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ROS-responsive PEGylated ferrocene polymer nanoparticles with improved stability for tumor-selective chemotherapy and imaging

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ABSTRACT

Ferrocene-based nanoparticles have garnered interest as reactive oxygen species (ROS)-responsive nanocarriers of anticancer drugs and imaging agents. However, their biomedical applications remain limited due to their poor physiological stability. PEGylation of nanocarriers improves their stability and biocompatibility. In this study, we aimed to develop novel PEG-ferrocene nanoparticles (PFNPs) with enhanced stability and ROS responsiveness for the delivery of paclitaxel (PTX) and imaging agents. PEGylation improved the stability of ferrocene nanoparticles, inhibiting their ROS-responsive destruction. Several PEG-ferrocene polymers containing different molar ratios of methacrylic acid and poly (ethylene glycol) methyl ether methacrylate was designed for optimization. ROS-responsive polymers with optimal monomer ratios were self-assembled into PFNPs with enhanced stability. The PFNPs distended, effectively releasing encapsulated PTX and imaging agents within 8 h in the presence of ROS. Furthermore, they remained stable, with no changes in their hydrodynamic diameters or polydispersity indexes after storage in an aqueous solution and biological buffer. The accumulation of PFNPs in a tumor model *in vivo* was 15-fold higher than a free dye. PTX-loaded PFNPs showed a substantial tumor-suppression effect, reducing tumor size to approximately 18% of that in the corresponding control group. These findings suggest a promising application of ROS-responsive PFNPs in tumor treatment as biocompatible nanocarriers of anticancer drugs and imaging agents.

1. Introduction

Cancer has attracted considerable research interest as one of the leading causes of death [1]. Researchers have explored various treatment methods to cure this life-threatening disease. Chemotherapy is a general strategy for cancer treatment, but its application remains limited due to the inherent limitations of anticancer drugs [2]. Drug delivery to target sites must be controlled to ensure therapeutic efficacy and prevent adverse effects on cells near tumor sites [3]. Nanotechnology for cancer therapy and diagnosis has achieved great development over the past few

decades along with a growing market of nanomedicines, and it is expected to be more personalized with an application of artificial intelligence [4]. Accordingly, different forms of nanocarriers have been developed for therapeutic and diagnostic delivery, including liposomes, dendritic polymer, micelles, inorganic nanoparticles, and quantum dots [5–7]]. Polymeric nanocarriers are simple, easy to prepare, biocompatible, and beneficial for drug solubilization [8]. They can also accumulate passively at tumor sites due to their enhanced permeability and retention (EPR) effect [9]. Furthermore, stimulus-responsive polymers sensitive to the tumor microenvironment have been designed to stop

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uncontrolled drug release during blood circulation and prevent toxicity against healthy cells [10]. A stimuli-responsive branched polymeric prodrug with imaging agents and a therapeutic agent have been developed for efficient imaging-guided antitumor therapy [11]. Selective drug release in the tumor cells achieved targeted elimination of 4T1 murine breast cancer *in vivo*, while healthy tissues remain safe.

Stimulus-responsive polymeric nanocarriers target unique tumor features, which normal tissues lack, including acidic pH, enzymes, proteins, and excessive reactive oxygen species (ROS) [12,13]. ROS are highly oxidative species derived from aerobic metabolism and include superoxide (O_2^-) , hydroxyl radicals (·OH), hydrogen peroxide (H_2O_2) , and singlet oxygen (102) [14]. The amount of ROS generated in inflammatory and tumor sites is approximately 100-fold higher than in normal tissues [15]. Therefore, elevated ROS levels in tumors are a good indicator of tumor-targeted drug delivery sites. As ROS homeostasis in tumor tissues is imbalanced and highly susceptible to overexpression, ROS-responsive drug delivery and ROS upregulation have been discussed for enhanced cancer therapy [16]. Functional groups such as boron, chalcogen, selenium, thioether, and ferrocene are important for the development of ROS-responsive nanocarriers [17,18]. The H₂O₂-responsive drug delivery and targeted anticancer efficacy have been achieved by polymers containing thioether moieties that switch from hydrophobic to hydrophilic states via oxidation [19]. Zhang et al. designed a phenylboronic ester-linked PEG-lipid conjugate for H₂O₂-responsive drug delivery [20]. Poly (propylene sulfide)-containing block copolymers were self-assembled into a ROS-responsive nanocarrier based on the hydrophobic-to-hydrophilic transition of the thioether groups [21].

Ferrocene is a useful ROS-responsive moiety with reversible redox activity and stability. Copolymerization of hydrophobic ferrocene with hydrophilic monomers generates amphiphilic block copolymers. The polymers can self-assemble into nanocarriers and disassemble upon oxidation following conversion from a hydrophobic state to a hydrophilic state. ROS-responsive nanocarriers containing hydrophobic ferrocene blocks and hydrophilic carboxyl groups have been developed for the controlled release of loaded drugs [22]. However, limitations such as low polymer yield, poor aqueous solubility, and uncontrollable physicochemical properties must be addressed to improve drug loading and enhance therapeutic activity. The advantage of block copolymers is the ease with which the properties of nanoparticles can be optimized by controlling the block composition and length [23]. In this study, ferrocene polymers were copolymerized with poly (ethylene glycol) (PEG) groups. PEG can provide stealth properties to nanoparticles due to its biocompatible and flexible nature [24]. Wang et al. discovered that the length of the PEG surface is an important factor that determines the properties of PEG-block-poly (ɛ-caprolactone) copolymers [25]. The circulatory time in the blood, uptake by tumor cells, and anticancer activity of the nanoparticles depend on the polymer length and density. However, enhanced stability can delay drug release; therefore, it is necessary to strike a compromise between stability and ROS responsiveness when developing nanoparticles [26].

In this study, we developed novel ROS-responsive PEG-ferrocene nanoparticles (PFNPs) using a series of amphiphilic PEGylated ferrocene polymers to control the stability and ROS-responsiveness of ferrocene. Their ROS responsiveness and dispersion stability were compared to determine the most appropriate nanocarriers for loading paclitaxel (PTX) and imaging agents. Drug release profiles were obtained based on their ROS sensitivity. Furthermore, the anticancer activities of free and encapsulated PTX were evaluated in murine normal fibroblast (NIH 3T3) and squamous cell carcinoma-7 (SCC7) cells. Finally, an *in vivo* study was performed to analyze the imaging effect and therapeutic efficacy of drug loaded PFNPs for cancer treatment.

2. Experimental section/methods

2.1. Reagents

Ferrocenylmethyl methacrylate (FMMA; 95%), methacrylic acid (MA; 99%), PEG methyl ether methacrylate (PEGMA; $M_n = 500$ g/mol), Nile Red (NR), tetrahydrofuran (THF, anhydrous, 99.9%), and N-acetyl-L-cysteine (NAC) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Hydrogen peroxide (30%) and 2,2-azobisisobutyronitrile (AIBN, 99%) were obtained from Junsei Chemical Co. (Tokyo, Japan) and Daejung (Seoul, Korea), respectively. PTX was obtained from the loading control (LC) Laboratories (Woburn, MA, USA). deionized water (DIW) and phosphate-buffered saline (PBS) were obtained from HyClone (Logan, UT, USA). Acetonitrile (ACN, High Performance Liquid Chromatography (HPLC) grade) was obtained from Honeywell (Charlotte, NC, USA). The fluorescent ROS indicator 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) was obtained from Invitrogen (Carlsbad, CA, USA). Solid DiIC18 (5) (DiD) and Hoechst 33342 were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Paraformaldehyde (4%) and formalin (10%) were obtained from Biosesang (Seongnam, Gyeonggi-do, Korea). The optimal cutting temperature (OCT) compound was obtained from Scigen Scientific Inc. (Gardena, CA, USA).

2.2. Synthesis and characterization of PEG-ferrocene polymers

Radical polymerization has been used to synthesize PEG-ferrocene polymers [22]. Before polymerization, MA and PEGMA monomers were passed over an inhibitor-removal column for 1 h. FMMA, MA, and PEGMA were dissolved in 2 mL of THF. Although the molar concentration of FMMA was fixed at 0.4 mM, that of MA and PEGMA varied from 0 to 2 mM. After the addition of AIBN (0.12 mM) as a radical initiator, the mixture was degassed by bubbling Ar gas for 5 min and then sealed. The polymerization reaction was conducted for 24 h at 70 °C with continuous stirring. Furthermore, the resulting product was cooled to 25 °C, purified via precipitation in hexane, dried, and then stored at 4 °C until use. The resulting PEG-ferrocene polymers were denoted as Poly-PC-1, Poly-PC-2, Poly-PC-3, and Poly-PC-4 based on the initial molar ratios of MA and PEGMA (MA:PEGMA = 2:0, 1.5:0.5, 1:1, and 0:2). Their physicochemical properties of polymers were analyzed at 25 °C using ¹H nuclear magnetic resonance (¹H NMR) (400 MHz, JNM-ECZ400S/L1, JEOL, Tokyo, Japan), with dimethyl sulfoxide $(DMSO-d_6)$ as the deuterated solvent. The weight average molecular mass (M_w; g/mol) and the molecular polydispersity index (PDI) (M_w/M_n) of the copolymers were analyzed using gel permeation chromatography (GPC) (1200S/miniDAWN TREOS, Agilent, CA, USA) in THF (flow rate of 1 mL/min, 35 °C).

2.3. Preparation and characterization of PEG-ferrocene nanoparticles

A simple nanoprecipitation method was used to prepare PFNPs, as previously reported [27]. Briefly, the PEG-ferrocene polymer solution (5 mg/mL) was added dropwise to 5 mL of DIW using a 30-G syringe. After 5 min of magnetic stirring at 530 rpm, the mixture was vacuum dried for 2 h to remove the organic solvent THF. The developed PFNPs were denoted as PFNP1, PFNP2, PFNP3, and PFNP4, based on the PEG-ferrocene polymers used for the preparation. Their hydrodynamic diameter, PDI, and zeta potential were analyzed using DLS (Zetasizer, ELSZ-2000 series; Otsuka Electronics Co., Ltd., Osaka, Japan).

Fifty microliters of H_2O_2 (0.4 M) were added to 1 mL of PFNP solution (1 mg/mL) to determine the ROS-responsiveness of PFNPs. After over 24 h of reaction with an oxidizing agent, changes in hydrodynamic diameter, PDI, and zeta potential of PFNPs were monitored at different time points (0, 2, 4, 8, and 24 h) using DLS analysis. The stability of each PFNP was evaluated under different conditions. First, the PFNP solutions were lyophilized for 3 days and then dispersed into DIW and PBS. The size and PDI of the PFNPs before and after lyophilization were analyzed

using DLS. The PFNPs were then stored at 37 °C and 100 rpm for 4 weeks, 5 days, and 72 h in DIW, PBS, and serum-containing PBS, respectively. Changes in these characteristics and the appearance of partial aggregation were monitored at each predetermined time point.

2.4. Preparation and characterization of PTX- and NR-loaded PFNP3

Amphiphilic polymers can load hydrophobic cargos when they selfassemble into micelles including an internal hydrophobic core and an external hydrophilic shell, above a certain concentration, called critical micelle concentration (CMC). The CMC of Poly-PC-3 was determined prior to PTX and NR loading using DLS (ZEN3600, Malvern Instruments, Malvern, UK) as previously described [28]. Initially, a series of diluted polymer solutions ranging from 4.5×10^{-10} to 2.7×10^{-6} mol/L was prepared in an aqueous solution. Then, DLS analysis was carried out in a polystyrene cell at 25 °C. The intensity of scattered light was detected at an optimized angle of 173° for high sensitivity and quality of detection. PTX was encapsulated in PFNP3 using the nanoprecipitation method. Briefly, different quantities of PTX (0, 100, 250, and 500 µg) were reacted with 1 mL of Poly-PC-3 (5 mg/mL) for 1 h under rotatory shaking. The mixture was then slowly added to 5 mL of DIW and stirred at 530 rpm for 5 min. After removing THF via vacuum drying, the PTX-loaded PFNP3 (PTX@PFNPs) was lyophilized for 3 days and stored at 4 °C before use. Although the theoretical loading contents of PTX were 0, 2, 5, and 10 wt%, DLS analysis was performed to determine the LC of PFNP3 with no changes in its characteristics.

Given that 2 wt% PTX is the optimal loading content of PFNP, PTX (LC = 2 wt%) and NR (LC = 0.1 wt%) were loaded into PFNPs using the abovementioned nanoprecipitation method. The as-prepared PTX- and NR-loaded PFNP3 (PTX/NR@PFNPs) were purified via spin-filtration for 10 min under 2000 rpm using the Amicon Ultra-15 centrifugal filter (molecular weight cutoff, 100 kDa; Merck Millipore, Billerica, MA, USA). The filtrate containing unloaded PTX and NR was analyzed using HPLC (Waters 2695; Waters Corp., Milford, MO, USA) and a microplate reader (SpectraMax iD3; Molecular Devices, CA, USA) to determine the LC and loading efficiency (LE) of PTX/NR@PFNPs. A C18 column HPLC $(5 \,\mu m, 4.6 \,mm \times 150 \,mm; SunFire \ensuremath{\mathbb{R}}$ C18 column; Waters, MO, USA) was performed to measure the quantity of unloaded PTX in 10 µL of filtrate using the mobile phase of 70% ACN for 20 min at a flow rate of 1 mL/ min. PTX was detected at a wavelength of 228 nm. The volume of unloaded NR was measured using a microplate reader at excitation and emission wavelengths of 530 and 635 nm, respectively. Equations (1) and (2) were used to calculate LC and LE of PTX/NR@PFNPs, as described previously [29].

$$LC (\%) = \frac{weight of fed drug - weight of unloaded drug}{weight of PFNP} \times 100$$
(1)

$$LE (\%) = \frac{weight of fed drug - weight of unloaded drug}{weight of fed drug} \times 100$$
(2)

The morphology of PTX/NR@PFNPs was observed using transmission electron microscopy (TEM) (JEM-2100Plus HR; JEOL, Tokyo, Japan) at an acceleration voltage of 200 kV. The copper grid for the analysis was prepared by adding two drops of 10 μ L of NP solution (1 mg/mL) and drying at 25 °C for 3 days. The size distribution, ROS responsiveness and stability of PTX/NR@PFNP were evaluated using DLS analysis as described above. Stability was analyzed in serum-containing PBS for 7 days at 37 °C. To further investigate the ROS-responsive behavior of PTX/NR@PFNP, changes in its morphology were observed using TEM after 8 h and 24 h of H₂O₂ treatment.

2.5. ROS-responsive PTX release from PTX/NR@PFNPs

To evaluate the ROS-responsive release of PTX from PFNPs, a Float-A-Lyzer G2 dialysis device (MWCO = 100 kDa, Spectrapro/Pro dialysis membrane; Repligen, MA, USA) containing nanoparticles (2.5 mg/mL) was incubated in 10 mL of PBS with or without 0.05, 0.1, 0.2, 0.4 M H_2O_2 at 37 °C for 24 h at 100 rpm. The release buffer was collected and replaced with a fresh medium at predetermined time points (20 min and 2, 4, 6, 8, and 24 h). A cumulative PTX-release profile was generated by measuring PTX concentrations using HPLC following the protocol described above.

2.6. Cell culture

NIH 3T3 and SCC7 cells from the Korean Cell Line Bank (Seoul, Korea) were cultured in Dulbecco's modified Eagle medium (DMEM) and Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Grand Island, NY, USA), respectively. Both cell culture media were supplemented with fetal bovine serum (FBS; Gibco) and an antibiotic-antimycotic mixture (AA; Thermo Fisher Scientific, Waltham, MA, USA). Cells were incubated under humidified conditions in a 5% CO_2 atmosphere at 37 °C.

2.7. In vitro cytotoxicity and anticancer activity of PTX/NR@PFNPs

NIH 3T3 cells were seeded in a 96-well plate (10,000 cells/well) to evaluate the cytotoxicity of PFNP3. Cell viability was measured using Cell Counting Kit-8 (CCK-8) solution (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions following treatment with 0–200 μ g/mL PFNP3 for 1 day. Cell viability was calculated using equation (3).

$$Cell \ viability \ (\%) = \left(\frac{\Delta A_{450} \ of \ sample}{\Delta A_{450} \ of \ control}\right) \times 100$$
(3)

SCC7 cancer cells were cultured in a 96-well plate at a density of 5000 cells/well. Cell viability was analyzed using the CCK-8 assay following treatment with bare PFNPs, PTX, and PTX/NR@PFNPs to evaluate their anticancer activity. The anticancer activity of these compounds was also evaluated in NIH 3T3 cells for comparison. Differences in ROS levels were determined using H₂DCFDA (10 μ M), which changes to a green fluorophore, DCF, in the presence of ROS. The fluorescence intensity was measured using a microplate reader, with excitation and emission wavelengths of 485 and 535 nm, respectively. Furthermore, SCC7 cells were treated with exogenous H₂O₂ (10 μ M) or an ROS inhibitor, NAC, to determine the ROS-responsive cytotoxicity of PTX/NR@PFNPs [30].

2.8. In vitro cellular uptake of PTX/NR@PFNPs

SCC7 tumor cells were seeded in a confocal dish at a density of 7 \times 10⁵ cells/dish. After incubation at 37 °C for 40 h, the cells were treated with NR@PFNPs (NR concentration = 2.5 µg/mL) and incubated for 1, 3, or 6 h. The cells were then counterstained with 10 µg/mL Hoechst 33342 for 15 min, washed several times with Dulbecco's PBS (DPBS), fixed with 4% paraformaldehyde at 25 °C, and washed again. Finally, images of NR and 4',6-diamidino-2-phenylindole (DAPI) fluorescence were obtained using a confocal laser scanning microscope (LSM800, Carl Zeiss). ImageJ software was used to quantify the fluorescence signals.

2.9. Animal and tumor model

All mouse experiments in this study were performed following the Laboratory Animal Care guidelines approved by an appropriate ethics committee (approval no. 2020–0359-05). SCC7 cells (2×10^6) were subcutaneously injected around the left femoral region of 6-week-old male *C3H/HeN* mice (Orient Bio, Inc., Republic of Korea) to establish a tumor mouse model.

2.10. In vivo and ex vivo imaging of PTX/DiD@PFNPs

When the tumors reached a size of approximately 250 mm³, free DiD or DiD@PFNP solution (2 mg/kg DiD in 100 μ L saline) was injected via the tail vein. Mice were anesthetized via inhalation of isoflurane 1, 3, 6, 12, or 24 h after injection. Whole-body and blood images were captured using an optical *in vivo* Imaging System-IVIS Lumina XRMS (IVIS, Per-kinElmer, Norwalk, CT, USA) with a DiD filter (Ex/Em = 660/710 nm) at each time point. The mice were euthanized after 24 h of observation, and the tumors and major organs (heart, lungs, liver, spleen, and kidneys) were excised. IVIS was used to obtain *ex vivo* fluorescence images. The excised tumors were fixed with 4% paraformaldehyde solution and

then frozen at -80 °C in a cryo-mold containing OCT compound. Furthermore, samples were cut into 10-µm-thick slices using a Cryocut Microtome (CM1850, Leica, Wetzlar, Germany) and stained for 10 min with Hoechst 33342. The fluorescence of the sliced tissues was observed using a Cy5.5 filter on an Olympus IX71 fluorescence inverted microscope (Olympus, Tokyo, Japan).

2.11. In vivo anticancer activity of PTX/DiD@PFNPs

The animal model was prepared as described in the imaging experiments. When the tumor reached a size of approximately 50–100 mm³, the mice were divided into three groups and treated with saline, free



Fig. 1. (a) Schema of the preparation of PFNPs with PEG-ferrocene polymers (Poly-PC-1, Poly-PC-2, Poly-PC-3, and Poly-PC-4) composed of different molar ratios of MA and PEGMA. (b) ¹H NMR spectra of PEG-ferrocene polymers. The peaks of ferrocene, MA, and PEG represent their actual quantities in PEG-ferrocene polymers. PEG, poly (ethylene glycol); PFNP, PEG-ferrocene nanoparticle; MA, methacrylic acid; PEGMA, poly(ethylene glycol) methyl ether methacrylate.

PTX, or PTX/DiD@PFNP (n = 5, PTX dosage of 2 mg/kg). All treatment agents were injected intravenously through the tail vein every 2 days (three times). Body weight and tumor size were measured every two days during the experimental period. Tumor volume was calculated using equation (4).

hepatotoxicity and nephrotoxicity, which were estimated by measuring alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), blood urea nitrogen (BUN), and creatinine (CRE) using DRI-CHEM NX 500 (Fujifilm Corporation, Tokyo, Japan).

2.12. Statistical analysis

$$Tumor volume = \frac{(longest tumor length) \times (shortest tumor length)^2}{2}$$
(4)

Mice were sacrificed 2 weeks after the initial injection. For histological analysis, the major organs and tumors were dissected and fixed in 10% formalin solution. They were then sectioned in paraffin, stained with Hematoxylin and eosin (H&E), and examined under an Axio Imager A1 optical microscope (Zeiss, Jena, Germany). Blood samples (500 μ L) were collected from the abdominal vena cava at 24 h after intravenous injection of NP. Plasma was isolated from the blood samples to evaluate

Each experiment was repeated three times (n = 3). The results are presented as mean \pm standard deviation. Differences between groups were analyzed using Student's *t*-test and one-way analysis of variance. Statistical significance was established at P < 0.05.



Fig. 2. Characterization of PFNPs. (a) Hydrodynamic diameter, (b) PDI, and (c) zeta potential of PFNPs. ROS responsiveness of PFNPs. (d) Hydrodynamic diameter, (e) PDI, and (f) zeta potential of PFNPs following oxidation. Analysis of PFNP stability. Changes in the hydrodynamic diameter of PFNPs after (g) lyophilization (FD), (h) 4 weeks in aqueous solution, and (i) 72 h in serum-containing PBS. Changes in the PDI of PFNPs after (j) lyophilization, (k) 4 weeks in aqueous solution, and (l) 72 h in serum-containing PBS. Changes in the PDI of PFNPs after (j) lyophilization, (k) 4 weeks in aqueous solution, and (l) 72 h in serum-containing PBS. Changes in the PDI of PFNPs after (j) lyophilization, (k) 4 weeks in aqueous solution, and (l) 72 h in serum-containing PBS. Partial aggregation of the nanoparticles is indicated by the symbol \blacktriangle . N/D represents the non-detectable measurement using DLS analysis. n = 3. PFNP, PEG-ferrocene nanoparticle; ROS, reactive oxygen species; PDI, polydispersity index; PBS, phosphate-buffered saline.

3. Results and discussion

3.1. Synthesis and characterization of PEG-ferrocene polymers

Ferrocene-conjugated block copolymers have been receiving increasing attention for biomedical applications due to their advantages in ROS-responsive drug delivery. Optimizing their structure and relative block lengths facilitates the construction of nanoparticles with desirable properties [31]. We synthesized four PEG-ferrocene polymers with different monomer ratios through random radical copolymerization (Fig. 1a). The ¹H NMR spectra indicated the conversion of the monomers MA and PEGMA to the designated polymer (Fig. 1b). Although the intensities of the monomer peaks at 5.38 and 6.21 ppm were negligible, the polymer peaks representing the protons of the MA, FMMA, and PEGMA blocks were observable at 12.3, 4.1, and 3.5 ppm, respectively, as previously reported [32,33]. Based on the initial molar ratios of the monomers, the NMR signals at the FMMA peak were similar in all spectra. The peak intensity of MA was the highest in Poly-PC-1 and gradually decreased in Poly-PC-2 and Poly-PC-3. In contrast, the peak intensity of PEGMA was the highest in Poly-PC-4 and decreased in Poly-PC-3 and Poly-PC-2, indicating that the initial monomer concentration determines the composition of PEG-ferrocene polymers. The molecular weight distribution of as-synthesized polymers was analyzed using GPC, as summarized in Table S1. The values of both M_n and M_w increased as the molar ratio of long-chain PEG increased. The ratio of M_w to M_n (PDI) of the polymers was approximately 2, indicating that the molecular weight distribution was acceptable.

3.2. Preparation and characterization of PFNPs

Amphiphilic PFNPs containing a hydrophobic ferrocene core and hydrophilic PEG shell were prepared by facile nanoprecipitation with four PEG-ferrocene polymers. When a polymeric solution in acetone was slowly dropped into an aqueous solution, polymer precipitates were formed in the form of nanoparticles as hydrophobic ferrocene tries to avoid water molecules [34]. The nanoparticles retained their stability, even after the removal of the organic phase, owing to their hydrophilic PEG chains. The average hydrodynamic diameter of the PFNPs was approximately 150 nm (Fig. 2a). Their uniform size distribution was confirmed using PDI to be below 0.3 (Fig. 2b). Nanoparticles with higher PEG ratios had higher PDI values due to the influence of PEG flexibility and folding on PDI of nanoparticles, as explained by Cruz et al. [35] Furthermore, their zeta potential became less negative due to the decreased number of MA segments with carboxyl groups (Fig. 2c). This finding indicates that the characteristics of PFNPs depend on the PEG-ferrocene polymers and their composition.

The ROS responsiveness and stability of the PFNPs were compared to optimize the nanoparticle for further experiments. In the presence of H₂O₂, the hydrophobic-to-hydrophilic transition of ferrocene triggered the destruction and subsequent partial aggregation of the ROSresponsive PFNPs, denoted by the symbol ▲. As shown in Fig. 2d and e, the size and PDI of PFNPs increased following treatment with H₂O₂, followed by non-detectable results in DLS (N/D). PFNP1 and PFNP2 were disassembled in 2 h, whereas the destruction of PFNPs with higher PEG ratios took longer time. Similarly, the decrease in surface charges to a neutral charge occurred faster in PFNPs with lower PEG ratios due to the transition of ferrocene transition to hydrophilic ferrocenium ions (Fe³⁺), suggesting that the PEG moieties make PFNPs less sensitive to ROS (Fig. 2f). In contrast, the stability of PFNPs was enhanced by PEGylation under various conditions (Fig. 2g-2l and Fig. S1). First, PFNPs containing PEGMA could be stored in a powder form and readily dispersed in both DIW and PBS without a change in their physical properties, including hydrodynamic diameter and PDI. As expected, PFNP3 and PFNP4 were more stable than those with lower PEG ratios, thus maintaining their characteristics with negligible changes in both size and PDI. Therefore, PFNP3 was identified as a potent candidate for

tumor-targeted delivery of anticancer drugs and imaging agents due to its considerable ROS responsiveness and excellent stability.

3.3. Drug loading and release profile of PTX/NR@PFNPs

The intensity of scattered light was measured as a function of the concentration of polymers to determine the CMC of Poly-PC-3 polymers (Fig. S2). At polymer concentrations below the CMC, the light intensity was almost constant. However, it began to increase when the polymer concentration approached the CMC, indicating that the number of micelles in the solution is considerably high. Therefore, the CMC of Poly-PC-3 was determined to be 0.063 \times 10⁻⁶ mol/L. These values are crucial for determining the concentration of block copolymers for the preparation of polymeric nanoparticles. Accordingly, the micelle sizes of polymer were measurable at concentrations above their CMC. PTX and NR were selected as hydrophobic model drugs and imaging agents, respectively. PFNP3 was used to encapsulate different quantities of PTX to determine its LC without considerable changes in its characteristics after drug loading. As shown in Fig. 3a and b, both diameter and PDI of PFNP3 increased significantly after loading 10 wt% PTX. The zeta potential of PFNP3 was not affected by PTX loading (Fig. 3c). Furthermore, PFNP3 containing over 5 wt% PTX presented poor lyophilization stability (Fig. 3d and e), indicating that PFNP3 could carry up to 2 wt% PTX without considerable changes in its properties. Therefore, PFNP3 containing 2 wt% PTX and 0.1 wt% NR was used for tumor chemotherapy and imaging. Based on HPLC results, the actual quantities of PTX and NR loaded into PFNP3 were 1.8 wt% and 0.09 wt%, respectively. This finding indicates that PFNP3 is a good nanocarrier, with a high LE of 92%. The average size of the as-developed PTX/NR@PFNPs was 116 nm, with a small size distribution (Fig. 3f). The TEM images in Fig. 3f show uniformly sized spherical nanoparticles. Similarly, to bare PFNPs, the drug-loaded nanoparticles were stable under normal physiological conditions and collapsed upon H_2O_2 treatment. The diameter and zeta potential of PTX/NR@PFNPs increased noticeably within 8 h of H2O2 treatment (Fig. 3g), indicating its ROS-responsive properties. Accordingly, destruction of nanoparticles was observed by TEM after 8 h and 24 h of H₂O₂ treatment as shown in Fig. S3. In contrast, the nanoparticles remained stable in the absence of H₂O₂. Therefore, the diameter and PDI remained constant during 7 days of storage in a biological buffer (Fig. 3h). As PFNP3 responded to ROS faster than PFNP4, their PTX-release profiles were compared (Fig. 3i). PFNP3 and PFNP4 released only 30% and 25% of PTX, respectively, in PBS without H₂O₂ within 24 h. However, a 2.5-fold higher release of PTX was observed with PFNP3 under ROS conditions. This result can be explained by the loss of π - π stacking interactions between ferrocenes, which results in a larger diffusion space in the nanoparticles and the release of encapsulated drugs [36,37]. PFNP4 treated with H₂O₂ also released a higher quantity of PTX than PFNP4 not treated with H₂O₂, although the difference between them was smaller than that observed for PFNP3 due to its weaker ROS responsiveness. This finding suggests that PFNP3 is more appropriate for drug delivery, particularly to ROS-rich tumor sites. To further investigate the ROS-responsive releases behavior of PFNP3, the drug release study was carried out at 3 different concentrations of H₂O₂. As expected, PTX release was faster in a biological buffer with a higher concentration of H2O2 (Fig. S4). While only 31.6% of PTX was released from PFNP3 in the absence of H₂O₂ within 24 h, respectively 42.3%, 48.3%, and 63.3% of PTX was released in a biological buffer with 0.05, 0.1, and 0.2 M H₂O₂. These findings correlate with previous results that drug release is dependent on ROS-responsive behavior of PFNP3.

3.4. In vitro cytotoxicity, anticancer efficacy, and intracellular uptake in SCC7 cells

The stability of the nanoparticles in a serum-containing culture medium was analyzed prior to *in vitro* assays. As shown in Fig. S5, we determined that PTX/NR@PFNP is stable for up to 7 days in a serum-



Fig. 3. Characterization of PFNP3 containing different quantities of PTX. (a) Hydrodynamic diameter, (b) PDI, and (c) zeta potential of PTX@PFNPs with different loading contents. Lyophilization stability of PFNP3 containing different amounts of PTX. (d) Hydrodynamic diameter and (e) PDI of PTX@PFNPs before and after lyophilization (FD). Characterization of PTX/NR@PFNPs. (f) TEM image and size distribution graph of PTX/NR@PFNPs. Scale bar, 50 nm. (g) ROS responsiveness of PTX/NR@PFNP. Changes in hydrodynamic diameter (black) and zeta potential (red) after 8 h of H₂O₂ treatment. (h) Analysis of PTX/NR@PFNP stability. Changes in the hydrodynamic diameter (black) and PDI (red) after 7 days of storage in a biological buffer at 100 rpm and 37 °C. (i) ROS-responsive release profiles of PTX loaded in PFNP3 and PFNP4 in biological buffer with and without 1.4% H₂O₂ at 100 rpm and 37 °C. *n* = 3. PFNP, PEG-ferrocene nanoparticle; PTX, paclitaxel; PDI, polydispersity index; TEM, transmission electron microscope; ROS, reactive oxygen species. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

containing culture medium without any change in their sizes and polydispersity indexes. The biosafety of PFNP3 was determined by analyzing the viability of NIH 3T3 cells. As shown in Fig. 4a, over 99% of cells remained viable after treatment with 10–200 μ g/mL PFNP3 (P > 0.05). This finding suggests that PFNP3 does not have considerable cytotoxicity when used as a nanocarrier for anticancer drugs or imaging agents. PFNP3 did not show anticancer activity in SCC7 cells (Fig. 4b). Free PTX killed approximately 30% of cells, regardless of the cell type. When the same amount of PTX was loaded into PFNP3, it exhibited greater anticancer activity (70%) in SCC7 cells due to efficient cellular uptake of nanoparticles (P < 0.01). This result is consistent with the finding of Xu et al., who reported that drug-loaded nanoparticles had stronger anticancer activity than free drugs [38]. ROS levels in SCC7 cells were higher than in NIH 3T3 cells (Fig. S6). Considering that normal cells have relatively lower ROS levels [39], a higher number of NIH 3T3 cells were viable after treatment with PTX/NR@PFNPs (P <0.05), indicating the ROS-responsive anticancer effect of the nanoparticles. Next, oxidative stress was induced by exogenous H₂O₂ to further explore the ROS-responsive anticancer activity of PTX/NR@PFNPs (Fig. S7). While PTX exhibited similar anticancer activity regardless of H₂O₂ treatment, the ROS-responsive PTX/NR@PFNP decreased the tumor cell survival rate more in presence of H_2O_2 (P < 0.001). This indicates a synergistic anticancer activity of PTX/NR@PFNP and exogenous H2O2. NAC was used as an ROS inhibitor in SCC7 cells to further investigate the influence of ROS on the anticancer activity of PTX/NR@PFNPs (Fig. 4c). PTX/NR@PFNPs lacking NAC exhibited strong anticancer activity (67%) under ROS-abundant conditions. In contrast, NAC-containing nanoparticles had anticancer activity similar to that of free PTX, demonstrating that PTX in PFNPs has a higher cytotoxicity due to ROS-responsive delivery to cancer cells. We also examined the cellular uptake of PFNPs. SCC7 cells were treated with NR@PFNPs and incubated at 37 °C. The fluorescence intensity gradually increased until 6 h (Fig. 4d and e). The fluorescence signal was barely observed at 4 °C, indicating that PFNP uptake occurred primarily by endocytosis, which requires energy. Cellular uptake of PFNP was also concentration-dependent (Fig. 4f and g).

3.5. In vivo biodistribution of DiD@PFNP in SCC7 tumor-bearing mice

Before the *in vivo* study, it was determined that DiD loading did not affect any of the characteristics of PFNP3 (Fig. S8). To investigate the biodistribution of PFNP, free DiD or DiD@PFNPs were injected intravenously into subcutaneously transplanted SCC7-tumor models. The whole-body fluorescence of DiD was observed under the near-infrared (NIR) wavelength using the IVIS Lumina XRMS system (Fig. 5a). The intensity of NIR fluorescence in tumor regions increased over time, and the accumulation of DiD@PFNPs was approximately 15-fold higher than free DiD 24 h post-injection. This difference can be attributed to the effect of EPR based on the nanoscale size of the PFNPs and the controlled



Fig. 4. Analysis of the *in vitro* cytotoxicity and anticancer activity of PTX/NR@PFNPs using the CCK-8 assay. (a) Analysis of PFNP3 cytotoxicity in NIH 3T3 cells. ROS-responsive anticancer efficacy of PTX/NR@PFNPs (b) in two cell lines and (c) after NAC treatment in SCC7 cancer cells (#P > 0.05, *P < 0.05, and **P < 0.01). Time- and dose-dependent uptake of NR@PFNPs by SCC7 tumor cells. (d) Confocal laser scanning microscopy (CLSM) of SCC7 cells after incubation with NR@PFNPs for 1, 3, or 6 h at 37 °C or 4 °C. (e) Quantification of the fluorescence intensity in (D). (f) Fluorescence images of SCC7 cells after incubation with different concentrations of NR@PFNPs. (g) Quantification of the fluorescence intensity in (f). n = 3. ROS, reactive oxygen species; PFNP, PEG-ferrocene nanoparticle; NAC, N-acetyl-L-cysteine.

drug release at the target site in response to increased ROS levels in the tumor region (Fig. 5b). Blood samples were collected at predetermined time points to analyze residual PFNPs. PFNPs showed over 6-fold higher fluorescence signal than free DiD in the blood at all time points, demonstrating stable blood circulation (Fig. 5c and d). Mice were

sacrificed 24 h after injection, and *ex vivo* imaging was used to assess the distribution of DiD@PFNPs and DiD in the major organs (heart, lungs, liver, spleen, and kidneys) and tumors. The fluorescence signal associated with DiD@PFNPs in tumor tissue was approximately 14-fold higher than free DiD (Fig. 5e and f). The overall lower fluorescence signal of



Fig. 5. *In vivo* biodistribution of DiD@PFNPs in SCC7 tumor-bearing mice following intravenous injection (n = 3). (a) Whole-body NIRF images of mice administered free DiD or DiD@PFNPs. Tumor sites are marked by circles with white dots. (b) Average fluorescence intensity of the tumor region in (a). ****P < 0.0001. (c) NIRF images of blood samples collected at preselected time points following intravenous injection. (d) Average fluorescence intensity of the blood samples in (c). *P < 0.05. (e) *Ex vivo* NIRF mages of major organs and tumors from groups of mice taken 24 h after intravenous injection of free DiD or DiD@PFNPs. (f) Average fluorescence intensity of the major organs and tumors in (e). *P < 0.05. (g) Fluorescence images of cryo-sectioned tumor tissues in (e). DiD, Solid DiIC₁₈ (5) (1,1'- dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt); PFNP, PEG-ferrocene nanoparticle; NIRF, near infrared fluorescence.

free DiD was due to its rapid clearance. Furthermore, we observed significant differences in fluorescence signals between the DiD@PFNP and free DiD groups in sliced tumor tissues (Fig. 5g). Fluorescence-based biodistribution studies do not yield quantitative measurements due to scattering and absorption caused by tissue heterogeneities [40]. The number of nanoparticles present *in vivo* does not exactly correlate with the fluorescence intensity detected. However, fluorescence imaging is a practical method to determine the whole-body distribution of nanoparticles in the early stage of research. Therefore, the findings of our fluorescence-based biodistribution study emphasize the potential of PFNPs as effective diagnostics in tumors.

3.6. In vivo therapy using PTX@PFNPs in SCC7 tumor-bearing mice

The therapeutic potential of PTX-loaded PFNPs was evaluated in

SCC7 tumor-bearing mice. When the tumor reached 50–100 mm³, the mice were randomly assigned to three groups: saline, free PTX, and PTX@PFNPs. The samples (2 mg/kg PTX) were administered through the tail vein on days 0, 2, and 4. Tumor volume and body weight were monitored every 2 days for 14 days. Fig. 6a shows the tumor-inhibition profile based on tumor volume. Tumors in the saline group grew rapidly during the experiment, with a tumor volume of approximately 1800 mm³ on the last day of observation. The best tumor-inhibition effect was achieved using PTX@PFNPs, with a mean tumor volume of approximately 300 mm³, which is significantly lower than that obtained using PTX alone (~800 mm³). The mice were sacrificed 10 days after the final injection, and tumor tissues were excised and weighed. Tumors were most effectively suppressed in the PTX@PFNP group (Fig. 6b). Mice in the PTX@PFNP group also presented the lowest tumor weight, indicating the superior therapeutic efficacy of PTX@PFNPs (Fig. 6c). H&E



Fig. 6. *In vivo* therapeutic effect of PTX@PFNPs in SCC7 tumor-bearing mice following intravenous injection (n = 5, PTX dosage of 2 mg/kg). (a) Tumor growth inhibition profiles of mouse models during 14 days of therapy. Injection points are indicated using black arrows. (b) Photograph of resected tumors from mice 14 days after the initial injection. (c) Graph showing the weight of tumors in (b). (d) H&E-staining images of tumor tissues after therapy. (e) Changes in the body weight of mice during 14 days of therapy. (n.s. non-specific differences with P > 0.05). (f) H&E-staining images of the major organs after therapy. **P < 0.01, ***P < 0.001. PTX, paclitaxel; PFNP, PEG-ferrocene nanoparticle.

staining analysis revealed that PTX@PFNP treatment damaged the tumor tissue (Fig. 6d). The body weight of the mice in the different groups is shown in Fig. 6e. No significant differences were observed among the groups, indicating that there were no severe adverse effects. Similarly, H&E staining analysis of the major organs (heart, lungs, liver, spleen, and kidneys) did not show abnormalities after PTX@PFNP treatment, similar to those of the saline group (Fig. 6f). To assess the biosafety of PTX@PFNP, we tested the biochemical indicators in blood samples after intravenous injection of PTX@PFNP into mice. We observed no significant differences in ALT, AST, ALP, BUN, and CRE between the saline and NP groups (Table S2). These compound datasets demonstrated PFNPs have good biocompatibility for *in vivo* application and effectively suppress tumor growth as an efficient drug carrier.

4. Conclusions

Efficient ROS-responsive delivery of the anticancer drug and imaging agents was achieved with the developed PFNPs. The properties of the nanoparticles depended on the initial molar ratios of MA and PEGMA moieties in the PEG-ferrocene polymers. The higher the PEG ratio, the better the stability of the PFNPs, but the lower the ROS responsiveness. PFNP3 was selected and optimized as the best compromise between the stability and ROS responsiveness of the nanoparticles. The optimized ferrocene nanoparticle successfully entered cancer cells and released the drug upon oxidation, resulting in the accumulation of fluorescent nanoparticles in the cells. It also exhibited excellent therapeutic efficacy in terms of tumor inhibition, both *in vitro* and *in vivo*. These findings demonstrate the promising application of PFNPs as a nano-platform for facilitating efficient ROS-responsive delivery of anticancer drugs and imaging agents during cancer therapy.

Credit author statement

Hyeryeon Oh and Eunjin Jeong: Conceptualization, Investigation, Methodology, Validation, Visualization, Writing – original draft. Jin Sil Lee, Jisu Kim, Donghyun Lee, and Byung Soo Kim: Investigation, Methodology, Formal analysis. Daekyung Sung, Heebeom Koo, Won Il Choi, and Giyoong Tae: Funding acquisition, Conceptualization, Project administration, Resources, Supervision, Validation, Writing – review & editing.

Associated content

Supporting Information is available free of charge.

Detailed information including GPC results of PEG-ferrocene polymers, stability of PFNPs, and characterization of PTX/DiD@PFNPs presented in this Article (PDF).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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References

- [1] H. Sung, J. Ferlay, R.L. Siegel, M. Laversanne, I. Soerjomataram, A. Jemal, F. Bray, Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries, CA Cancer J. Clin. 71 (2021) 209–249, https://doi.org/10.3322/caac.21660.
- [2] G. Wei, Y. Wang, G. Yang, Y. Wang, R. Ju, Recent progress in nanomedicine for enhanced cancer chemotherapy, Theranostics 11 (2021) 6370–6392, https://doi. org/10.7150/thno.57828.
- [3] J. Majumder, T. Minko, Multifunctional and stimuli-responsive nanocarriers for targeted therapeutic delivery, Expert Opin. Drug Deliv. 18 (2021) 205–227, https://doi.org/10.1080/17425247.2021.1828339.
- [4] P. Tan, X. Chen, H. Zhang, Q. Wei, K. Luo, Artificial intelligence aids in development of nanomedicines for cancer management, Semin. Cancer Biol. 89 (2023) 61–75, https://doi.org/10.1016/j.semcancer.2023.01.005.
- [5] C. Yu, L. Li, P. Hu, Y. Yang, W. Wei, X. Deng, L. Wang, F.R. Tay, J. Ma, Recent advances in stimulus-responsive nanocarriers for gene therapy, Adv. Sci. 8 (2021), 2100540, https://doi.org/10.1002/advs.202100540.
- [6] V. Singh, S. Md, N.A. Alhakamy, P. Kesharwani, Taxanes loaded polymersomes as an emerging polymeric nanocarrier for cancer therapy, Eur. Polym. J. 162 (2022), 110883, https://doi.org/10.1016/j.eurpolymj.2021.110883.
- [7] H. Li, J. Sun, H. Zhu, H. Wu, H. Zhang, Z. Gu, K. Luo, Recent advances in development of dendritic polymer-based nanomedicines for cancer diagnosis, WIREs Nanomed, Nanobiotechnol 13 (2021), e1670, https://doi.org/10.1002/ wnan.1670.
- [8] S. Sur, A. Rathore, V. Dave, K.R. Reddy, R.S. Chouhan, V. Sadhu, Recent developments in functionalized polymer nanoparticles for efficient drug delivery system, Nano-Struc. Nano-Objects 20 (2019), 100397, https://doi.org/10.1016/j. nanoso.2019.100397.
- [9] M. Sharifi, W.C. Cho, A. Ansariesfahani, R. Tarharoudi, H. Malekisarvar, S. Sari, S. H. Bloukh, Z. Edis, M. Amin, J.P. Gleghorn, T.L.M.T. Hagen, M. Falahati, An updated review on EPR-based solid tumor targeting nanocarriers for cancer treatment, Cancers 14 (2022) 2868, https://doi.org/10.3390/cancers14122868.
- [10] M.A. Rahim, N. Jan, S. Khan, H. Shah, A. Madni, A. Khan, A. Jabar, S. Khan, A. Elhissi, Z. Hussain, H.C. Aziz, M. Sohail, M. Khan, H.E. Thu, Recent advancements in stimuli responsive drug delivery platforms for active and passive cancer targeting, Cancers 13 (2021) 670, https://doi.org/10.3390/ cancers13040670.
- [11] H. Cai, X. Dai, X. Wang, P. Tan, L. Gu, Q. Luo, X. Zheng, Z. Li, H. Zhu, H. Zhang, Z. Gu, Q. Gong, K. Luo, A nanostrategy for efficient imaging-guided antitumor therapy through a stimuli-responsive branched polymeric prodrug, Adv. Sci. 7 (2020), 1903243, https://doi.org/10.1002/advs.201903243.
- [12] S. Wang, Z. Wang, G. Yu, Z. Zhou, O. Jacobson, Y. Liu, Y. Ma, F. Zhang, Z.-Y. Chen, X. Chen, Tumor-specific drug release and reactive oxygen species generation for cancer chemo/chemodynamic combination therapy, Adv. Sci. 6 (2019), 1801986, https://doi.org/10.1002/advs.201801986.
- [13] H. Park, G. Saravanakumar, J. Kim, J. Lim, W.J. Kim, Tumor microenvironment sensitive nanocarriers for bioimaging and therapeutics, Adv. Healthcare Mater. 10 (2021), 2000834, https://doi.org/10.1002/adhm.202000834.
- [14] K. Nakai, D. Tsuruta, What are reactive oxygen species, free radicals, and oxidative stress in skin diseases? Int. J. Mol. Sci. 22 (2021), 10799 https://doi.org/10.3390/ ijms221910799.
- [15] M. Kyu Shim, S. Yang, I.-C. Sun, K. Kim, Tumor-activated carrier-free prodrug nanoparticles for targeted cancer immunotherapy: preclinical evidence for safe and effective drug delivery, Adv. Drug Deliv. Rev. 183 (2022), 114177, https://doi. org/10.1016/j.addr.2022.114177.
- [16] Y. Ding, Q. Pan, W. Gao, Y. Pu, K. Luo, B. He, Reactive oxygen species-upregulating nanomedicines towards enhanced cancer therapy, Biomater. Sci. 11 (2023) 1182–1214, https://doi.org/10.1039/D2BM01833K.
- [17] Z. Fan, H. Xu, Recent progress in the biological applications of reactive oxygen species-responsive polymers, Polym. Rev. 60 (2020) 114–143, https://doi.org/ 10.1080/15583724.2019.1641515.
- [18] S. Li, F. Song, C. Sun, J. Hu, Y. Zhang, Amphiphilic methoxy poly(ethylene glycol)b-poly(carbonate-selenide) with enhanced ROS responsiveness: facile synthesis and oxidation process, Eur. Polym. J. 159 (2021), 110752, https://doi.org/10.1016/j. eurpolymj.2021.110752.
- [19] F. Cheng, T. Su, K. Luo, Y. Pu, B. He, The polymerization kinetics, oxidationresponsiveness, and in vitro anticancer efficacy of poly(ester-thioether)s, J. Mater. Chem. B 7 (2019) 1005, https://doi.org/10.1039/C8TB02980F.
- [20] T. Zhang, X. Chen, C. Xiao, X. Zhuang, X. Chen, Synthesis of a phenylboronic esterlinked PEG-lipid conjugate for ROS-responsive drug delivery, Polym. Chem. 8 (2017) 6209–6216, https://doi.org/10.1039/C7PY00915A.
- [21] J. Liang, B. Liu, ROS-responsive drug delivery systems, Bioeng. Transl. Med. 1 (2016) 239–251, https://doi.org/10.1002/btm2.10014.
- [22] Y. Na, J.S. Lee, J. Woo, S. Ahn, E. Lee, W.I. Choi, D. Sung, Reactive oxygen species (ROS)-responsive ferrocene-polymer-based nanoparticles for controlled release of

H. Oh et al.

drugs, J. Mater. Chem. B 8 (2020) 1906–1913, https://doi.org/10.1039/ C9TB02533B.

- [23] J.-M. Rabanel, J. Faivre, S.F. Tehrani, A. Lalloz, P. Hildgen, X. Banquy, Effect of the polymer architecture on the structural and biophysical properties of PEG–PLA nanoparticles, ACS Appl. Mater. Interfaces 7 (2015) 10374–10385, https://doi. org/10.1021/acsami.5b01423.
- [24] S.R. Mane, I. Hsiao, M. Takamiya, D. Le, U. Straehle, C. Barner-Kowollik, C. Weiss, G. Delaittre, Intrinsically fluorescent, stealth polypyrazoline nanoparticles with large Stokes shift for in vivo imaging, Small 14 (2018), 1801571, https://doi.org/ 10.1002/smll.201801571.
- [25] J.-L. Wang, X.-J. Du, J.-X. Yang, S. Shen, H.-J. Li, Y.-L. Luo, S. Iqbal, C.-F. Xu, X.-D. Ye, J. Cao, J. Wang, The effect of surface poly(ethylene glycol) length on in vivo drug delivery behaviors of polymeric nanoparticles, Biomaterials 182 (2018) 104–113, https://doi.org/10.1016/j.biomaterials.2018.08.022.
- [26] Q. Zhou, L. Zhang, T. Yang, H. Wu, Stimuli-responsive polymeric micelles for drug delivery and cancer therapy, Int. J. Nanomed. 13 (2018) 2921–2942, https://doi. org/10.2147/IJN.S158696.
- [27] W.I. Choi, N. Kamaly, L. Riol-Blanco, I.-H. Lee, J. Wu, A. Swami, C. Vilos, B. Yameen, M. Yu, J. Shi, I. Tabas, U.H. Von Andrian, S. Jon, O.C. Farokhzad, A solvent-free thermosponge nanoparticle platform for efficient delivery of labile proteins, Nano Lett. 14 (2014) 6449–6455, https://doi.org/10.1021/nl502994y.
- [28] A. Gupta, A.P. Costa, X. Xu, S.-L. Lee, C.N. Cruz, Q. Bao, D.J. Burgess, Formulation and characterization of curcumin loaded polymeric micelles produced via continuous processing, Int. J. Pharm. 583 (2020), 119340, https://doi.org/ 10.1016/j.ijpharm.2020.119340.
- [29] H. Zhang, L. Zhang, Z. Cao, S. Cheong, C. Boyer, Z. Wang, S.L.J. Yun, R. Amal, Z. Gu, Two-dimensional ultra-thin nanosheets with extraordinarily high drug loading and long blood circulation for cancer therapy, Small 18 (2022), 2200299, https://doi.org/10.1002/smll.202200299.
- [30] Q. Pan, B. Zhang, X. Peng, S. Wan, K. Luo, W. Gao, Y. Pu, B. He, A dithiocarbamatebased H2O2-responsive prodrug for combinational chemotherapy and oxidative stress amplification therapy, Chem. Comm. 55 (2019) 13896–13899, https://doi. org/10.1039/C9CC05438C.
- [31] M. Hasannia, A. Aliabadi, K. Abnous, S.M. Taghdisi, M. Ramezani, M. Alibolandi, Synthesis of block copolymers used in polymersome fabrication: application in drug delivery, J. Control. Release 341 (2022) 95–117, https://doi.org/10.1016/j. jconrel.2021.11.010.

- [32] J. Woo, H. Seo, Y. Na, S. Choi, S. Kim, W.I. Choi, M.H. Park, D. Sung, Facile synthesis and coating of aqueous antifouling polymers for inhibiting pathogenic bacterial adhesion on medical devices, Prog. Org. Coat. 147 (2020), 105772, https://doi.org/10.1016/j.porgcoat.2020.105772.
- [33] J. Woo, Y. Na, W.I. Choi, S. Kim, J. Kim, J. Hong, D. Sung, Functional ferrocene polymer multilayer coatings for implantable medical devices: biocompatible, antifouling, and ROS-sensitive controlled release of therapeutic drugs, Acta Biomater. 125 (2021) 242–252, https://doi.org/10.1016/j.actbio.2021.02.038.
- [34] A. Zielińska, F. Carreiró, A.M. Oliveira, A. Neves, B. Pires, D.N. Venkatesh, A. Durazzo, M. Lucarini, P. Eder, A.M. Silva, A. Santini, E.B. Souto, Polymeric nanoparticles: production, characterization, toxicology and ecotoxicology, Molecules 25 (2020) 3731, https://doi.org/10.3390/molecules25163731.
- [35] L.J. Cruz, P.J. Tacken, R. Fokkink, C.G. Figdor, The influence of PEG chain length and targeting moiety on antibody-mediated delivery of nanoparticle vaccines to human dendritic cells, Biomaterials 32 (2011) 6791–6803, https://doi.org/ 10.1016/j.biomaterials.2011.04.082.
- [36] R.H. Staff, M. Gallei, M. Mazurowski, M. Rehahn, R. Berger, K. Landfester, D. Crespy, Patchy nanocapsules of poly(vinylferrocene)-based block copolymers for redox-responsive release, ACS Nano 6 (2012) 9042–9049, https://doi.org/ 10.1021/nn3031589.
- [37] Y. Xu, L. Wang, Y.-K. Li, C.-Q. Wang, Oxidation and pH responsive nanoparticles based on ferrocene-modified chitosan oligosaccharide for 5-fluorouracil delivery, Carbohydr. Polym. 114 (2014) 27–35, https://doi.org/10.1016/j. carbpol.2014.08.003.
- [38] X. Peng, Y. Jia, L. Zhen, W. Meng, Z. Linghui, Z. Guansheng, Z. Yi, L. Na, Z. Zhen, Y. Si, W. Ying, Z. Dai, D. Zhijun, Therapeutic effect of doxorubicin-chlorin E6loaded mesoporous silica nanoparticles combined with ultrasound on triplenegative breast cancer, Int. J. Nanomed. 15 (2020) 2659–2668.
- [39] H. Ye, Y. Zhou, X. Liu, Y. Chen, S. Duan, R. Zhu, Y. Liu, L. Yin, Recent advances on reactive oxygen species-responsive delivery and diagnosis system, Biomacromolecules 20 (2019) 2441–2463, https://doi.org/10.1021/acs. biomac.9b00628.
- [40] Y. Liu, Y.-C. Tseng, L. Huang, Biodistribution studies of nanoparticles using fluorescence imaging: a qualitative or quantitative method? Pharm. Res. (N. Y.) 29 (2012) 3273–3277, https://doi.org/10.1007/s11095-012-0818-1.