WILEY-VCH

DOI: 10.1002/((please add manuscript number)) Article type: Communication

Self-assembled nanoparticles from phenolic derivatives for cancer therapy

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Keywords: polyphenols, nanoparticles, platinum prodrug, tumor therapy, self-assembly

Abstract

Therapeutic nanoparticles hold clinical promise for cancer treatment by avoiding limitations of conventional pharmaceuticals, Herein, we introduce a facile and rapid method to assemble PEGylated Pt prodrug nanocomplexes through metal-polyphenol complexation and combined with emulsification results in ~100 nm diameter nanoparticles (PtP NPs) that exhibit high drug loading (0.15 fg Pt per nanoparticle) and low fouling characteristics. The PtP NPs were characterized for potential use as cancer therapeutics. Mass cytometry was used to quantify uptake of the nanoparticles and the drug concentration in individual cells in vitro. The PtP NPs have long circulation times, with an elimination half-life of 17 h in healthy mice. The in vivo anti-tumor activity of the PtP NPs was systematically investigated in a human prostate cancer xenograft mouse model. Mice treated with the PtP NPs demonstrated four times better

inhibition of tumor growth than either free prodrug or cisplatin. This study presents a promising strategy to prepare therapeutic nanoparticles for biomedical applications.

Cancer remains a major cause of mortality in both developed and developing countries, with chemotherapy a mainstay of cancer treatment.^[1] Chemotherapy utilizes anti-cancer drugs to inhibit or eliminate cancer cells;^[2] however, many clinically used anti-cancer drugs have significant side effects and limited therapeutic efficacy due to a non-specific biodistribution and a resulting low delivery efficiency.^[3,4] Therefore, substantial efforts have been directed toward developing nanomedicines to reduce side effects and improve therapeutic performance.^[5-7] Nanoparticles show particular promise as candidates for cancer treatment because of their high surface to volume ratio and tunable size, shape, composition, and surface chemistry, which in combination allow for improved tumor targeting and enhanced therapeutic efficacy.^[8-11] A wide range of nanoparticles, including quantum dots,^[12,13] rare earth nanoparticles (NPs),^[14,15] polymer NPs,^[16-18] carbon NPs,^[19] mesoporous silica NPs,^{[20-} metal-organic framework NPs^[23,24] and liposomes,^[25] have been investigated. 22] Nanoparticles for cancer therapy are often engineered to be in the 100 nm (or sub-100 nm) diameter range, with low fowling surface properties to enable prolonged circulation, and with high drug loading capacity to improve drug efficacy. A central goal has been to design, assemble and apply nanoparticles with these properties so that they can accumulate efficiently in certain tumors through the enhanced permeability and retention (EPR) effect, increasing the local drug concentration and efficacy, while decreasing side effects and non-specific accumulation in healthy tissue.

Polyphenol-based nanocomplexes are promising candidates for anticancer nanomedicines owing to their flexible design aspects: for example, rapid self-assembly process, metal and

polyphenol modularity, and controlled responsiveness.^[26-28] Herein, we engineer polyphenol nanocomplexes into ~100 nm PEGylated nanoparticles containing a Pt prodrug (PtP NPs) for cancer therapy. Pt(II)-based drugs, although potent, are generally associated with drug resistance and have adverse side effects, such as nephrotoxicity, neuropathy and hearing loss.^[29,30] We use the Pt prodrug as a model drug to demonstrate the improved efficacy and reduced side effects of phenol-based nanocomplexes. The PtP NP building blocks are assembled by a rapid emulsion-based co-assembly process with multifunctional synthetic polyphenols conjugated either to the Pt(IV) prodrug, PEG, or an imaging agent in under 1 h. We demonstrate that the PtP NPs can be internalized by cells and that the tetravalent Pt prodrug is reduced to divalent cisplatin intracellularly, leading to cellular apoptosis. The PtP NPs exhibited improved tumor accumulation and reduced tumor growth reduction *in vivo*, as well as a longer blood half-life and reduced side effects, when compared to free Pt prodrug and cisplatin. As emulsion based processes are industrially used and readily scalable, this approach offers a promising route for rapidly producing efficacious nanomedicines and theranostics with appropriate sizes and surface properties for nanoparticle-drug delivery.

Therapeutic Pt prodrug-polyphenol derivatives and low-fouling PEG-polyphenol derivatives were co-assembled into nanocomplexes by cross-linking with Fe³⁺ ions (**Figure 1a**). The Pt prodrug, namely c,c,t-Pt(NH₃)₂Cl₂(O₂CCH₂CH₂COOH)₂ with two free carboxyl functionalized axial ligands, was synthesized by oxidizing commercial cisplatin with H₂O₂ followed by modification with succinic anhydride (see Figure S1 in the Supporting Information), with the structure determined by ¹H NMR analysis (Figure S2) and ESI-MS (Figure S3). Two galloyl groups were introduced at the axial position of the Pt(IV) prodrug via reacting c,c,t-Pt(NH₃)₂Cl₂(O₂CCH₂CH₂COOH)₂ with 5-hydroxydopamine hydrochloride (Figures S4 and S5), leading to the final Pt prodrug-polyphenol. The PEG-polyphenol was synthesized by conjugating catechol groups onto each terminus of an 20 kDa 8-arm-PEG

(Figure S6).^[31] It is well known that the stoichiometry of polyphenol-Fe³⁺ complexes is controlled by pH.^[32] and therefore the Pt prodrug-polyphenol and PEG-polyphenol were first mixed with FeCl₃. The pH of the mixture was <5 due to the acidity of the FeCl₃ solution, resulting in primarily polyphenol-Fe³⁺ mono-complexes.^[32] Water-in-oil nanoemulsions were formed by mixing the complexed solution with an oil phase containing a mixture of hexane, TritonTM X-100 and hexanol. The polyphenol-Fe³⁺ mono-complex emulsions were then stabilized by raising the pH through the addition of Tris buffer (pH 8.5). This coalescence processes resulted in homogeneous nanoemulsions with a pH of ~8.5, with the pH increase resulting in polyphenol-Fe³⁺ bis- and tris-complexes. Stable PtP NPs were then obtained after washing with ethanol and water. Transmission electron microscopy (TEM), atomic force microscopy (AFM), high-angle annular dark-field scanning transmission electron microscopy (HAADF-STEM) and dynamic light scattering (DLS) demonstrated that the PtP NPs were ~100 nm in diameter and the polydispersity is 0.31 (Figure 1 and Figure S7). AFM height analysis (Figure 1g) revealed that the PtP NPs particles collapsed following air drying (height ~15-20 nm). The PtP NP structure was further elucidated by synchrotron small-angle X-ray scattering (SAXS) experiments (Figure S8), where the distinct peak at ~0.1 Å-1 suggests a repeated molecular pattern of size 5.6-6.0 nm, which likely corresponds to the individual constituent nanocomplexes making up the larger PtP NPs. Energy-dispersive X-ray spectroscopy (EDS, Figure 1f) confirmed the presence of Fe and Pt in the PtP NPs. Further, the amount of Pt in each PtP NP was 0.15 fg or 9.8 w/w%, as determined by a combination of flow cytometry (Figure S9) and inductively coupled plasma optical emission spectrometry (ICP-OES).

Cell association experiments were performed by incubating AF488-labeled PtP NPs with human prostate cancer (PC3) cells for 1, 4, 12 and 24 h. The cell association was less than 20% after 24 h for AF488-labeled PtP NPs at 1 mg mL⁻¹ (**Figure 2a**). This increased sharply

to ca. 80% when the PtP NP concentration was increased to 10 mg mL-1. Similar to PC3 cells, higher concentrations of PtP NPs resulted in a higher cell association with HeLa cells (Figure S10). The cell association also increased sharply, likely due to the increased probability of particle association at higher concentrations. Mass cytometry was used to quantify cellassociated heavy metals at the level of single cells.^[33] The PtP NPs were internalized through endocytic processes,^[34] which was observable as the median mass of Pt per cell increased from 1.8 to 9.8 fg, and the number of nanoparticles per cell increased from 12 to 65 as the incubation time progressed from 4 to 24 h (Figure 2b, Figure S11, Table S1). Parallel experiments with cisplatin at an equivalent Pt dose showed that cisplatin diffused into cells quickly and reached equilibrium (median of 11.2 fg cell⁻¹) after 12 h (Figure 2c, Table S1). To elucidate if the internalization mechanism influenced the cytotoxicity, MTT bioassays were conducted for the PtP NPs, free platinum prodrug (Pt prodrug polyphenol) and cisplatin at different concentrations. The cell viability of PC3 cells decreased with increasing concentration of the PtP NPs (Figure 2d), with the PtP NPs showing comparable cytotoxicity to platinum prodrug and cisplatin. In addition, HeLa cells and luciferase-expressing PC3 cells demonstrated similar results for the PtP NPs (Figures S12 and S13). In order to further investigate the mechanism of cytotoxicity, a flow cytometry cell apoptosis study was performed using Annexin V 488 and propidium iodide (PI) staining (Figure 2e-g). At a Pt dosage of 5 µg mL-1, the PtP NPs induced cell apoptosis and death after 24 h, and the apoptotic ratio increased from 64 to 90% when the incubation was extended to 48 h. Compared with treatment using free cisplatin (Figure S14), the PtP NPs showed significantly enhanced apoptotic ratios both at 24 and 48 h incubation. Confocal microscopy was then used to investigate the cellular interactions between AF488-labeled PtP NPs and cells, and consistent with the cell association and mass cytometry results, more PtP NPs were

internalized by PC3 and HeLa cells as the incubation time increased (Figure 2h-k and Figure S15).

A pharmacokinetic study was performed in healthy mice (C57BL/6) by monitoring the Pt concentration in the bloodstream post-intravenous injection of the PtP NPs (**Figure 3a**). We used healthy mice for the nanoparticle biodistribution studies, as the xenograft cancer mice have compromised immune systems. The Pt concentration versus time follows a two-compartment mode, and pharmacokinetic parameters were determined (Table S2). The distribution half-lives and elimination half-life were 10.2 and 17.6 h, respectively. However, the half-life of cisplatin is approximately 20 to 30 min,^[30] which is much shorter than that for the PtP NPs. The biodistribution of the PtP NPs was further investigated by determining the Pt concentration in different organs and in the bloodstream. The nanoparticles rapidly clear via the kidney renal pathway (Figure 3b), likely because the prodrugs' ester linkages are hydrolysable.^[35] From 1 h post injection onward, most of the PtP NPs remained in the bloodstream until further clearance.

Penetration of Chlorin e6 labeled-PtP NPs (Ce6-PtP NPs) was assessed in a multicellular 3D HeLa cell tumor spheroid model. Confocal microscopy images demonstrated that, after incubation with Ce6-PtP NPs for 24 h, the multicellular spheroid has a spherical structure with a diameter of ~300 μ m (Figure 3c-e). The Ce6-PtP NP fluorescence was observed not only on the periphery, but also throughout the middle of the spheroid. Quantitative intensity analysis of the spheroid (Figure 3f) showed that the fluorescence intensity in the middle was of similar magnitude to that seen in the periphery. These results indicate that PtP NPs are able to penetrate into solid tumors.

In vivo anti-tumor inhibition studies were performed using a luciferase PC3 cell line as the xenograft tumor model in nude mice. Healthy normal mice were chosen instead of nude mice for the biodistribution studies because these mice have functioning immune systems that can

interact with the PtP NPs.^[36] The mice were treated with PtP NPs, cisplatin, or free prodrug (Pt prodrug polyphenol) on day 1, 3 and 5 by tail vein injection for three times with the dosages normalized to the amount of Pt (0.8 mg kg⁻¹). A PBS treatment group was used as the control, and the tumor volume and weight of the mice were monitored twice a week (Figure S16). As shown in Figure 4a, the free cisplatin and free prodrug exhibited negligible antitumor efficacy, whereas the PtP NPs significantly inhibited tumor growth (Figure S17). The volume of tumor after PtP NP treatment was 4 times smaller than all the other control groups after 21 days (Figure 4a). Notably, three mice in the PtP NPs group did not have detectible tumors, while only a single mouse from the PtP NPs group had a tumor of comparable size to the other treatment groups. Using whole-body representative bioluminescence images of luciferase-expressing PC3 tumors on day 10 post treatment (Figure 4b) and photographs of mice from various groups on day 21 (Figure S18), it was observed that the size of the tumors in PtP NPs treated mice was smaller than all of the other groups. Photos of the PC3 tumors from the four different groups further confirmed that the PtP NPs could effectively inhibit tumor growth. These results are likely to be a result of the long circulation time of the PtP NPs, which could allow them to accumulate in the tumor (presumably by the EPR effect), whereas the free cisplatin and prodrug are both cleared quickly from circulation. In addition, no mice from any of the groups lost a significant amount of weight during and post treatment (Figure S19), indicating that there were no obvious side toxic effects of the PtP NPs in mice. The relative effects of PtP NPs on different organs were also examined histologically in haematoxylin and eosin (H&E) stained sections (Figure S20). Compared with the other treatment groups, the tumor tissue was disrupted in mice treated with PtP NPs. However, there were no obvious pathological changes in any of the other main organs. These data further support that the PtP NPs can specifically kill tumor cells, while leaving healthy cells unaffected.

Tumor accumulation of the PtP NPs was confirmed by in vivo imaging (Figure 4d), where Ce6-PtP NPs were injected intravenously into luciferase PC3 cancer tumor-bearing mice at a Pt dose of 1 mg kg⁻¹. At 0.5 h post injection, the fluorescence signal was primarily located in the tumor and liver, and at 5 h post injection the fluorescence signal in the tumor was significantly enhanced. At 24 h and beyond, the fluorescence signal in the tumor remained strong; however, the signal in the liver decreased after 24 and 48 h. Based on the total radiant efficiency in the tumor site, the tumor accumulation of PtP NPs reached a peak after 5 h, and dropped slightly after 24 and 48 h post injection (Figure 4e). In combination with the in vivo biodistribution data in healthy mice, these results are consistent with a model in which PtP NPs can passively target the tumor due to prolonged circulation times. Further, microanatomical localization of the PtP NPs within the tumors was examined, where tumor tissue was dissected from mice 48 h post-intravenous injection of the Ce6-PtP NPs. As anticipated, the Ce6-Ptp NPs were evident in the blood vessels (mouse-anti-rat endothelial cell antigen (RECA) labeled endothelia cells, green) feeding the tumor (Figure 4f-i). The Ce6-PtP NPs (red) were located both in the tumor blood vessel and also the tumor cells. These results indicate that the Ce6-PtP NPs can accumulate in the tumor via blood circulation and also can be internalized into the cancer cells. In the intercellular environment, the Pt prodrug is reduced to a highly toxic drug, which induces cell apoptosis.

In conclusion, combining metal polyphenol coordination with nanoemulsification leads to PtP NPs with circulation times of around 18 h, and after internalization in the tumor cells, the Pt prodrug was reduced in the intercellular environment to cisplatin, subsequently leading the cell apoptosis by binding with DNA in the nucleus.^[37] The PtP NPs exhibited superior tumor inhibition with a fixed dose of Pt compared with free Pt prodrug and cisplatin. Furthermore, no obvious side effects of the PtP NPs were observed. Our modular approach demonstrates the phenol-based loading of a Pt prodrug, low-fouling PEG moieties, and the imaging agent

Ce6. We are currently studying the use of other metal ions (replacing Fe^{3+}) to tailor the properties of the PtP NPs properties for applications such as positron emission tomography (64Cu²⁺), magnetic resonance imaging (Mn²⁺ and Gd³⁺), and X-ray computed tomography (Yb³⁺ and Au³⁺).

Experimental Section

Experimental details including materials, synthesis of Pt prodrug polyphenol and 8-arm PEG polyphenol, assembly of PtP NPs, in vitro and in vivo experiment, three-dimensional multicellular spheroids model, mass cytometry assay, synchrotron experiment, and instrumentation are documented in the supporting information.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

This research was conducted and funded by the Australian Research Council (ARC) Centre of Excellence in Convergent Bio-Nano Science and Technology (project number CE140100036). This work was also supported by the ARC under the Australian Laureate Fellowship (F.C., FL120100030) and Discovery Project (F.C., DP130101846) schemes and the National Health and Medical Research Council under project grant APP1098867. This work was performed in part at the Materials Characterisation and Fabrication Platform (MCFP) at the University of Melbourne and the Victorian Node of the Australian National Fabrication Facility (ANFF). Part of this research was conducted at the SAXS/WAXS beamline at the Australian Synchrotron. J.J.R. thanks the OCE funding scheme at CSIRO. We acknowledge Danzi Song and Ka Fung (Leo) Noi (Department of Chemical and Biomolecular Engineering, the University of Melbourne, Australia), for helpful discussions on pharmacokinetic studies. We also acknowledge A/Prof. Clare Parish (Florey Institute of Neuroscience and Mental Health) for providing access to the microtome.

Received: ((will be filled in by the editorial staff)) Revised: ((will be filled in by the editorial staff)) Published online: ((will be filled in by the editorial staff))

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Figure 1. (a) Schematic of the PtP NP self-assembly process. (b, c) TEM images of PtP NPs. (d) AFM image of air-dried PtP NPs. (e) DLS of PtP NPs in water. (f) EDS analysis of PtP NPs. (g) Height measurement along the line in the AFM image (d). The scale bars are 2 μ m (b), 200 nm (c) and 1 μ m (d).



Figure 2. (a) PC3 cell association of PtP NPs at 1, 4, 12 and 24 h incubation and different Pt concentrations. The data represent the mean \pm SD (n=3). (b, c) Pt concentration in PC3 cells after incubation with PtP NPs or cisplatin for 4, 12 and 24 h, as measured by mass cytometry. (d) PC3 cytotoxicity of PtP NPs, free prodrug and cisplatin as a function of Pt concentration, as evaluated by MTT assay after 48 h incubation. Data represent mean \pm SD (n=4). (e, f) Apoptosis of PC3 cells after treatment with PtP NPs for 24 and 48 h (g, control). (h-k) Confocal microscopy images of PC3 cells incubated with AF488 labeled PtP NPs at 4, 8, 12 and 24 h, Green corresponds to AF488 labeled PtP NPs, red to the cell membrane stained by WGA594, and blue to nuclear staining by Hoechst 33342, the scale bars are 25 µm.



Figure 3. (a) Pharmacokinetics of intravenously injected PtP NPs in healthy mice. (b) Tissue distributions of PtP NPs at different times following intravenous injection. The data are expressed as mean \pm SD (n=3). (c-f) Penetration of Ce6 labeled PtP NPs after 24 h incubation with a HeLa cell spheroid. Red corresponds to Ce6 labeled PtP NPs (c), bright field of the HeLa cell spheroid (d), merged image of the two channels (e) and fluorescence intensity of the surface plot (f). The scale bars are 50 µm.



Figure 4. (a) Tumor growth inhibition of luciferase-expressing PC3 cells treated with different groups at the same Pt dose. Data presented as mean \pm SD (n = 7 for PBS and n = 8 for other groups). (b) In vivo bioluminescence images of representative mice bearing luciferase PC3 cell xenograft tumors post 10 days treatment with different groups. (c) Resected luciferase-expressing PC3 tumors from experimental groups at the 21st day. (d) Time dependent in vivo fluorescence imaging of Ce6 labeled PtP NPs intravenously injected into mice with luciferase-expressing PC3 xenograft tumors, the blue dashed circle is the location of the tumor. (e) Fluorescence intensity of the tumor region. Data are presented as mean \pm SD (n=2). (f-i) Photomicrographs illustrates DAPI stained nuclei (f), RECA+ endothelial cells (g) and Ce6 labeled PtP NPs in the tumor (h), and the merged image (i).