0.25 μmol/L of drug. Empty polymersomes were used at the same copolymer concentration as in the polymersome–paclitaxel sample. For the 6-hour time point, the medium was removed from the wells after 6 hours of incubation at 37°C, and fresh medium was added. After 18 hours of incubation at 37°C, the medium was aspirated and 10 μL of MTT reagent was added at a concentration of 5 mg/mL in PBS. After 2 hours of incubation at 37°C, the solution was aspirated and the absorbance was measured at 570 nm with a microplate reader (Tecan Austria GmbH). Alternatively, for the 24- and 48-hour time point, the cells were incubated for 24 or 48 hours with the samples and the MTT assay was followed as described before.

**Cellular uptake and cargo release experiments**

A total of 50,000 MKN-45P, CT26, or SKOV-3 cells were seeded in a 24-well plate with a coverslip. After 24 hours, polymersomes loaded with Rho or with doxorubicin were added to the cells at a concentration of 0.8 mg/mL and incubated for 24 more hours. The cells were washed with PBS, fixed with paraformaldehyde, stained with DAPI, and observed under fluorescence confocal microscopy (Zeiss LSM 510). The images were analyzed with the ZEN lite 2012 image software.

**In vivo biodistribution studies**

Nude mice were intraperitoneally injected with 10⁶ MKN-45P cells and Balb/c mice received an intraperitoneal injection of 2 × 10⁶ CT26 cells. Alternatively, nude mice were injected intraperitoneally with 10⁶ MKN-45P cells and subcutaneously with 10⁵ MKN-45P cells in the right flank. The MKN-45P tumors were grown for 2 weeks and the CT26 tumors for 1 week. For the ex vivo imaging, FAM-labeled polymersomes were injected intraperitoneally (0.5 mg in 500 μL of PBS) or intravenously (0.5 mg in 100 μL of PBS) and after 24 hours the animals were perfused with 10 mL of PBS. The tumors and organs were excised for fluorescence imaging using Illumatool light source (Lightools Research) and fluorescence quantification by ImageJ software. Tissues were snap-frozen with liquid nitrogen, and kept at −80°C for further analysis.

For the in vivo imaging, the same amount of Rho-labeled polymersomes were injected intraperitoneally into the MKN-45P-bearing mice and the animals were imaged in vivo after 24 hours using the Optix MX3 (Art Advanced Research Technologies Inc.).

**Immunofluorescence and microscopic imaging**

The excised tumor and organs were cryosectioned, fixed with paraformaldehyde, and immunostained with anti-fluorescein rabbit IgG fragment (Life Technologies) and rat anti-mouse CD31 (BD Biosciences) as primary antibodies, and Alexa fluor 488 goat anti-rabbit IgG and Alexa fluor 647 goat anti-mouse IgG (Invitrogen) as secondary antibodies. The nuclei of cells were stained with DAPI. Images of the tissue sections were taken with confocal microscopy (Zeiss LSM 510) and the images were analyzed with the ZEN lite 2012 image software.

**Experimental tumor therapy**

Athymic nude mice were injected intraperitoneally with 10⁶ MKN-45P-luciferase (MKN-45P-luc) cells and after 8 days, the tumor burden was measured in vivo. Luciferin Firefly (Biosynth) was injected intraperitoneally at a concentration of 15 mg/mL in PBS and 10 μL/g of body weight, and the bioluminescence was detected after 10 minutes using the IVIS imaging system.
The animals were distributed in 4 groups with same tumor burden average in each group. The mice were treated every other day with intraperitoneal injections of 1 mL of paclitaxel-Cremophore EL, Abraxane, or paclitaxel-polymersomes at the same drug concentration, or PBS. The total dose of paclitaxel was 7 mg/kg. The tumor burden was monitored every 4 days. After 21 days of tumor induction, the animals were perfused with 10 mL of PBS, and the tumors and organs were excised.

**Results**

**Formation and characterization of polymersomes and paclitaxel-loaded polymersomes**

The formation of POEGMA–PDPA (Supplementary Fig. S1) polymersomes and loading of paclitaxel were carried out by the hydration of a polymer film at neutral pH. Briefly, the copolymer and the paclitaxel were combined to form a film of polymer/drug, followed by hydration to form vesicles. The loading capacity of the polymersomes was found to be 0.5 to 0.9 moles paclitaxel/mol polymer, corresponding to 4–7 × 10⁴ molecules of drug per vesicle (Supplementary Fig. S2B).

TEM was used to characterize the structure of the polymeric vesicles. As shown in Fig. 1, POEGMA–PDPA copolymer alone or in combination with paclitaxel formed vesicles at pH 7.4, whereas no vesicles were formed at pH 6. The preservation of polymersome structure and size after the loading of the drug was studied by measuring the polymersome size by DLS. We found that the hydrodynamic diameter of paclitaxel-polymersomes [225 nm, polydispersity index (PDI) 0.2] was very similar to the empty polymersomes (219 nm with a PDI of 0.19), indicating no significant differences in the structure (Fig. 1A) or size (Fig. 1B).

![Figure 1](image)

**Stability of polymersomes and paclitaxel release**

The stability of polymersomes in aqueous solutions was investigated by monitoring the particle size at different time points after vesicle formation. After incubation of polymersomes in PBS (pH 7.4) at 37°C for 24 hours, there were no significant changes in the average hydrodynamic diameter of the vesicles (Fig. 1B). To evaluate long-term stability of polymersomes, their size was measured after storage at 4°C for 4 months. There was no aggregation of the polymersome suspension, and the mean diameter of the vesicles remained unchanged over time (Fig. 1B), indicating that polymersomes in aqueous solutions remain thermodynamically stable. The release of drug from polymersomes over time was evaluated by incubating the paclitaxel-polymersomes in PBS (pH 7.4) at 37°C, followed by centrifugation to separate polymersomes from free drug, and quantifying paclitaxel in the supernatant using UPLC. After 72 hours of incubation, only 0.7% of drug was released (Supplementary Fig. S2B) and no visible aggregation of the particles was observed. After 4 months at 4°C, about 12% of the drug was released from paclitaxel-polymersomes (Supplementary Fig. S2B). When the empty or paclitaxel-polymersomes were exposed to pH below 6, the colloidal dispersion turned clear, indicating disassembly of the vesicles. The paclitaxel in the solution was quantified by UPLC to estimate the encapsulation efficiency and to confirm the quantitative drug release (Supplementary Fig. S2B and S2C).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Size (nm)</th>
<th>PDI</th>
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<tbody>
<tr>
<td>PS</td>
<td>219</td>
<td>0.19</td>
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<tr>
<td>PS-PTX, 24 h at 37°C</td>
<td>226</td>
<td>0.19</td>
</tr>
<tr>
<td>PS-PTX, 4 months at 4°C</td>
<td>221</td>
<td>0.2</td>
</tr>
<tr>
<td>PS-PTX</td>
<td>225</td>
<td>0.2</td>
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<tr>
<td>PS-PTX, 24 h at 37°C</td>
<td>231</td>
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**Figure 1.** Characterization of empty polymersomes (PS) and paclitaxel (PTX)-loaded polymersomes (PS-PTX). A, TEM images of freshly prepared empty and paclitaxel-polymersomes. Polymersome samples were deposited on copper grids and stained with phosphotungstic acid at pH 7.4. B, DLS determination of hydrodynamic diameter and PDI values of empty and paclitaxel-polymersomes after incubation for indicated time and temperature.
We also evaluated the stability of polymersomes in cell culture medium in the presence of cultured cells. Paclitaxel-polymersomes (10 μmol/L paclitaxel) were incubated with MKN-45P and CT26 cells at 37 °C for 24 hours and the medium was analyzed by UPLC for the presence of free and polymersome-encapsulated paclitaxel. No free paclitaxel was detected in the medium for either cell line (Supplementary Fig. S2D), demonstrating that paclitaxel is not released from the polymersomes prior to cellular uptake.

**In vitro activity of paclitaxel-polymersomes in tumor cell lines**

To assess the suitability of polymersomes for drug delivery to peritoneal cancer, we first studied *in vitro* cellular internalization and subcellular distribution of polymersomes loaded with fluorescent cargoes. We used MKN-45P cells, a human gastric cancer cell line with a high potential for peritoneal dissemination (30), CT26 cells, a mouse colon carcinoma cell line, and SKOV-3 cells, a human ovarian adenocarcinoma. All the cells showed robust uptake of polymersomes loaded with rhodamine B octadecyl ester (Rho) or with doxorubicin as judged by confocal microscopy after 24-hour incubation (Supplementary Fig. S4). Whereas Rho fluorescence remained limited to the cytosol, doxorubicin fluorescence was also observed in the nuclei of the cells. Nuclear accumulation of doxorubicin suggests that polymersome cargo is released from the endosomes, possibly through endosomal membrane disruption by proton sponge effect (26, 27).

We next investigated *in vitro* cytotoxicity on a panel of tumor cell lines by the MTT assay (Fig. 2 and Supplementary Fig. S5A). Treatment of MKN-45P cells with paclitaxel-polymersomes (0.5 μmol/L of paclitaxel, 24 hours of incubation) decreased the cellular viability to about 50%, significantly lower than the viability of cells treated with Abraxane or with free drug (Fig. 2A and B). Likewise, CT26 cells treated with paclitaxel-polymersomes had significantly lower viability than cells treated with Abraxane, free paclitaxel, or nonloaded polymersomes. We tested the toxicity of paclitaxel formulations in MKN-45P and CT26 cells over a range of concentrations (0.25–10 μmol/L of paclitaxel) at different time points (6, 24, and 48 hours). We found paclitaxel-polymersomes to be the most toxic formulation across the paclitaxel concentration range at the 6- and 24-hour time points (Supplementary Fig. S5A). At the 48-hour time point, paclitaxel-polymersomes were significantly more toxic than other formulations at 10 μmol/L of paclitaxel, with less pronounced differences at lower doses. To distinguish cellular toxicity from cytostatic effects, we stained treated cells with trypan blue vital stain (Supplementary Fig. S5B). After 24 hours of treatment, the count of viable MKN-45P and CT26 cells was similar in all groups (Supplementary Fig. S5B). However, paclitaxel-polymersomes were significantly more cytostatic (as judged on the basis of total cell count) than Abraxane or free paclitaxel. The inhibition of cell proliferation observed in our results is in agreement with the results of the MTT assay and the mechanism of action of paclitaxel, known to target tubulin and interfere with the cell division.

In addition to studies on MKN-45P and CT26 cells, we tested the effect of the paclitaxel formulations on the viability of the SKOV-3 ovarian carcinoma cells, and observed a significant decrease in the viability of cells treated with paclitaxel-polymersomes compared with the other paclitaxel formulations (Fig. 2).

**Homing and penetration of POEGMA–PDPA polymersomes in peritoneal carcinomatosis lesions**

To determine the potential of the polymersome platform for *in vivo* targeting of peritoneal carcinomatosis lesions, we used MKN-45P peritoneal xenograft and CT26 syngeneic peritoneal tumor models in mice. Animals bearing disseminated peritoneal tumors were intraperitoneally injected with 0.5 mg of polymersomes labeled with fluorescein (FAM-polymersomes). Macroscopic assessment of resected organs revealed accumulation of fluorescent polymersomes in both MKN-45P (Fig. 3A) and CT26 (Fig. 3B) tumors, after 24 hours of injection. In the MKN-45P model, the fluorescence in the tumor was 3.7-fold higher than in the lungs, 5.3-fold higher than in the kidneys, and 12 times higher than in the liver (Fig. 3C). For the CT26 tumor model, the accumulation of polymersomes in the tumor was about three times higher than in the lungs and liver, and 4.5 times higher than in kidneys (Fig. 3C). In tumors and organs resected from noninjected animals bearing MKN-45P and CT26 tumors, no signal over background was observed (Supplementary Fig. S6).

In addition, we used *in vivo* imaging to study accumulation of polymersomes labeled with rhodamine (Rho-polymersomes) to MKN-45P tumors expressing luciferase (Supplementary Fig. S7). At 24 hours postinjection, the fluorescence of Rho-polymersomes overlapped with the luminescence emitted by the luciferase-expressing tumor.

During macroscopic FAM imaging, intrinsic tissue autofluorescence is mapped along with fluorescein signal and intensity of the FAM fluorescence might be affected by the local microenvironment. To confirm polymersome homing using an alternative assay, we immunostained tissue sections of tumors and control organs with antifluorescein antibody. Confocal imaging of stained tissue sections confirmed a strong signal in tumors and...
Confocal fluorescence microscopy of both CT26 and MKN-45P tumors demonstrated selective accumulation and penetration of polymersomes in the peritoneal lesions (Fig. 3D). Twenty-four hours after intraperitoneal injection of FAM-polymersomes into CT26 tumor-bearing mice, FAM signal was detected both near the surface of the tumors and also deep inside tumor tissue, indicating effective tumor penetration by polymersomes (Fig. 3D). In MKN-45P tumors, polymersomes colocalized with tumor vessels (Fig. 3D, white arrow). In addition, we observed in both MKN-45P and CT26 models polymersomes in areas of the tumors with no detectable CD31-positive blood vessels, in tumor periphery, and deep within the tumor’s parenchyma (Fig. 3D, pink arrow).
This suggests that polymersomes entered into the tumor nodules using dual mode: directly, in a circulation-independent manner, and via a systemic route. To dissect the tumor entry pathways of polymersomes, we injected mice bearing dual peritoneal and subcutaneous MKN-45P tumors with FAM-polymersomes using either intraperitoneal or intravenous route. In subcutaneous tumors, both administration routes resulted in accumulation of polymersomes in the blood vessels and adjacent areas (Supplementary Fig. S9). Peritoneal tumors from mice intravenously injected with polymersomes did not show polymersome accumulation in the periphery and poorly vascularized areas, and showed overall lower accumulation of polymersomes than seen after the intraperitoneal administration (Supplementary Fig. S10). The amount of polymersomes trapped in the liver was higher when polymersomes were administrated systemically compared with intraperitoneal injection (Supplementary Fig. S8).

These observations support that intraperitoneal polymersomes target peritoneal carcinomatosis using both direct penetration and systemic routes leading to more robust delivery than is possible with intravenous route.

Antitumor efficacy of paclitaxel-polymersomes in mice bearing peritoneal MKN-45P gastric tumors

Bioluminescence-based imaging of live mice bearing MKN-45P-luc peritoneal tumors was used to measure tumor burden during experimental therapy. Tumor growth was compared in groups of mice that received intraperitoneal injections of paclitaxel-polymersomes, Abraxane, or paclitaxel solubilized with Cremophor EL (paclitaxel-Cremophor). In all treatment groups, the dose of paclitaxel used was 7 mg/kg.

Twelve days after initiation of the treatment, the luminescence detected from the tumor in mice treated with paclitaxel-polymersomes was more than 12 times lower than in the PBS control group (Fig. 4A), whereas tumor luminescence in the PBS, Abraxane, and paclitaxel-Cremophor groups did not differ significantly.

Analysis of locally disseminated tumor nodules revealed a trend toward a lower number of nodules in the paclitaxel-polymersome treatment group compared with the paclitaxel-Cremophor and Abraxane groups (Fig. 4B). Interestingly, the tumors in the paclitaxel-polymersome group started to shrink after the first day of treatment and also after 10 days of treatment, and continued to do so until day 12, showing that the tumors not only grow more slowly, but also tend to shrink, in contrast to the tumors in other treatment groups.

Discussion

A critical goal in cancer therapy is to increase the tumor selectivity of chemotherapeutics to improve the balance between anticancer activity and side effects of the drug. In the current study, we assessed intraperitoneal administration of drug-loaded pH-sensitive polymersomes as a treatment for peritoneal cancer. We showed that polymersomes allow efficient payload delivery in tumor cells in vitro and in vivo, using mouse models of peritoneal carcinomatosis. Polymersomes are specifically taken up and accumulate in peritoneal tumor lesions, and increase the antitumor activity of a chemotherapeutic drug. Whereas several reports describe applications of polymersomes for anticancer drug delivery to cultured cells, the novelty of this work is the in vivo application of polymersomes for intraperitoneal cancer chemotherapy. We demonstrated here that the pH-sensitive polymersomes loaded with paclitaxel are more effective than Abraxane, a nanoformulation in clinical use, and paclitaxel-Cremophor, at a very low drug dose in the treatment of MKN-45P gastric tumor. We hypothesized that this efficacy is a result of a drug delivery to tumors by both direct (intraperitoneal contact of polymersomes with cells) and indirect (via the vasculature) mechanisms, thus the encapsulated drug has effect on both large vascularized tumors and small avascular nodules. To test suitability of polymersomes for payload delivery to intraperitoneal tumors, we chose to use the intraperitoneal administration route, as the peritoneal barrier maintains a positive gradient of the drug in the peritoneum, thereby increasing the local effects and reducing the systemic toxicity (1). Moreover, access of systemic drugs to nonvascularized tumor foci, often present in the peritoneal carcinomatosis, is limited in the case of systemic therapy (36). Intraperitoneally administered FAM-labeled POEGMA–FDPA polymersomes showed robust accumulation and penetration in malignant lesions in both MKN-45P peritoneal xenografts and CT26 syngeneic peritoneal tumors in mice, and only a background signal in the control organs. In contrast, when polymersomes were administered systemically, their accumulation was lower in peritoneal tumors.
Polymersomes for Peritoneal Carcinomatosis Treatment

Tumors and higher in normal organs. Microscopic analysis of the distribution of polymersomes in peritoneal and subcutaneous tumors suggested that polymersomes target peritoneal tumors through a combination of direct local penetration and indirect circulation-mediated homing. Such dual targeting is important, as a number of clinical studies indicate that combination of intravenous and intraperitoneal chemotherapy for peritoneal carcinomatosis results in better outcome (decreased rate of metastasis and decreased peritoneal recurrence) than either therapy alone (37). Paclitaxel is very poorly absorbed into systemic circulation when administered by the intraperitoneal route, with peak levels in the peritoneal cavity of 1,000-fold higher than in the plasma (38), necessitating supplementation of intraperitoneal paclitaxel with systemic chemotherapies. Our data suggest that formulation of paclitaxel in polymersomes overcomes this limitation. In a previous reported work, doxorubicin-loaded PMPC–poly(2-(methacryloyloxy)ethyl phosphorylcholine)–PDPA vesicles showed rapid cellular uptake and cytotoxicity in melanoma cells, but not in normal human dermal fibroblasts (12). In another in vitro study, cultured head and neck cancer spheroids, PMPC-PDPA polymersomes loaded with paclitaxel and/or doxorubicin were found to penetrate into the spheroids and to trigger extensive cell death (13). In the latter study, an interaction between the PMPC block displayed on the polymersome surface and the scavenger receptor of tumor cells was described. However, in the current work, we used pH-sensitive polymersomes made by the copolymer POEGMA–PDPA, that lack phosphorylcholine groups in the PMPC chains involved in high affinity interactions with class B scavenger receptors. It is possible that tumor tropism of polymersomes may be further improved by active targeting, conjugation of polymersomes to ligands with affinity to malignant cells and cells in tumor stroma. We have previously shown that tumor homing of Abraxane nanoparticles that possess intrinsic tumor selectivity can be further improved by functionalization with tumor homing and penetrating peptides (39, 40). The unique flexibility of polymersomes that allows them to pass through pores much smaller than their size is likely to contribute to enhanced tissue penetration (17). Indeed, the enhanced penetration of polymersomes has been demonstrated in vitro on human mucosa (41), human skin (17), and tumor spheroids (12), and also in vivo (42).

As a drug for experimental therapy with polymersomes we used paclitaxel, a poorly water-soluble potent anticancer agent known to be rendered more soluble and bioactive by incorporation in nanoparticles (43). Our paclitaxel-polymersomes were more efficient than Abraxane in inhibiting MKN-45P and CT26 cell growth, suggesting that tumor cell uptake and/or cytoplasmic release of cytotoxic cargo is more efficient for polymersomes compared with albumin-paclitaxel nanoparticles. When paclitaxel-polymersomes were used for experimental therapy of mice bearing intraperitoneal MKN-45P gastric tumors, they showed potent antitumor activity, superior to Abraxane or paclitaxel-Cremophor. It has been reported that in the treatment of MKN-45P mice with intraperitoneally administered NK105, a paclitaxel-incorporating micellar nanoparticle formulation (8), or a copolymer conjugated with paclitaxel (10), the weight of peritoneal tumor nodules was approximately 5 times lower than in mice treated with paclitaxel-Cremophor. In another study of intraperitoneal treatment of ovarian tumors, dendrimer-based nanoparticles loaded with paclitaxel reduced the growth of tumors about 5-fold more than Abraxane (7). In these studies, the total paclitaxel dose used was between 20 and 40 mg/kg. In our case, tumor burden of the mice treated with paclitaxel-polymersomes was 8 times lower than in paclitaxel-Cremophor group and 7 times lower compared with Abraxane. Remarkably, the total dose of paclitaxel we used in our treatment was only 7 mg/kg, the lowest reported paclitaxel dose used in experimental tumor treatment with free paclitaxel or Abraxane (7, 8, 10, 44–46). The rationale for using the paclitaxel dose below the MTD, with which the paclitaxel-polymersomes are still effective, was to demonstrate that it is possible to reduce the paclitaxel side effects while preserving therapeutic antitumor activity. The high antitumor activity of intraperitoneally administered paclitaxel-polymersomes may be due to a combination of factors. On a biodistribution level, intrinsic tumor tropism of polymersomes in combination with dual targeting (direct and circulation-mediated) and ability to pass through narrow pores may allow more extensive tumor accumulation than is possible with other formulations. Loading of a drug in pH-sensitive polymersomes may improve its pharmacokinetics, with bioactive drug being released exclusively at endosomal low pH inside the tumor cell.

In summary, pH-sensitive POEGMA-PDPA polymersomes loaded with paclitaxel internalized and released the drug into cultured malignant cells in a more efficient manner than other paclitaxel formulations. In mouse models of peritoneal carcinomatosis in vivo, intraperitoneally administered polymersomes specifically targeted and penetrated into vascularized and nonvascularized peritoneal tumors, and paclitaxel-loaded polymersomes exhibited higher antitumor efficacy than Abraxane or free paclitaxel. These observations warrant future preclinical and clinical research aimed at the development of polymersome-based drug delivery systems for the treatment of peritoneal carcinomatosis.

Disclosure of Potential Conflicts of Interest

E. Ruoslahti has ownership interest (including patents) in DrugCendR LLC. T. Teesalu has ownership interest (including patents) in DrugCendR Inc. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

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