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Cellular Delivery of Nanoparticles Revealed with Combined Optical and Isotopic Nanoscopy

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Abstract

Direct polymerization of an oxaliplatin analogue was used to reproducibly generate amphiphiles in one pot, which consistently and spontaneously self-assemble into well-defined nanoparticles (NPs). Despite inefficient drug leakage in cell-free assays, the NPs were observed to be as cytotoxic as free oxaliplatin in cell culture experiments. We investigated this phenomenon by super-resolution fluorescence structured illumination microscopy (SIM) and nanoscale secondary ion mass spectrometry (NanoSIMS). In combination, these techniques revealed NPs are taken up *via* endocytic pathways before intracellular release of their cytotoxic cargo. As with other drug-carrying nanomaterials, these systems have potential as cellular delivery vehicles. However, high-resolution methods to track nanocarriers and their cargo at the micro- and nanoscale have been underutilized in general, limiting our understanding of their interactions with cells and tissues. We contend this type of combined optical and isotopic imaging strategy represents a powerful and potentially generalizable methodology for cellular tracking of nanocarriers and their cargo.

Graphical Abstract

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The authors declare no competing financial interest.

Supporting Information

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Additional figures (PDF)

Movie (AVI)

Keywords

NanoSIMS; SIM; drug-loaded nanoparticles; drug delivery; platinum(II) complexes; cytotoxicity; fluorescence

The design of new nanomedicines depends on an intimate understanding of the nanostructure itself and how it interacts with cells and tissues. Most frequently, intracellular trafficking and distribution of nanomaterials within cells and tissues are analyzed by fluorescence techniques that rely on labeling the materials and various organelles for colocalization analysis.^{1,2} However, correlation between trafficking of the carrier and the cargo separately and in combination remains challenging, where distinct labeling of components and orthogonal, correlated imaging strategies are desirable.

Herein, we describe a proof-of-concept drug delivery system where orthogonal, combined imaging strategies were required to understand performance and behavior in cells. Specifically, we study a nanoparticle (NP) with a covalently linked Pt(II)-based anticancer therapeutic in its hydrophobic core (Figure 1). This nanomaterial is very capable of delivering a cytotoxic form of Pt(II) intracellularly, despite observations that the drug leaks inefficiently in cell-free assays. To gain an understanding of the drug and nanocarrier behavior once in a biologically relevant environment, their uptake by cancer cells was investigated.

The uptake and subsequent intracellular distribution of Pt drugs is generally studied by quantifying the amount of Pt associated with cells or within intracellular organelles by quantitative techniques such as atomic absorption spectroscopy (AAS) or inductively coupled plasma mass spectrometry (ICP-MS).^{3,4} Besides technical difficulties associated with loss of the analyte during sample preparation, these techniques provide little information about the state of the carrier with respect to the drug being associated or dissociated. To probe these types of parameters, the system described here was monitored with nanoscale resolution *via* combined fluorescence and isotopic microscopy. This was achieved by mapping drug and NP distribution collectively and separately using structured illumination microscopy (SIM) and nanoscale secondary ion mass spectrometry (NanoSIMS). Although other element-specific imaging techniques such as X-ray fluorescence microscopy have previously, successfully been used to track Pt drugs intracellularly,^{5,6} the high spatial resolution (~50 nm), sensitivity (ppm in element imaging), and mass resolution (¹⁵N/¹⁴N) offered by NanoSIMS make the technique stand out for this sort of high-resolution correlated imaging study. This has recently been demonstrated by

Legin *et al.* for a ^{15}N -labeled cisplatin analogue. Aided by fluorescent staining of cellular organelles, the authors show the potential of these techniques combined together to study metal vs ligand intracellular distributions.⁷ Moreover, the fact that this concept (abbreviated as COIN, standing for correlated optical and isotopic nanoscopy) had recently been successfully thoroughly applied for the study of protein turnover in neurones encouraged us to use this technique to investigate the intracellular fate of nanomaterials.⁸

While technically any drug/NP combination could be isotopically enriched and possibly tagged with fluorophores, the Pt(II)-based carrier system studied here lends itself to this type of combined analysis as the drug is Pt based, having no natural abundance within the cells. This allowed facile, orthogonal isotopic enrichment of the carrier material and labeling with fluorophore. We reason that any study of uptake processes and mechanisms for nanomaterials in general will be greatly aided by correlated optical and isotopic nanoscopy techniques,⁸ coupled together to provide information regarding drug-nanoparticle-environment interactions at the micro- and nanoscale.^{7,9–12}

RESULTS AND DISCUSSION

Polymers and NP Synthesis and Characterization.

Drug-loaded nanomaterials are generally obtained by encapsulation of the drug during formulation of the particle *via* noncovalent adsorption or passive entrapment.^{13,14} A limitation of these loading strategies is the burst release of drug.¹⁵ In addition, these approaches typically result in low and uncontrolled encapsulation efficiency. Regardless, NPs loaded with Pt(II)-containing drugs have been reported to possess advantages compared to monomeric small-molecule Pt(II) complex formulations.^{16–19} Our approach was to incorporate a Pt(II) drug directly into an NP delivery system, circumventing the need for multistep loading protocols and increasing both reproducibility and loading efficiency (Figure 1). Thus, we synthesized an analogue containing a polymerizable moiety as the first example of a Pt(II) prodrug capable of being directly incorporated into a growing polymer *via* ring-opening metathesis polymerization (ROMP).^{20,21} In order to provide efficient polymerization by avoiding steric problems, the Pt(II) monomer (**2**) was copolymerized with a phenyl monomer (**1**) as a hybrid 1:2 mixture. To obtain the desired amphiphile, an oligoethylene-glycol (OEG)-modified monomer (**3**) was subsequently polymerized to ensure hydrophilicity before adding ethyl vinyl ether to terminate the elongation reaction. Time-dependent ^1H NMR confirmed complete polymerization of the first block by absence of the olefin peaks of the Pt(II) and phenyl monomers (6.33 and 6.14, respectively) and the presence of broad *trans* and *cis* olefin peaks of the polymer backbone at 5.81 and 5.59 ppm, respectively (see Figure S1). Polymerization of the second block was analogously confirmed by disappearance of the olefin peaks of the OEG monomer at 6.30 ppm. All polymers were further characterized by size exclusion chromatography with multiangle light scattering analysis (SEC MALS, Figure S2).

To investigate the reproducibility of the synthetic technique, four batches of polymers were synthesized (**P_{B1-4}**, Figure 1b). Additionally a control polymer (**P0**) lacking platinum drug, but containing a phenyl core and an OEG shell with similar ratios to the drug-containing polymers, was synthesized (Figure 1a). A ratio of hydrophobic (**1** and **2**) to hydrophilic

monomer (**3**) of 30:3 was used for all polymers prepared. In all cases the polymers showed monomer incorporation that was similar to what was expected with a narrow molecular weight distribution (Table 1).

The amphiphilic nature of the block copolymers was utilized to generate micellar NPs, prepared by dialysis from the common solvent DMF into water.²² The NPs obtained (Table 1, Figure 2) over multiple, reproducible syntheses had similar sizes (hydrodynamic diameter of 130–140 nm) and narrow particle-size distributions ($\sigma_{DLS} < 0.05$). The ζ -potential of the NPs ranged from –23 to –31 mV.²³ The particles were characterized in solution by dynamic light scattering (DLS, Figures S3, S4), NP tracking analysis (Figure S5),²⁴ dry state transmission electron microscopy (TEM, Figure 2), and cryo- and *in situ* liquid TEM (Figure S6),²⁰ from which the concentration, morphology, and size distribution could be determined. Moreover, each technique allowed us to confirm the complete dispersion of the particles in suspension, a highly desirable characteristic of NPs intended for *in vivo* applications (Video S1). Furthermore, we note the excellent stability of the suspension at room temperature over months or after freezing and defrosting as monitored by TEM, demonstrating the ability of the system to be stored for long periods of time (Figure S7). It should be noted, the particles exhibited high and similar loading efficiencies across preparations with drug content ranging between 38 and 43 wt %.

For imaging purposes a polymer was designed including three labels detectable by either fluorescence microscopy or NanoSIMS: ¹⁹⁵Pt (from the drug), a ¹⁵N version of monomer **1** (**1***), and a Cy5.5 (**Cy**) tag. The polymer block sizes (2.09×10^4 g mol^{–1}; [**1,2**]₅₂-*b*-**3**₃-*b*-**Cy**₁) and the particles prepared from this polymer (**Cy-¹⁵N-NP**; D_{DLS} 150 nm) showed no significant differences compared to the other polymer batches (Figure 2).

Drug Release Studies.

Pt release from the NPs was monitored in cell-free assays under a variety of conditions expected to increase the penetration of nucleophiles, such as chloride ions and water, into the core of the polymeric NP to promote ligand exchange reactions that displace the labile carboxylate ligands (Figure 3). Initial release studies conducted in phosphate buffer solutions (PBS) demonstrated less than 20% Pt release over 7 days and no change in the size and distribution of the particles (Figure 3a). Additionally, no statistical difference in the Pt release between NP batches was observed ($p = 0.3$, ANOVA) and no change in their hydrodynamic diameter was observed (Figure S4). As expected, in acidic (pH < 5) and basic (pH = 8) buffer solutions the Pt release increased, probably due to an acid- or base-facilitated hydrolysis. However, the increase was no more than 30% after 4 days of incubation (Figure 3b). A similar situation was observed when NPs were incubated in blood plasma, where after 4 days only 40% of the Pt present within the particles was released (Figure 3d). These results were also confirmed by DLS measurements. NPs suspended in PBS showed no signs of aggregation nor a change in their hydrodynamic diameter. However, when incubated in serum-containing cell culture media, the size of the NPs tended to increase with the appearance of large aggregates (Figure S4). The NPs were also exposed to liver microsomes, a rich source of metabolizing enzymes, in an attempt to mimic intracellular lysosomal conditions. Again, less than 30% of the loaded Pt was

released over 4 days (Figure 3c). In all cases the Pt release was significantly different from the Pt signal obtained for free oxaliplatin, which can freely diffuse through the dialysis tubing ($p < 0.05$, t test). The NPs were incubated in tissue culture media and imaged by cryo-TEM at different time points in order to detect any morphological changes (Figure S8). Although no size changes were observed after 2 days of incubation, after 7 days the size of the NPs increased and the morphology appeared to be bicontinuous.^{25,26} This morphology is particularly interesting for drug delivery, as there is a continuous water phase penetrating the structures, providing access to the hydrophobic core, which may facilitate drug release. At 7 days NPs showed hollow zones, which likely correspond to NP swelling and phase separation. These results, together with the previously described DLS studies, show that NPs are susceptible to external conditions and change their morphology while releasing a fraction of the drug load. However, release occurs at a much slower rate than uptake rates into cells *in vitro* and the rate required for efficacy in terms of cell cytotoxicity.

Cytotoxicity Studies.

Despite inefficient leakage of Pt(II) from loaded NPs, we analyzed their *in vitro* cytotoxicity to human cervical cancer (HeLa) and human lung carcinoma (A549) cell lines and determined average IC₅₀ values for **NP_{B1-4}** of 5.5(±1.2) and 2.6(±0.6) μ M or approximately 1000 and 500 NP/pl, respectively (Figure 4). When compared to the parent drugs, cisplatin and oxaliplatin, the NPs were significantly less potent against HeLa cells. Against A549 cells the antiproliferative potencies were in most cases (**NP_{B1}**, **NP_{B3}**, **NP_{B4}**) in the same range as that of oxaliplatin and greater than that of cisplatin (**NP_{B1}**, **NP_{B2}**). Since the active drug in the NPs is an oxaliplatin analogue, it is expected that their activity is in the same range. However, the differences in NP potency as cytotoxic agents against the different cell lines demonstrates the possibility of differential uptake and/or different intracellular trafficking pathways. Either a significant amount of the drug was being released within the cells at a sufficient rate to kill the cells or the NP scaffold is able to deliver small amounts of the active Pt complex to the target site much more effectively than the parent small molecule.

Cellular Uptake Studies.

The *in vitro* assays demonstrate that our understanding of NP structure (*e.g.*, covalent linkers or nonbiodegradable polymers) and its relationship toward the function of this sort of nanomedicine is limited. Therefore, we chose to rely on a multimodal imaging approach to investigate the uptake and intracellular trafficking of Pt(II)-loaded NPs. More specifically, to test whether particles were taken up into cells and then released their cargo, we combined SIM with NanoSIMS imaging techniques. Using the NanoSIMS we can gain both chemical and spatial information about the location and relative state of the NP. Previously, NanoSIMS has been utilized to qualitatively visualize extracellular and intercellular processes directly with ~100 nm resolution and without the need for non-native labels (*i.e.*, fluorophores).^{10,11,27} More recently, NanoSIMS has enabled the direct visualization of the subcellular distribution of Pt complexes by tracking isotopically labeled ligands as well as the metal.^{7,9} We designed polymers including three labels detectable by either fluorescence microscopy or NanoSIMS: ¹⁹⁵Pt (from the drug), a ¹⁵N version of monomer **1** (**1***), and a Cy5.5 (Cy) tag introduced on the polymer (Figure 1).

Particles prepared from this polymer (**Cy-¹⁵N-NP**) were incubated with HeLa cells (3 μ M with respect to Pt) for 4 h. Staining of the plasma membrane followed by fixation and observation *via* SIM showed internalization of **Cy-¹⁵N-NP** and high levels of colocalization with fluorescently labeled vesicles suggesting an uptake mediated by endocytic vesicles that originate from the plasma membrane (Figure 5, top panels, and Figure S9). Additionally, immunostaining to distinguish between early endosomes and lysosomes showed further trafficking of the NP mainly to lysosomes (Figure 5, bottom panels, and Figure S10). These results were indicative of NPs or more specifically polymers bearing the Cy dye being taken up by endocytic pathways. However, they lack information regarding the location of platinum.

To elucidate the behavior of the nanocarrier and cargo within the complex cellular milieu, HeLa cells were incubated with **Cy-¹⁵N-NP** for 4 or 24 h. They were then subjected to SIM followed by NanoSIMS, and the colocalization of the different labels in the intra- and extracellular space was investigated (Figure 6). Since the **Cy-¹⁵N-NP** were ¹⁹⁵Pt rich and ¹⁵N enriched, ¹²C¹⁴N⁻, ¹²C¹⁵N⁻, and ¹⁹⁵Pt counts were measured to determine regions where ¹⁵N was present in an amount exceeding natural abundance (NA) and appeared colocalized with the ¹⁹⁵Pt signal. Surface morphological features of a HeLa cell could be observed *via* a secondary electron (SE) image overlaid with enriched ¹⁵N (defined here as ¹²C¹⁵N⁻/¹²C¹⁴N⁻ > 5 $\times$ NA) and ¹⁹⁵Pt maps produced from NanoSIMS (Figure 6). This image shows colocalization of the signals suggesting Pt(II)-loaded particles both adsorbed to the cell and attached to the glass surface outside the cell.

To determine how Pt is localized with respect to the NP scaffold, we imaged multiple planes of the cell with NanoSIMS, where the destructive nature of the ion beam was used to remove layers of material from the sample surface (Figure 7). We note that eroding the cell by sputtering with the NanoSIMS does not provide reconstruction of three-dimensional information in a linear fashion, as is possible by SIM, where obtaining three-dimensional information is more straightforward.^{7,9} As the ablation of the cell proceeds, images are captured that map out patterns of ions coming from different depths within the cell. In this manner, images obtained from HeLa cells incubated with **Cy-¹⁵N-NP** for 4 h (Figures 7b,d and S11,S12) or 24 h (Figures 7c,e and S13,S14) were obtained. In samples incubated for 4 h, the first scan shows particles adsorbed to the surface of the cells. It is not until multiple layers are removed from the surface by the ion beam that internalized NPs can be imaged. Images from plane 10 and beyond revealed colocalization (yellow) of ¹⁵N enrichment (green) and ¹⁹⁵Pt signal (red) providing clear evidence of Pt-loaded NPs present intracellularly. Therefore, we reason that any colocalization of Pt with NPs at 24 h or lack thereof would either constitute evidence that the particles remain intact or provide direct evidence that Pt is released intracellularly over time. In an attempt to analyze relative levels of colocalization of ¹⁵N (arising from the polymer itself) and ¹⁹⁵Pt (arising from the Pt drug) within the cell, the ¹⁹⁵Pt signal from all planes was summed, and two types of regions of interest (ROIs) were defined: the first one delineating regions with detectable Pt and the second showing regions lacking detectable Pt signal but still within the cell boundaries (Figure S15). For both time points, the ¹⁵N enrichment appeared to be at least 10 $\times$ greater in areas colocalized with Pt (on particle) than in areas not colocalized (cell background)

(Figure S16). Most notably, as it can be observed in Figure 7b,c, after 24 h incubation a slight decrease of the enriched ^{15}N signal was observed for regions with high ^{195}Pt counts, suggesting specific metabolism of the NPs causing a separation of the carrier polymer from its cargo. This suggests that even covalently linked drugs within the hydrophobic domain of a nonbiodegradable NP can undergo significant release after cell internalization within 1 day. Nontreated HeLa cells were also analyzed by NanoSIMS in a similar fashion to confirm the absence of the signals that were used to detect the NPs. As expected, full ion images and ion count plots (Figures S17, S18, and S19) show $^{15}\text{N}/^{14}\text{N}$ ratios indicative of NA and that no ^{195}Pt was detected.

Combined Optical and Isotopic Studies.

With a detailed analysis of NanoSIMS data in hand, we endeavored to couple fluorescence data obtained by SIM to construct combined images. For these experiments, fluorescence microscopy was performed prior to NanoSIMS analysis. Overlays of SIM images on planes 10 and 6 with NanoSIMS isotopic maps of the cells on planes 28 and 54 are shown in Figure 8 (4 and 24 h NP incubation, respectively). The combined images show, in addition to the isotopic labels (enriched ^{15}N and ^{195}Pt), significant accumulation of the fluorescent tag from the NP in the cells. This combined imaging analysis, accounting for the drug (^{195}Pt), polymer backbone (^{15}N), and orthogonal label (fluorescent dye), is consistent with data analyses shown in Figures 7 and S16, where the presence of a significant ^{195}Pt signal (blue, Figure 8) at 24 h is only weakly correlated with signals associated with the original carrier system. By contrast, at the earlier time point, significant colocalization is observed for drug and carrier. NanoSIMS data and combined nanoscopy analysis (Figures 7 and 8), together with lysosomal and endosomal tracking data, prove the particles (and/or polymers) are trapped in these intracellular vesicles and that drug release yielding favorable cytotoxicity is the result of release from the carrier once internalized.

CONCLUSIONS

We studied drug-loaded polymeric NPs wherein a Pt(II)-prodrug-based cytotoxin is covalently bound to well-defined NPs. The materials exhibit reproducible morphologies and high levels of Pt drug loading and yielded NPs with consistent morphological characteristics (Figure 1).^{19,28,29} Although cell-free stability studies demonstrated only limited drug release, the cytotoxic potency of the NPs to selected cancer cell lines was in the same range as free cisplatin and oxaliplatin. The high cytotoxic activity of this type of NP highlights the lack of relationship between *in vitro* activity and cell-free drug release profiles, as well as our basic understanding of how structure and function are related for nanomedicines. To probe this observation, drug release from the cargo *after* cell internalization was confirmed through a multimodal imaging strategy that combined fluorescence SIM with NanoSIMS to separately track the NP polymer (^{15}N and Cy5.5) and the cargo (^{195}Pt). We note that NPs are generally and frequently tracked in biological environments by the incorporation of fluorescent tags.^{30,31} It is clear from the studies presented here that such an approach would only have revealed the presence and localization of the fluorophore with endosomal labels, but not location of the cargo and disposition with respect to the polymeric nanocarrier. Therefore, while further advances are needed, we believe that these combined techniques

should be generalizable for many nanoscale drug delivery systems, where it is of tremendous importance to characterize and track the composition and integrity of the delivery system and the cargo at each stage of the uptake and release process.

METHODS

Monomer Synthesis.

Monomers **1**, **2**, and **3** were synthesized following previously published protocols.^{20,32} Briefly, monomer **1*** was synthesized as follows: a mixture of 5-norbornene-*exo*-2,3-dicarboxylic anhydride (5 mmol) and ¹⁵N-urea (6 mmol) was heated at 145 °C for 4 h. Water (20 mL) was added, and the solution was heated until it was homogeneous. A white-gray solid precipitated upon cooling. It was filtered, washed with cold water, and dried to obtain ¹⁵N-5-norbornene-*exo*-2,3-dicarboxyimide. ¹⁵N-5-norbornene-*exo*-2,3-dicarboxyimide (5 mmol), benzyl bromide (6 mmol), and K₂CO₃ (5.5 mmol) in 20 mL of acetone were refluxed for 45 h. The solvent was removed, and the product was washed with water and dried under vacuum to obtain ¹⁵N-benzyl-5-norbornene-*exo*-2,3-dicarboxyimide (**1***). Synthesis of the Cy5.5 monomer proceeds as follows: 3-(1,1,2-trimethyl-1*H*-benzo[*e*]indol-3-ium-3-yl)propane-1-sulfonate (4.9 mmol), 6-(1,3-dioxopropan-2-yl)nicotinic acid (2.2 mmol), and sodium acetate (16.1 mmol) was dissolved in 125 mL of a 1:1 mixture of acetic anhydride and acetic acid and heated to reflux for 5 h. Once complete, the solvent was removed by rotary evaporation and the blue solid was dissolved in water with 0.5% TFA and purified by reverse phase HPLC to obtain 3-(2-((1*E*,3*Z*,5*E*)-3-(5-carboxypyridin-2-yl)-5-(1,1-dimethyl-3-(3-sulfopropyl)-1,3-dihydro-2*H*-benzo[*e*]indol-2-ylidene)penta-1,3-dien-1-yl)-1,1-dimethyl-1*H*-benzo[*e*]indol-3-ium-3-yl)propane-1-sulfonate. Then, the Cy5.5 analogue (0.13 mmol), HATU (0.13 mmol), and DIPEA (0.75 mmol) were added to 2 mL of anhydrous DMF. After 10 min, 2-(2-aminoethyl)-3a,4,7,7a-tetrahydro-1*H*-4,7-methanoisindole-1,3(2*H*)-dione (0.37 mmol) was added. After 45 min, cold diethyl ether was added to the reaction and the precipitate was collected and dried under vacuum to obtain the Cy5.5 monomer.

Polymer Synthesis.

Polymer **P0** was synthesized as follows: to a stirred solution of **1** (0.0612 mmol) in dry DMF was added a solution of modified second-generation Grubbs' ruthenium catalyst (0.002 mmol) in dry DMF. The reaction was left to stir under a N₂ atmosphere for 2 h. An aliquot was removed and quenched with ethyl vinyl ether for SEC-MALS analysis. A solution of the hydrophilic OEG monomer, **3** (0.006 mmol), in DMF was added to the remaining reaction mixture. The mixture was left to stir under a N₂ atmosphere for an additional 30 min, followed by ethyl vinyl ether quenching. After 25 min the polymer was crashed out over cold ether to give an offwhite solid. Polymers **P_{B1-4}** were synthesized as follows: to a stirred solution of **1** (0.0210 mmol) and **2** (0.0105 mmol) in dry DMF was added a solution of modified second-generation Grubbs' ruthenium catalyst (0.001 mmol) in dry DMF. The reaction was left to stir under a N₂ atmosphere for 2 h. An aliquot was removed and quenched with ethyl vinyl ether for SEC-MALS analysis. A solution of the hydrophilic OEG monomer, **3** (0.003 mmol), in DMF was added to the remaining reaction mixture.

The mixture was left to stir under a N₂ atmosphere for an additional 30 min, followed by ethyl vinyl ether quenching. After 25 min the polymer was crashed out over cold ether to give an offwhite solid. **Cy-¹⁵N-P** polymer was synthesized as follows: to a stirred solution of **1*** (0.0210 mmol) and **2** (0.0105 mmol) in dry DMF was added a solution of modified second-generation Grubbs' ruthenium catalyst (0.001 mmol) in dry DMF. The reaction was left to stir under a N₂ atmosphere for 2 h. An aliquot was removed and quenched with ethyl vinyl ether for SEC-MALS analysis. A solution of the hydrophilic OEG monomer, **3** (0.003 mmol), in DMF (100 μ L) was added to the remaining reaction mixture, and the mixture was left to stir under a N₂ atmosphere for an additional 30 min. The living polymer was then split into two pots. While the first portion was quenched with ethyl vinyl ether, one equivalent of Cy5.5 monomer was added to the second portion and left to stir for 1 h before quenching with ethyl vinyl ether as well. The polymers were crashed out over cold ether.

Spherical NP Formation.

Each block copolymer (0.7 mg) was dissolved in DMF (1000 μ L) and dialyzed against H₂O through a 3.5 kDa MWCO dialysis tubing for 2 days. The H₂O was changed twice over that time.

Pt Release Studies.

For the *in vitro* Pt-release profiles, a suspension of the NPs containing a known concentration of Pt(II) (set as 100%) was added to a dialysis cup (Slide-A-Lyzer MINI Dialysis Unit 3500 MW, Thermo Scientific), and sealed into a plastic tube containing the desired release condition. These were placed in an incubator shaker at 37 °C. Samples (the dialysates) were collected. The samples were lyophilized, digested with an ultrapure 1:3 concentrated HCl/HNO₃ solution, placed on an oil bath at 60 °C, dried, and resuspended in 0.01 M NaCN. Finally, the suspensions were analyzed for their Pt concentration by ICP-OES. Three replicates were measured for each ICP-OES determination.

Cytotoxicity Studies.

HeLa and A549 cancer cell lines were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, 1% nonessential amino acids, 1% sodium pyruvate, and 1% glutamine. The cells were grown in 5% CO₂ at 37 °C. The cytotoxicity of the Pt(II)-loaded NPs was determined by their effect on growth rate as quantified by crystal violet staining at the end of drug exposure. HeLa and A549 (3×10^4 to 4×10^4 cells/well) cells were seeded in 100 μ L of media in 96-well plates. After overnight incubation at 37 °C the cells were exposed to various concentrations of NP-containing media for 96 h. Cell cultures were fixed with 4% paraformaldehyde, washed three times with water, and stained with crystal violet. The absorbance at 595 nm of each well was measured using a microtiter plate reader scanning spectrophotometer and analyzed relative to the absorbance of drug-free control wells. Each experiment was performed in triplicate.

Cellular NP Uptake Studies.

To investigate NP cellular uptake using SIM, HeLa cells were plated in glass-bottom cell culture dishes (precoated with fibronectin) 12 h prior to incubation with 3 μM Cy- ^{15}N -NP suspension in DMEM for 4 h. After incubation, the media containing NPs was removed and fresh media with WGA-AF-488 was added. After treatment, cells were washed three times with heparin and twice with PBS. Cells were fixed with 2% paraformaldehyde and washed three times with PBS. DAPI was added and incubated overnight. Cells were imaged in PBS with SIM. Analogously, for immunostaining, cells were plated and incubated with NP as previously described. After fixation, cells were permeabilized with saponin and incubated with blocking solution (0.2% saponin and 1% BSA in PBS). The primary antibodies mouse LAMP1 and rabbit EEA1 were added in a 1/100 and 1/800 dilution, respectively, in blocking buffer and incubated. Cells were washed with PBS, and the secondary antibodies, goat anti-mouse-AlexaFluor488 and goat anti-rabbit-AlexaFluor546, were added in blocking buffer. DAPI was added and incubated overnight. Cells were imaged in PBS with SIM. To investigate NP cellular trafficking *via* SIM and NanoSIMS, HeLa cells were plated in culture dishes in which were placed an 18 mm² ITO coverslip precoated with fibronectin 12 h prior to incubation with a 15 μM Cy- ^{15}N -NP suspension in DMEM for 4 or 24 h. After incubation, the media containing NPs was removed and fresh media with WGA-AF-488 and 2 drops of NucBlue was added and incubated for 10 and 30 min, respectively. After treatment, cells were incubated three times with heparin and washed twice with PBS. Cells were fixed with 2.5% glutaraldehyde and washed three times with PBS. The cells were then subjected to a series of dehydration washes with 30%, 50%, and 75% and three times with 100% ethanol (30 min each). The dry cells on the ITO glass were then first imaged by SIM followed by NanoSIMS. The X and Y coordinates of the sample stage were recorded for every SIM image and were used to locate the cells for the following NanoSIMS imaging.

NanoSIMS Imaging Conditions.

The samples were coated with 10 nm of Au prior to analysis to minimize sample charging.²⁷ High beam current sputtering was performed to remove ~15 nm of material from the surface of the sample and to assist in reaching sputter equilibrium.³³ After this, images with either 35 $\mu\text{m} \times 35 \mu\text{m}$ and 256 pixel $\times$ 256 pixel or 40 $\mu\text{m} \times 40 \mu\text{m}$ raster areas and 512 pixel $\times$ 512 pixel were acquired with a ~1 pA Cs⁺ primary ion beam (width ~115 nm) using magnetic peak switching, where in the first scan of a plane $^{12}\text{C}^-$, $^{16}\text{O}^1\text{H}^-$, $^{12}\text{C}^{14}\text{N}^-$, and $^{12}\text{C}^{15}\text{N}^-$ were collected (1 ms/pixel) and in the second scan of the plane $^{12}\text{C}^{15}\text{N}^-$ and $^{195}\text{Pt}^-$ were collected (3 ms/pixel). Each scanning plane resulted in greater than 1 nm of removal and analysis.³⁴ To determine if $^{12}\text{C}^{15}\text{N}^-/^{12}\text{C}^{14}\text{N}^-$ enrichment was associated with the $^{195}\text{Pt}^-$ signal in the cell (as opposed to substrate), all planes of the $^{195}\text{Pt}^-$ ion image were summed to determine what regions $^{195}\text{Pt}^-$ was detected throughout the imaging experiment. Regions of interest were then created of pixels that were and were not colocalized with the $^{195}\text{Pt}^-$ signal. The $^{12}\text{C}^{15}\text{N}^-/^{12}\text{C}^{14}\text{N}^-$ ratio was then determined for each of these ROIs and plotted as a function of image plane.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Synthetic route to polymeric NPs. The particles were prepared by assembly of copolymer amphiphiles in which Pt(II) complexes are part of the hydrophobic block. (a) Polymerization of **1** as a first hydrophobic block, followed by **3** as the hydrophilic block, yielded polymer **P0**. Dialysis into water formed **NP0**, the drug-free control NPs. (b) Copolymerization of **1** and **2** as the hydrophobic block, followed by **3** as the hydrophilic block, yielded polymers **P_{B1}**, **P_{B2}**, **P_{B3}**, and **P_{B4}**. Dialysis into water formed **NP_{B1}**, **NP_{B2}**, **NP_{B3}**, and **NP_{B4}**, respectively, the drug-containing NPs. (c) Copolymerization of a ¹⁵N-modified version of **1** (**1***) and **2** as the hydrophobic block followed by **3** as the hydrophilic block and a Cy5.5-functionalized norbornene monomer as a third block generated polymer **Cy-¹⁵N-P**. Dialysis into water yielded **Cy-¹⁵N-NP**, the isotopic and fluorescently labeled drug-containing NPs.

Figure 2.

TEM images of NP0, the four batches of nonlabeled NP (B1-B4), and Cy-¹⁵N-NP.

Two different magnifications are used to show the bulk (top) and zoomed-in (bottom)

morphologies of each of the particles in turn. The control particles had to be stained with uranyl acetate for their visualization (left panels show the two magnifications of NP0).

The high-contrast Pt(II)-loaded NPs were visualized without the need of a staining process because of the high-*Z* element core.

Figure 3.

(a) Release of Pt from the four batches of NP in phosphate-buffered saline solution (PBS, pH 7.4) at 37 °C after 7 days of incubation. (b) Pt release from unlabeled NP_{B1} after 4 days of incubation at different pHs. (c) Pt release from NP_{B1} after treatment with lysosomal extract. (d) Time-dependent Pt release form unlabeled NP_{B1} in blood plasma. Pt content was determined by ICP-OES.

Figure 4.

Cytotoxic activity of the four batches of NPs against HeLa and A549 cells determined by the crystal violet assay after 96 h of incubation and expressed as IC₅₀ in μM . Note: the IC₅₀ of the control particles NP0 was $>25 \mu\text{M}$.

Figure 5.

SIM analysis of the subcellular localization of Cy-¹⁵N-NP (red) in HeLa cells examined using specific fluorescent probes. (a) Cells stained with a probe specific for the cell membrane (wheat germ agglutinin, WGA AF-488, green) and nucleus (DAPI, blue). Arrows show NP colocalized with endocytic vesicles that originated from the cell membrane as indicated by their green fluorescence. (b) Cells were double immunostained for early endosomes (EEA1-Alexa546, green) and lysosomes (LAMP1-Alexa488, blue) as well as for DNA (DAPI, white). Arrows show NP colocalized with lysosomes. Left panels show lowest magnification images of cells (a and b) with successive zoom-in images shown in a1,2 and b1,2.

Figure 6. Composite NanoSIMS image of a HeLa cell incubated with $15\ \mu\text{M}$ Cy- ^{15}N -NP where ^{195}Pt (red) and ^{15}N enrichment (green) are overlaid on the secondary ion (SE) image. Yellow regions indicate colocalization of signals. The scale bar represents $10\ \mu\text{m}$.

Figure 7.

(a) Scheme summarizing the time-dependent NanoSIMS experiments carried out in HeLa cells treated with Cy-¹⁵N-NP. (b and c) HeLa cells incubated with Cy-¹⁵N-NP for 4 h (top three panels, b1–3) or 24 h (bottom three panels, c1–3) and imaged by NanoSIMS. Removal of layers of organic matter from the cell surface followed by imaging shows colocalization (yellow) of the ¹⁹⁵Pt (red) and ¹⁵N (green) of the NP inside the cell. The cell surface is represented by the ¹²C¹⁴N⁻ ion map (blue). For full images and other isotopic ion plots see Figures S11 and S13. For similar data captured for different cells, see Figures S12 and S14. (d and e) Summed observed ¹⁹⁵Pt signals (white pixels) in HeLa cells incubated for 4 h (d) and 24 h (e). Red circles are the ROIs selected for ¹⁵N/¹⁴N quantification where ¹⁹⁵Pt counts are observed. Cell boundaries (yellow line) were delimited from the corresponding ¹²C¹⁴N⁻ ion images. The averaged ¹⁵N and ¹⁹⁵Pt signals per selected ROIs at each of the selected planes are shown for panels b and c. For full count plots see Figure S16. For $t = 0$ h data see Figures S17, S18, and S19. The scale bars represent 10 μm.

Figure 8.

Overlaid NanoSIMS and SIM images show the colocalization of the fluorescent tag (red) with ^{195}Pt (blue) and ^{15}N (green) after 4 (left) and 24 h (right) incubation of Cy- ^{15}N -NP with HeLa cells. Overlay of ^{195}Pt and the fluorescent tag is shown in magenta, and overlay of all three (^{195}Pt , ^{15}N , and fluorescence tag) is shown in white. Cell boundaries (white line) were delimited from the corresponding $^{12}\text{C}^{14}\text{N}^-$ NanoSIMS ion images. For individual SIM and NanoSIMS images see Figure S20.

Table 1.

Physical Characteristics of the Control Polymer (P0), the Four Batches of Pt(II)-Containing Polymers (P_{B1-4}), and the Respective Particles (NP0 and NP_{B1-4})^a

polymer	identity	M_n [g mol ⁻¹]	M_w [g mol ⁻¹]	M_w/M_n	nanoparticle	D_{DLS}^c [nm]	D_{NTA}^e [nm]	f_{NTA}^f	ζ^g [mV]	% drug ^h [wt %]
P0	1 ₂₈ -b-3 ₃	9.35×10^3	9.50×10^3	1.01	NP0	184	101	0.4	-18 ± 9	0
P _{B1}	1 ₂₃₂ -b-3 ₃	1.14×10^4	1.19×10^4	1.06	NP _{B1}	134	101	0.4	-31 ± 11	43
P _{B2}	1 ₂₂₇ -b-3 ₄	1.13×10^4	1.21×10^4	1.07	NP _{B2}	132	96	0.2	-26 ± 9	41
P _{B3}	1 ₂₃₃ -b-3 ₆	1.48×10^4	1.58×10^4	1.07	NP _{B3}	127	77	0.3	-24 ± 9	38
P _{B4}	1 ₂₃₉ -b-3 ₅	1.6×10^4	1.71×10^4	1.07	NP _{B4}	138	89	0.3	-23 ± 9	41

^aIntended ratio of monomers **1** to **2** (**1:2**) in the hydrophobic polymer is 20:10. Intended ratio of hydrophobic to hydrophilic monomers (**1:2:3**) in the amphiphilic polymer is 30:3.

^bDetermined *via* SEC-MALS.

^cIntensity weighted mean hydrodynamic diameter obtained *via* DLS.

^dDispersity from the DLS measurement.

^eNumber weighted mean diameter determined *via* NTA with NanoSight.

^fDispersity from the NTA measurement.

^gZeta potential measured by phase analysis light scattering.

^h% Oxaliplatin analogue = $M_wPt(II)\text{-monomer}/M_w\text{polymer}$.