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Lipid-coated nanoscale coordination polymers for targeted cisplatin delivery

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Abstract

Nanoscale coordination polymers (NCPs) containing a Pt(IV) cisplatin prodrug, disuccinatocisplatin, were formed by a surfactant-templated synthesis and were shown to have a prodrug loading of 8.2 wt% and a diameter of ~133 nm by dynamic light scattering. These NCPs were stabilized by coating with a DOPC/cholesterol/DSPE-Peg_{2K} lipid layer; a release profile in phosphate buffered saline showed an initial drug release of ~25% within the first hour and no more release observed up to 192 h. The NCP was rendered target-specific for sigma receptors by addition of an AA-DSPE-Peg_{2K} conjugate (AA = anisamide) in the lipid formulation. The AA-containing NCP showed a statistically significant decrease in IC₅₀ (inhibitory concentration, 50%) compared to the non-targeted NCP. Enhanced uptake of the AA-containing NCP was further supported by confocal microscopy and competitive binding assays.

Introduction

Coordination polymers are hybrid materials formed by linking exo-multidentate ligands with metal-connecting points and possess a number of beneficial properties including porosity, high surface areas, and versatile functionalities. As a result, coordination polymers have been explored for a number of applications such as catalysis,^{1–3} gas storage,^{4–7} nonlinear optics,⁸ chemical sensing,⁹ and separations.^{10, 11} When scaled down to the nano-regime, the resulting nanoscale coordination polymers (NCPs) can not only exhibit the same beneficial properties as their bulk counterparts but also possess interesting functions that are unique to nanomaterials. In particular, because of their synthetic tunability and intrinsic degradability, NCPs have shown great promise in biomedical applications such as diagnostic imaging^{12–16} and drug delivery.^{17–20}

Small molecule chemotherapeutics are used to treat many forms of cancer, but their efficacy is compromised by a number of drawbacks, including poor pharmacokinetics, high systemic toxicity, poor patient tolerance, and small therapeutic windows.²¹ Nanoparticulate therapeutics can overcome many of these drawbacks and preferentially deposit in tumors by taking advantage of the enhanced permeability and retention (EPR) effect²² and the ability to actively target cancer cells. The EPR effect results from the ability of nanoparticles to penetrate the leaky vasculatures of tumors and be retained due to poor lymphatic drainage. Cisplatin, one of the three FDA-approved platin chemotherapeutics, is used to treat a variety of cancers but has many side effects such as nephrotoxicity and neurotoxicity. We are interested in designing novel strategies for targeted cisplatin delivery using NCPs. Although

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an earlier silica-coated NCP formulation was developed to deliver cisplatin at very high drug loadings, this system suffers from the burst release of significant amounts of drug from the particles.¹⁷ Other nanoscale platforms have been developed for cisplatin delivery but suffer from low drug loadings, poor pharmacokinetics, and lower cytotoxicity than the free drug.^{23–30} A PLGA-PEG-based nanoparticle system has been developed for delivery of a cisplatin prodrug and has shown promising efficacy against prostate cancer cells.^{31, 32} Herein we report the synthesis and characterization of new cisplatin-containing NCPs which have been stabilized by lipid coatings and functionalized with the targeting ligand, anisamide (AA), a small molecule that displays an affinity for sigma receptors (Scheme 1). The present NCP system shows enhanced efficacy compared to cisplatin in non-small cell lung cancer (NSCLC) cell lines; increased uptake is further demonstrated by confocal microscopy imaging and competitive binding assays.

Results and discussion

We first sought to synthesize a Zr-based NCP containing the Pt(IV) cisplatin prodrug, disuccinatocisplatin (DSCP), in hope of taking advantage of the strong Zr-carboxylate bond.³³ Zr-DSCP NCPs (**1a**) were synthesized by acetone-induced precipitation of a solution of ZrCl₄ and DSCP in N,N-dimethylformamide (DMF). Particles of **1a** are ~190 nm in diameter by dynamic light scattering (DLS) and 100–200 nm by transmission electron microscopy (TEM, Figure 1a). **1a** has a high drug loading (27.1 wt%) by inductively-coupled plasma mass spectrometry (ICP-MS) analysis of Pt, but rapidly releases the cisplatin cargo in phosphate buffered saline (PBS) with a half-life ($t_{1/2}$) of ~30 min. Several attempts to stabilize this material with surface coatings were unsuccessful (SI), presumably due to the strong driving force of **1a** to form Zr-phosphate particles in PBS.

In order to enhance the stability of cisplatin-containing NCPs under biologically relevant conditions, we synthesized a La-DSCP NCP using La(III) ions as the metal-connecting points. Lanthanide ions tend to have a better balance between particle stability and the driving force to form phosphates.¹⁹ NCPs of La-DSCP (**2a**), capped with the lipid, 1,2-dioleoyl-*sn*-glycero-3-phosphate (DOPA) were prepared by a surfactant-templated heating technique. A water in cyclohexane reverse microemulsion of $W = 7.4$ (W = water:surfactant molar ratio) containing TritonX-100, 1-hexanol, LaCl₃, DSCP, and DOPA was heated in a microwave at 70 °C for 10 min to afford **2a** with a diameter of 50–150 nm by TEM (Figure 1b) and ~133 nm by DLS in tetrahydrofuran (THF), with a polydispersity index (PDI) of 0.12 (Figure 1c, Table 1). **2a** has a DSCP loading of 8.2 wt% by ICP-MS. Thermogravimetric analysis (TGA) indicates a high amount of organic weight loss for **2a**, indicating the presence of the DOPA coating (Figure S8, SI). **2a** has a $t_{1/2}$ of ~35 min in phosphate buffered saline (PBS) at 37 °C.

Nanomaterials can be stabilized by lipid coatings.³⁴ **2a** was coated with a lipid layer composed of cholesterol (chol), 1,2- dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), and 1,2- distearoyl-*sn*-glycero-3-phosphoethanolamine-N-(polyethylene glycol) (DSPE-Peg_{2K}) in a 2:2:1 molar ratio following a reported protocol.³⁵ The size and morphology of lipid-coated NCPs (**2b**) did not differ from those of **2a** by TEM. **2b** has a DLS diameter of ~163 nm in PBS, and is highly stable in PBS buffer, with ~25% Pt release within the first hour and no additional release observed up to 192 h. The enhanced stability was supported by time-dependent DLS measurements in the presence of bovine serum albumin in which the particle size only decreases by ~20 nm and the PDI remains constant over 18 h (Figure S15, SI).

2b was also modified with DSPE-Peg_{2K}-AA (where AA is anisamide) to be rendered cancer-specific. AA is a benzamide known to target sigma receptors, which are

overexpressed on a number of epithelial tumor cells.^{36–38} AA-modified particles (**2c**) were prepared by replacing DSPE-Peg_{2K} with DSPE-Peg_{2K} and DSPE-Peg_{2K}-AA in a 9:1 molar ratio during the lipid coating procedure for **2a**. **2c** has the same size and morphology as **2b** and displays similar stability characteristics as **2b** by DLS. As cisplatin is the first-line treatment for NSCLC, we subjected two NSCLC lines (NCI-H460 and A549) to *in vitro* efficacy studies (Figure 2). NCI-H460 cells overexpress the sigma receptor and A549 have lower expression, so studies on A549 cells were used as a negative control.

In vitro efficacies of **2a–c** were determined over 48 h using the trypan blue exclusion assay. In NCI-H460 cells, **2c** showed superior efficacy to that of cisplatin with IC₅₀ values of cisplatin = $0.53 \pm 0.14 \mu\text{M}$, **2a** = $5.57 \pm 0.40 \mu\text{M}$, **2b** = $0.67 \pm 0.06 \mu\text{M}$, and **2c** = $0.32 \pm 0.03 \mu\text{M}$. There is a statistically significant difference between the IC₅₀ values for **2b** and **2c** by the t test ($p = 0.05$). Additionally, there was not a statistical difference in efficacy between **2b** and **2c** in A549 cells by the t test ($p = 0.32$), likely due to a low level of sigma receptor expression in this cell line. Additionally, analysis by the t test also showed that there was not a significant difference between IC₅₀ values for cisplatin and **2b** in either NCI-H460 ($p = 0.21$) or A549 ($p = 0.12$) cells.

Control vehicles, La NCP formulations that lack the cisplatin prodrug, were prepared from succinic acid (Suc) using the same synthetic strategies for **2a**. The La-Suc particles **3a** were lipid-coated (**3b**) and AA-targeted (**3c**) using the same method as **2a** and display similar size and morphology as the **2a–c**. These NCPs were tested against NCI-H460 cells to show that cytotoxicity of **2a–c** originates from the cisplatin prodrug, not the La(III) metal or the lipid coating. No cytotoxic effects were observed in the relevant particle concentration range (Figure S21, SI).

Enhanced uptake of AA-targeted formulations was confirmed by laser scanning confocal microscopy. Previously, we have shown that Ru(bpy)₃²⁺ is an efficient optical imaging agent.¹⁵ Particles of **3c** were doped with 10 mol% Ru(bpy)₃²⁺ by a surfactant-templated synthesis, and were used for optical imaging by laser scanning confocal microscopy. Particles of Ru(bpy)₃²⁺-doped **3a–c** were incubated with NCI-H460 cells for 30 min and imaged on a confocal microscope. Images show almost no fluorescence for **3a**, as these particles likely disintegrate before they are internalized. Slightly more fluorescence is observed for **3b**, but intense fluorescence is observed for **3c**, indicating enhanced uptake due to active targeting. Additionally, the mode of uptake was probed by confocal microscopy in which NCI-H460 cells labeled with LysoTracker green were treated with Ru(bpy)₃²⁺-doped **3c**. Co-localization of the green fluorescence from LysoTracker with red fluorescence from **3c** indicates entrapment of the NCPs within lysosomes (Figure 4). In order to further verify enhanced uptake of the targeted particles, competitive binding assays were conducted in which the sigma receptors of NCI-H460 cells were saturated with NH₃-Peg_{5K}-AA, then incubated with either **2b** or **2c**. As shown in Figure 3, the uptakes of both **2b** or **2c** are the same when the cells have been saturated with an equal amount of NH₃-Peg_{5K}-AA, which means that the active targeting ability of **2c** is silenced. Additionally, even less **2c** uptake is observed when the cells are treated with a higher concentration of NH₃-Peg_{5K}-AA. When a similar assay is done with A549 cells, no difference in NCP uptake between cells incubated with **2b** or **2c** is observed after treatment with NH₃-Peg_{5K}-AA (Figure S22, SI).

Conclusions

We have developed a NCP formulation containing a cisplatin prodrug for targeted delivery to non-small cell lung cancer cell lines. The NCP was stabilized with a cholesterol/DOPC/DSPE-Peg lipid coating and made to actively target sigma receptors by doping with a DSPE-Peg-anisamide conjugate. This formulation proved more potent than free cisplatin

against NSCLC cell lines, and enhanced uptake was further demonstrated by confocal microscopy and competitive binding assays. The generality of the present strategy should allow the preparation of lipid-coated NCPs containing other chemotherapeutics for efficacious treatment of cancers.

Experimental Section

Materials and General Procedures

All chemicals, unless otherwise noted, were purchased from Fisher Scientific or Sigma Aldrich and used without further purification. Cisplatin (for synthesis of DSCP) was purchased from AK Scientific. DOPA, DOPC, and DSPE-Peg_{2K} were purchased from Avanti Polar Lipids. Cell culturing supplies were purchased from Fisher Scientific. LysoTracker green lysosomal marker was purchased from Life Technologies. Microwave reactions were carried out in a CEM MARS 5 digestion microwave system. SEM images were obtained on a Hitachi 4700 Field Emission Scanning Electron Microscope, and TEM images were obtained on a JEM 100CX-II Transmission Electron Microscope. A Cressington 108 Auto Sputter Coater equipped with a Au/Pd (80/20) target and MTM-10 thickness monitor was used to coat samples before SEM imaging. SEM micrographs were obtained on glass slides, and TEM micrographs were obtained on carbon-coated copper grids. TGA was performed on a Shimadzu TGA-50 equipped with a platinum pan, and samples were heated at a rate of 3 °C/min under air. Size and zeta potential information was obtained on a Malvern ZetaSizer dynamic light scattering instrument. Fluorescence spectra were obtained on a Shimadzu RF-530PC spectrofluorophotometer. Confocal microscopic images were obtained on glass slides and imaged on the Olympus FlowView confocal microscope at the UNC-CH Microscope and Imaging Facility. Images were analyzed using ImageJ (with the UCSD plugin) and PhotoShop.

Synthesis of Zr-DSCP NCP (1a)

NCPs of **1a** were prepared by an acetone-induced precipitation technique. In a 150 mL beaker, 34.8 mg (0.149 mmol) of ZrCl₄ was dissolved in 13.5 mL of DMF, followed by the addition of 80.4 mg (0.151 mmol) of DSCP and 270 µL of 3M (0.75 mmol, 5 eq.) acetic acid. Stirring rapidly, ~100 mL acetone was poured into the DMF solution, resulting in a white dispersion which was stirred at room temperature for 1 h, in the dark. The particles were isolated by centrifugation and underwent subsequent wash/centrifugation steps once with acetone and once with ethanol. The particles were dispersed in ethanol. Yield: 24 mg (21.0 %).

Synthesis of La-DSCP@DOPA (2a)

Particles of **2a** were prepared by a surfactant-assisted heating method. Two reverse (water in oil) microemulsions were prepared in two 25 mL round bottom flasks by adding 5 mL 0.3 M TritonX-100/1.5 M 1-hexanol in cyclohexane. To one flask was added (while stirring) 200 µL of aq. LaCl₃·7H₂O at a concentration of 25 mg/mL (5 mg, 0.0135 mmol, pH = 5.5), and to the other flask was added 200 µL of aq. DSCP at a concentration of 25 mg/mL (5 mg, 0.01 mmol, pH = 4) and 30 µL of DOPA in CHCl₃ at a concentration of 200 mg/mL (6 mg, 0.008 mmol). Both reverse microemulsions (*W* = 7.4) became clear instantly, were combined in a microwave vessel, and microwaved at 70 °C for 10 min using 400 W, 100% power, and a 5 min ramping time. The white product was isolated by centrifugation, subsequently washed/centrifuged once with THF, and dispersed in THF. Yield: 13.5 mg (84.4 %).

Synthesis of La-Suc@DOPA (3a) and Ru(bpy)₃²⁺-doped 3a

Particles of **3a** were prepared by a similar surfactant-assisted heating method to **2a** in 64.3% yield. Particles of **3a** doped with Ru(bpy)₃²⁺ were prepared by a similar surfactant-templated method except that [Ru(5,5'-CO₂H-bpy)(bpy)₂](PF₆)₂ was added to the reverse microemulsion.

Release Profiles

All release profiles were carried out in 5 mM PBS at 37 °C. This concentration was chosen because it exceeds the blood phosphate concentration. Between 2–4 mg NCPs were prepared in 500 µL PBS or 30 % v/v EtOH/H₂O and added to a length of 10K molecular weight cutoff dialysis tubing. The dialysis tubing was submerged in a beaker containing 400 mL 5 mM PBS at 37 °C. Aliquots of 1 mL each were removed from the beaker at designated time intervals. Samples were digested in concentrated HNO₃, diluted with H₂O, and analyzed by ICP-MS for Pt content. Pt content (in ppb) was converted to % drug released.

Cytotoxicity Assay of 2a-c against NCI-H460 or A549 Cells

Confluent NCI-H460 or A549 cells were trypsinized and a cell density was obtained from a hemocytometer. Six-well plates were seeded with 5.0x10⁴ cells/well and a total of 3 mL media. Plates were incubated at 37°C and 5% CO₂ overnight. Amounts of cisplatin or NCP suspensions in RPMI-1640 medium were added to wells, along with additional media, resulting in various cisplatin concentrations (µM). Plates were incubated at 37°C and 5% CO₂ for 48 h and viability was determined via the trypan blue exclusion assay.

Viability Assay of 3a-c against A549 Cells

Confluent A549 cells were trypsinized and a cell density was obtained from a hemocytometer. Six-well plates were seeded with 5.0x10⁴ cells/well and a total of 3 mL media. Plates were incubated at 37°C and 5% CO₂ overnight. Media was removed from wells, and each well was washed with 2 mL PBS. Amounts of NCP suspensions in RPMI-1640 medium were added to wells, along with additional media, resulting in various amounts equivalent to µM cisplatin concentrations tested in the cytotoxicity assays. Plates were incubated at 37°C and 5% CO₂ for 24 h and viability was determined via the trypan blue exclusion assay.

Confocal Microscopy Using NCI-H460 Cells

Confluent NCI-H460 cells were trypsinized and counted with a hemocytometer. Glass coverslips were placed in the wells of six-well plates, followed by 2.0x10⁵ cells/well and a total of 3 mL media/well. Plates were incubated at 37 °C and 5 % CO₂ overnight. Media was removed from wells, and each well was washed with 2 mL PBS. Amounts of Ru(bpy)₃²⁺-doped **3a-c** (0.2 mg/well) were added with a total of 2 mL media/well. Wells were incubated at 37 °C and 5 % CO₂ for 30 min, media removed, wells washed with 1 mL PBS, and coverslips adhered onto glass slides with antifade mounting medium. Images were obtained on an Olympus FlowView500 confocal microscope.

Co-Localization Assay Using Confocal Microscopy

Confluent NCI-H460 cells were trypsinized and counted with a hemocytometer. Glass coverslips were placed in the wells of six-well plates, followed by 2.0x10⁵ cells/well and a total of 3 mL media/well. Plates were incubated at 37 °C and 5 % CO₂ overnight. A 100 nM solution of LysoTracker green lysosomal marker was prepared in RPMI-1640 complete growth medium. Media was removed from wells, and each well was washed with 2 mL PBS. Each well received 3 mL dye solution and incubated for 2 h. Media was removed from

wells, and each well was washed with 2 mL PBS. Ru(bpy)₃²⁺-doped **3c** (0.2 mg/well) was added with a total of 3 mL dye solution per well. Wells were incubated at 37 °C and 5 % CO₂ for 1 h, media removed, wells washed with 1 mL PBS, and coverslips adhered onto glass slides with antifade mounting medium. Images were obtained on an Olympus FlowView500 confocal microscope.

Competitive Binding Assay Using NCI-H460 or A549 Cells

Confluent NCI-H460 or A549 cells were trypsinized and counted with a hemocytometer. Five T25 culture flasks were seeded with 1x10⁶ cells/flask and 6 mL of complete growth medium containing FBS. The flasks were incubated at 37 °C and 5 % CO₂ for three days, changing the media once during this time. Media was removed from the flasks, and each flask was washed with 2 mL PBS. The flasks were given 3 mL media containing different amounts of NH₃-PEG_{5K}-AA and incubated for 30 min. The media was removed, each flask washed with 2 mL PBS, and 0.5 mg **2b** or **2c** were added to each flask with a total of 6 mL media. The flasks were incubated for 30 min, trypsinized, and cell pellets were digested in concentrated HNO₃ for 2 days. Samples were diluted with H₂O for a total of 4 mL 2% HNO₃ and analyzed by ICP-MS for La and Pt content.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig 1. TEM images of (a) **1a** and (b) **2a**. (c) DLS plots of **2a**, **2b**, and **2c**. (d) Release profiles of **2a** and **2b** in PBS buffer at 37 °C.

Fig. 2. IC₅₀ curves showing efficacy of free cisplatin and **2a–c** against (a) NCI-H460 and (b) A549 cells. *In vitro* viability results showing no cytotoxic effects on (c) NCI-H460 and (d) A549 cells after treatment with **3a–c**.

Fig 3.

Laser scanning confocal microscopy images showing DIC (b, d, f, h) and red fluorescence (a, c, e, g) attributed to particle fluorescence: (a, b) control (no particles), (c, d) **2a**, (e, f) **2b**, and (g, h) **2c**. Scale bars = 20 μ m. Competitive binding assays with NCI-H460 cells for (i) La content and (j) Pt content.

Fig 4. Laser scanning confocal microscopy images showing co-localization of Ru(bpy)₃²⁺-doped **3c** (red fluorescence) with lysosomes labeled with LysoTracker green. (a) Red channel only, (b) green channel only, (c) DIC, and (d) overlay of red and green channels. Scale bars = 20 μm.

Name	Metal	Ligand	Coating
1a	Zr ⁴⁺	DSCP	none
2a	La ³⁺	DSCP	none
2b	La ³⁺	DSCP	DOPC/Chol/DSPE-Peg
2c	La ³⁺	DSCP	DOPC/Chol/DSPE-Peg/DSPE-Peg-AA
3a	La ³⁺	Succinate	none
3b	La ³⁺	Succinate	DOPC/Chol/DSPE-Peg
3c	La ³⁺	Succinate	DOPC/Chol/DSPE-Peg/DSPE-Peg-AA

Scheme 1.

Schematic showing the lipid-coated and anisamide-targeted NCP formulations. Cisplatin release from the NCP is presumably triggered by the acidic lysosomal pH followed by *in situ* reduction of DSCP to cisplatin by endogenous thiol-based reductants such as glutathione.

Table 1

Characterization Data for DSCP NCPs.

Particle	Z _{avg} (nm) ^a	#avg (nm) ^a	PDI ^a	Potential (mV) ^b	Drug Loading (wt%) ^c
1a	190	169	0.08	-9.8	27.1
2a	133	93	0.12	-	8.2
2b	163	125	0.14	-8.9	-
2c	164	84	0.17	-2.4	-

^aTaken in EtOH for **1a**, THF for **2a**, and PBS for **2b** and **2c**.^bTaken in PBS.^cDetermined by ICP-MS analysis of Pt.

Table 2IC₅₀ Values for Cisplatin and DSCP NCPs.

Drug/Particle	NCI-H460 Cells IC ₅₀ (μM)	A549 Cells IC ₅₀ (μM)
Cisplatin	0.53±0.14	2.20±0.17
2a	5.57±0.40	5.90±1.03
2b	0.67±0.06	1.20±0.53