

ORIGINAL ARTICLE

Cisplatin delivery vehicles based on stabilized polymeric aggregates comprising poly(acrylic acid) chains

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Stabilized polymeric aggregates (SPAs) comprising poly(acrylic acid) (PAA) chains were studied as a delivery platform for cisplatin. SPAs were prepared by blending a poly(ethylene oxide)-block-poly(propylene oxide)-block-poly(ethylene oxide) triblock copolymer with a poly(acrylic acid)-block-poly(propylene oxide)-block-poly(acrylic acid) triblock copolymer and additional loading and photocrosslinking of pentaerythritol tetraacrylate (PETA). Dynamic light scattering analysis revealed particles with a hydrodynamic diameter of 176 nm and a monomodal particle size distribution. The stabilized polymeric aggregates were loaded with cisplatin by a ligand exchange reaction, achieving a high loading efficiency of 76%. A study on the release of complexes of platinum(II) from the particles in phosphate-buffered saline (PBS) and citrate buffer solution (CBS) at 37 °C revealed a sustained release profile. More than 90% and nearly 80% of the loaded drug were released within 312 h in PBS and CBS, respectively. The *in vitro* cell viability assay indicated that cisplatin immobilized in the SPAs is less cytotoxic than the non-immobilized agent. The intracellular accumulation of the entrapped complex was comparable to that of the free drug. *Polymer Journal* (2017) 49, 607–615; doi:10.1038/pj.2017.29; published online 7 June 2017

INTRODUCTION

The use of polymeric nanocarriers for drug delivery has shown significant therapeutic potential to improve the efficiency and specificity of the drug action. Cis-dichlorodiamminoplatinum(II) (cisplatin) is one of the most potent anticancer drugs and is widely used in the treatment of many solid tumors.¹ However, disadvantages, including severe side effects, are a short circulation period in the blood due to glomerular excretion and intrinsic or acquired resistance of some tumors to the drug, limit its clinical application.^{2,3} The drawbacks of therapy with cisplatin have stimulated an intensive search for less toxic alternatives, mainly focusing on the development of various drug delivery systems, including water-soluble polymers,^{4–7} long-circulating liposomes^{8–10} and polymeric micelles.^{11–13}

Core-shell polymeric micelles formed from amphiphilic block copolymers have been actively studied as delivery systems of low-molecular-mass drugs, proteins, nucleic acids and imaging agents.¹⁴ Their main advantages include small size (<200 nm in diameter), ability to entrap hydrophobic drugs into the core and to increase their solubility, controlled pH- and temperature-responsive drug release, systemic stability and unique disposition characteristics in the body, resulting in superior performance compared to other colloidal delivery systems.¹⁵ It has also been established that polymeric micelles accumulate to a higher extent in solid tumors than larger nanoparticles like liposomes.¹⁶

The potential for drug delivery of the polymeric micelles obtained from commercially available poly(ethylene oxide)-block-poly(propylene oxide)-block-poly(ethylene oxide) (PEO-PPO-PEO) triblock copolymers (Pluronics, Synperonics, Poloxamers) has been extensively investigated due to specific advantages, such as resistance of the PEO shell to protein adsorption and cellular adhesion, ability of the PPO core to solubilize water-insoluble compounds, and availability of hydroxyl groups to which receptor-specific ligands can be attached. Moreover, pluronic block copolymers are recognized pharmaceutical excipients listed in the US and British Pharmacopoeia.^{17,18}

Pluronic copolymers have been used for the delivery of cisplatin and other cytostatic platinum complexes. Sonoda *et al.*¹⁹ reported that treatment with a cisplatin-pluronic combination resulted in a pronounced tumor reduction and did not cause apparent damage to the liver tissue.

In particular, polymeric micelles comprising acid moieties are attractive carriers of cisplatin because the drug can be easily immobilized within the micelle via ligand exchange reaction. Nishiyama *et al.* prepared micelles from a block copolymer composed of poly(aspartic acid) and poly(ethylene glycol) blocks. The drug was bound to the aspartic residues of the copolymer via exchanging the chloride ligands with the carboxylate ones. The metal complex immobilized in the polymeric micelles exhibited lower cytotoxicity *in vitro* than the free drug. Moreover, the micellar system caused much less renal damage than the unbound cisplatin. Nevertheless, the

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structural decay of the micelles caused liver and spleen accumulation despite restrained nephrotoxicity.^{12,13} A next-generation cisplatin carrier derived from PEG-*b*-poly(L-glutamic acid) offered improved stability and tumor selectivity due to the compact packaging of ordered α -helical bundles of the loaded polypeptide segments in the micelle core.²⁰ These advantageous features resulted in reduced nephro- and neurotoxicity in rats²¹ and ototoxicity in guinea pigs.²² Phase I/II clinical trials ascertained that there were mild adverse effects and acceptable efficacy of the cisplatin-loaded PEG-*b*-poly(L-glutamic acid) micelles, alone or in combination with gemcitabine in patients with pancreatic cancer, allowing translation into phase III studies.²³ The progress in clinical studies encourages searching for carrier alternatives with the structural stability to provide effective and safe drug action.

Polymeric micelles with cross-linked cores from block copolymer comprising poly(ethylene glycol) and poly(methacrylic acid) were prepared and tested as a delivery platform for cisplatin. The micelles effectively incorporated cisplatin with a high loading capacity (up to 42% w/w). A sustained release of platinum(II) complexes from the polymer carrier in phosphate-buffered saline (PBS) was observed. The released complexes retained their pharmacological activity—they reduced the thermal stability of a model DNA and formed adducts with the DNA of tumor cells.²⁴

In our recent study, we reported results concerning the design of a star-shaped PEGylated core-shell type polymer and its evaluation as a delivery system for cisplatin. The shell consisted of poly(acrylic acid) arms and reversibly linked PEG chains. The PEGylation of the carrier resulted in an increase in the drug-loading capacity and solution stability on storage. The PEGylated conjugates displayed a sustained manner of platinum(II) complex release without the initial burst effect. They demonstrated an enhanced growth inhibitory activity compared with the nonPEGylated system. A certain limitation of the developed system was the amount of the released platinum(II) complexes from the carrier—less than half of the loaded drug was released over a period of 12 days.²⁵

The present study was aimed at evaluating the applicability of stabilized polymeric aggregates (SPAs), comprising poly(acrylic acid) (PAA) chains, for effective loading and delivery of cisplatin. To the best of our knowledge, this is the first study on the complexation of cisplatin with stabilized 'pluronic' aggregates, containing functional poly(acrylic acid) moieties, for drug delivery application. The content of PAA in the aggregates is ~ 32 wt.%, which provides the possibility to exploit the ligand exchange reaction for cisplatin conjugation to acrylic acid units in the hydrated shell. This study was mainly focused on the determination of the drug-loading capacity, release profile, cytotoxicity and cellular uptake. Different analytical methods, such as dynamic light scattering (DLS), atomic force microscopy (AFM), inductively coupled plasma optical emission spectrometry (ICP-OES) and inductively coupled plasma mass spectrometry (ICP-MS), were applied for the characterization of the investigated polymeric micelles.

MATERIALS AND METHODS

Materials

Cis-dichlorodiamminoplatinum(II) (cisplatin) (99.9%) was purchased from Merck (Darmstadt, Germany). Dialysis membranes with a molecular weight cut-off (MWCO) of 3500 and 12 000 were supplied by SpectraPor (USA). Poly(propylene glycol) (Fluka, USA, MW 2000 g mol⁻¹; PPG₃₅) was dried by azeotropic distillation with toluene. Triethylamine, 2-bromoisobutyl bromide, CuBr, trifluoroacetic acid, pentamethyldiethylenetriamine and pentaerythritol tetraacrylate (PETA) were purchased from Aldrich (St Louis, MO, USA) and were used as received. *tert*-Butyl acrylate (*t*BA, BASF, Germany) was stirred

over calcium hydride (Merck) overnight and was vacuum distilled just before use. All of the solvents were purified by standard procedures. Silica-60 gel (Merck) was used as supplied to remove residues of the atom transfer radical polymerization (ATRP) catalysts. 'Pluronic' P65 was kindly donated by BASF (Germany). Dulbecco's modified Eagle's medium was purchased from Gibco-Invitrogen (Inchinnan, UK). Dimethyl sulfoxide and trypsin were obtained from AppliChem (Darmstadt, Germany); thiazolyl blue tetrazolium bromide (MTT) and purified agar were from Sigma-Aldrich Chemie GmbH (Germany).

Synthesis of PAA₁₈PPO₃₅PAA₁₈ triblock copolymer

First, poly(*tert*-butyl acrylate)-block-poly(propylene oxide)-block-poly(*tert*-butyl acrylate) (PrBA-PPO-PrBA) triblock copolymer was synthesized via atom transfer radical polymerization (ATRP) of *t*BA initiated by Br-PPO₃₅-Br in acetone using the pentamethyldiethylenetriamine/CuBr catalytic system as described elsewhere.²⁶ The ATRP macroinitiator was synthesized by reacting PPG₃₅ with 2.5 mol equiv of 2-bromoisobutyl bromide in dry toluene in the presence of triethylamine (2.5 mol equiv) at 20 °C for 24 h, as reported elsewhere.^{27–29} ATRP was carried out at 50 °C for 6 h. The copolymer was precipitated in hot water (60 °C) and filtered out. It was dissolved in tetrahydrofuran (THF), and the solution was eluted through a silica gel column to remove the Cu(II) complex. Finally, THF was removed under reduced pressure, and the copolymer was dried.

Next, the PrBA-PPO-PrBA copolymer was dissolved in freshly dried and distilled dichloromethane. Trifluoroacetic acid in a fivefold molar excess with respect to the *tert*-butyl groups was added. The reaction mixture was stirred at room temperature for 24 h, and then it was dialyzed against CHCl₃ for 72 h. The solvent was removed under reduced pressure, and the resulting PAA-PPO-PAA copolymer was dried.

Preparation of stabilized polymeric aggregates

Mixed polymeric aggregates were prepared by blending 1.05 g of PEO₁₉PPO₂₉-PEO₁₉ (P65) and 0.945 g of PAA₁₈PPO₃₅PAA₁₈ (1.5:1 molar ratio) triblock copolymer in 20 ml of THF under stirring. THF was then evaporated under reduced pressure, and 200 ml of deionized water (pH=9, NaOH) was added. Next, 0.3 g of PETA (15 wt.% of copolymers) was dissolved in 1 ml of acetone and was added to the colloid solution under stirring at 20 °C. Argon was bubbled through the solution for 45 min, followed by irradiation with a full-spectrum ultraviolet (UV) light (TQ 150 Original Hanau high-pressure 150 W mercury lamp provided with a quartz tube and a cooling quartz jacket) for 45 min. SPAs were purified by dialysis against water using a dialysis bag with an MWCO of 12 000 for 14 days, and then the cross-linking efficiency was gravimetrically determined as follows:

$$\text{Cross-linking efficiency (\%)} = \frac{\text{weight after dialysis}}{\text{weight before dialysis}} \times 100$$

Loading of SPAs with cisplatin

First, SPAs (0.05 g bearing 0.0165 g (1.75×10^{-4} mol) acrylate units) and cisplatin (0.0131 g, 0.44×10^{-4} mol) were separately dissolved in deionized water. The molar ratio of cisplatin to carboxylate groups was 1:4. Next, the solutions were mixed, and the pH was adjusted to 8. After 24-h stirring at room temperature in the dark, the yellowish and turbid sample became transparent and colorless. Unbound cisplatin was removed by dialysis against deionized water for 48 h using a membrane with an MWCO of 3500.

A second loading of SPAs was performed similarly using more cisplatin—that is, the molar ratio of cisplatin to carboxylate groups was 1:2. Unbound cisplatin was removed by dialysis against deionized water for 48 h using a membrane with an MWCO of 3500. During the dialysis, white sediment was formed. The loaded micelles were assigned as SPA-Pt.

Release of complexes of platinum(II) from the loaded SPAs

The release of complexes of platinum(II) from the polymeric carrier was studied by the dialysis method using a membrane with an MWCO of 3500 in PBS (0.01 M, pH 7.4, 0.14 M NaCl) and in citrate buffer solution (CBS; 0.1 M, pH 5). A solution (10 ml) of the SPAs-Pt with a known platinum(II)

Table 1 Molecular characteristics of the triblock copolymers used in this study

Copolymer composition (¹ H NMR)	DP of the outer blocks (¹ H NMR)	M _n (¹ H NMR)	M _n (SEC)	M _w /M _n (SEC)
PPO ₃₅ -b-(PtBA ₁₈) ₂	18	6600	7800	1.13
PPO ₃₅ -b-(PAA ₁₈) ₂	18	4600	—	—
PPO ₂₉ -b-(PEO ₁₉) ₂	19	3400	2100	1.10

Abbreviations: DP, degree of polymerization; ¹H NMR, nuclear magnetic resonance spectrometry; PAA, poly(acrylic acid); PPO, poly(propylene oxide); PEO, poly(ethylene oxide); PtBA, poly(tert-butyl acrylate); SEC, size exclusion chromatography.

concentration was placed into a dialysis bag and dialyzed against PBS (200 ml) at 37 °C and gentle stirring. Another 10 ml of the solution of the loaded carrier with a known platinum(II) concentration was also placed into a dialysis bag and was dialyzed against CBS (200 ml) at 37 °C and gentle stirring. Aliquots of 10 ml were taken from both buffer solutions outside of the dialysis bag at defined time periods, and fresh PBS or CBS of the same volume was added. The concentrations of the platinum(II) complexes present in the dialysate aliquots were measured, and the amount of platinum(II) released from the micelles was expressed as an accumulative percentage of the total platinum(II) available and plotted as a function of time.

Analytical methods

Nuclear Magnetic Resonance Spectrometry (¹H NMR): The ¹H NMR spectra were recorded in CDCl₃ and acetone-*d*₆ using a 250-MHz Bruker AC-spectrometer.

Size exclusion chromatography (SEC): PtBA-PPO-PtBA copolymer was analyzed by SEC at room temperature using PSS SDV-gel columns (5 μm, 60 cm, 1 × linear (10²–10⁵ Å), 1 × 100 Å), with THF as an eluent (flow rate = 1.0 ml min⁻¹) and a refractometer for the detection. Molecular weights and dispersity were determined using polystyrene calibration. SEC measurement of the PEO-PPO-PEO copolymer was performed using a Waters chromatograph equipped with a UV detector and a Waters Styragel column eluted with 0.5 wt.% LiBr containing dimethylformamide at 70 °C at a flow rate of 1 ml min⁻¹. PEO standards were used for calibration.

DLS measurements were performed at 25 °C using the Malvern Zetasizer in aqueous solution at a scattering angle 90°. ζ-Potential values were determined using the Smoluchowski equation relating the ionic mobilities with surface charge and are reported as averages of five repeated measurements.

UV spectra of cisplatin and SPAs-Pt solutions in deionized water were performed using a UV–vis spectrometer DU 800 (Beckman Coulter, Brea, CA, USA).

AFM: a multimode instrument equipped with a NanoScope 3D controller (MultiMode, Veeco Instruments Inc., Plainview, NY, USA) operating in the tapping mode in air with standard 125 μm single-crystal silicon cantilevers (Model TESP; Veeco Instruments Inc.) was used. The piezoelectric scanner had a scan range of ~10 × 10 μm². The aqueous solutions were spin-coated onto mica wafers in air at 1500 r.p.m. for 8 min. All samples were imaged at room temperature and were measured 24 h after coating.

Atomic spectrometric methods: the concentrations of platinum(II) present in the dialysate or loaded polymer solutions were determined by ICP-OES (HORIBA Jobin–Yvon ULTIMA 2, France). The intracellular levels of platinum were measured by ICP-MS (XSERIES 2; Thermo Scientific, Waltham, MA, USA).

Cell lines and culture conditions

The human cell lines K-562 (chronic myeloid leukemia) and HUT-78 (T-cell lymphoma) were purchased from the German Collection of Micro-organisms and Cell Cultures (DSMZ GmbH, Braunschweig, Germany). The cells were maintained as suspension cultures in a controlled environment using 10% fetal bovine serum and 0.002 M L-glutamine-supplemented RPMI-1640 liquid medium in cell culture flasks housed at 37 °C in a 'BB 16-Function Line' Heraeus incubator (Kendro, Hanau, Germany) with a humidified atmosphere and 5% CO₂. The exponential growth of the cell cultures was maintained by

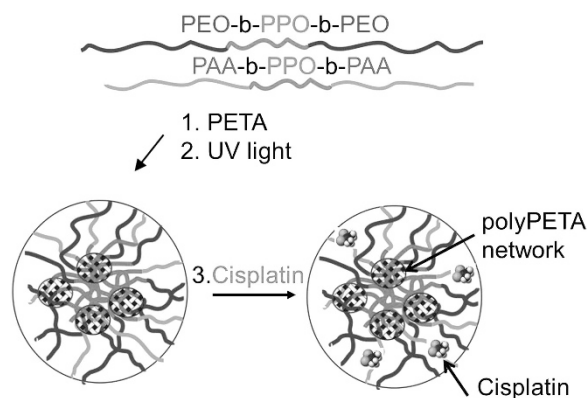


Figure 1 Sketch of stabilized polymeric aggregates (SPA; simplified structure) and their conjugates with cisplatin. A full color version of this figure is available at *Polymer Journal* online.

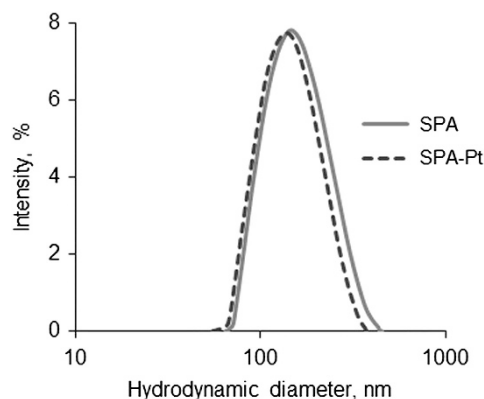


Figure 2 Particle size distribution of unloaded (solid line) and loaded (dotted line) nanocarriers determined by the dynamic light scattering (DLS) technique; the ratio [acrylate units]: [cisplatin] = 4:1. A full color version of this figure is available at *Polymer Journal* online.

removal of the cellular suspension and supplementation with fresh medium aliquots two or three times weekly.

The human cell lines MDA-MB-231 (estrogen-, progesterin- and HER-2/Neu-negative breast cancer) and HT29 (colon cancer) were obtained from the cell culture collection of IEMPAM-BAS. The cell cultures were grown in Dulbecco's modified Eagle's medium supplemented with 5–10% fetal bovine serum, 100 U ml⁻¹ penicillin and 100 mg ml⁻¹ streptomycin. The cell number and viability were determined by Trypan blue dye exclusion using a Countess Automated Cell Counter (Invitrogen). The cultures were kept in a humidified incubator (Thermo Scientific, HEPA Class 100) at 37 °C with 5% CO₂ in the air. For routine passages, adherent cells were detached using a mixture of 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid. The cell lines were passaged two or three times per week (1:2 to 1:3 split). The experiments were performed during the exponential phase of cell growth.

Cytotoxicity assays (MTT-dye reduction assay)

The cells were seeded in 96-well flat-bottomed microplates at a concentration of 1 × 10⁴ cells per well. After the cells were grown for 24 h to a sub-confluent state (~60–70%), the cells from monolayers were washed with PBS (pH 7.2) and were covered with media modified with a solution containing different concentrations (5, 10, 50, 100 and 200 μmol l⁻¹) of the compounds tested. Each solution was applied into 4–6 wells. Samples of cells grown in non-modified medium served as controls. After 72 and 120 h of incubation, the effect of the compounds on cell viability and proliferation was examined by the MTT colorimetric assay of cell survival, which was performed as described by

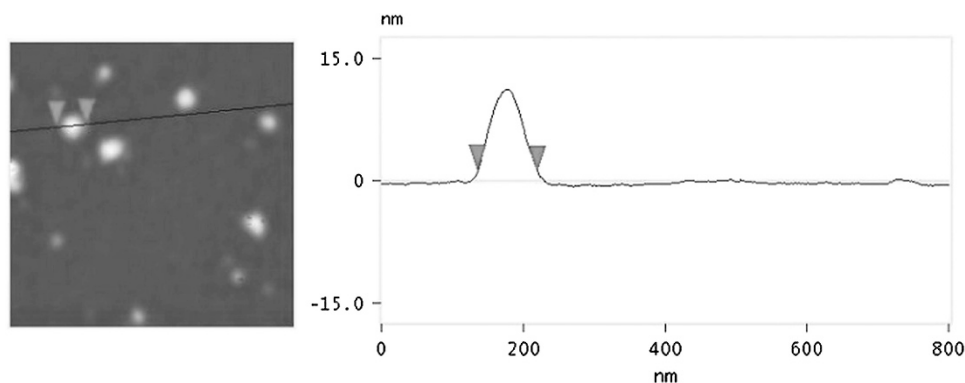


Figure 3 AFM image of loaded SPAs-Pt. A full color version of this figure is available at *Polymer Journal* online.

Mosmann.³⁰ The method consisted of 3 h of incubation with MTT solution (5 mg of MTT in 10 ml of Dulbecco's modified Eagle's medium) at 37 °C under 5% carbon dioxide and 95% air, followed by extraction with a mixture of absolute ethanol and dimethyl sulfoxide (1:1 vol/vol) to dissolve the blue MTT formazan.

The data are presented as the mean \pm s.e.m. Statistical differences between the control and treated groups were assessed using one-way analysis of variance followed by the Dunnett *post hoc* test and Origin 6.1TM.

Cellular uptake

Exponentially growing K-562, Hut-78 cells and MDA-MB-231 cells were seeded in sterile Petri dishes and were exposed to cisplatin as free drug and macromolecular conjugate SPAs-Pt at equimolar concentrations (corresponding to 20 and 50 μ M cisplatin, respectively) for 3 and 24 h. Thereafter, the cells were spun at 15 000 r.p.m. for 15 min, and the liquid cell medium was discarded. The treated cells were then washed three times with PBS and once with L-cysteine. Each wash was performed using the following procedure: 1 ml of the washing agent was added to the cells, which were then shaken by vortexing for one min and spun at 15 000 r.p.m. for 5 min. Next, the cells were resuspended in 50 μ l of conc. nitric acid and were heated at 90 °C for 1 h. Following the complete decomposition of the cells, the volume of the samples was adjusted to 200 μ l, and the intracellular levels of platinum were determined by ICP-MS and were presented as ng Pt/10⁶ cells.

RESULTS AND DISCUSSION

Synthesis of stabilized polymeric aggregates

An amphiphilic ABA triblock copolymer with a central PPO block and two outer poly(acrylic acid) blocks was synthesized by ATRP of *t*BA and subsequent hydrolysis of *Pt*BA blocks. First, the Br-PPO₃₅-Br macroinitiator was synthesized by the reaction of PPG₃₅ (MW 2000 g mol⁻¹) with 2-bromoisobutryl bromide as described elsewhere.²⁶ Next, a *Pt*BA-PPO-*Pt*BA copolymer was obtained via ATRP of *tert*-butyl acrylate, initiated by Br-PPO₃₅-Br, in the presence of the CuBr/pentamethyldiethylenetriamine catalytic system in acetone. The *Pt*BA-PPO-*Pt*BA copolymer was derivatized into PAA-PPO-PAA by hydrolysis of the outer *Pt*BA blocks with five equivalents of trifluoroacetic acid with respect to the *t*BA-units according to a known procedure.³¹

The number-average degree of polymerization of *Pt*BA and copolymer composition were calculated by ¹H NMR analysis (Table 1, Supplementary Figure S1). The determinations were based on the integrals of the peaks assigned (i) to the PPO protons at $\delta = 3.55$ p.p.m. (2H, -O-CH₂-CH-) and $\delta = 3.39$ p.p.m. (1H, -O-CH₂-CH-), (ii) to the *Pt*BA protons at $\delta = 1.53$ (2H, -CH₂-C(=O)H-) and $\delta = 1.44$ (9H, O-C(CH₃)₃). The hydrolysis of *Pt*BA with trifluoroacetic acid under mild conditions allowed for a quasi-quantitative

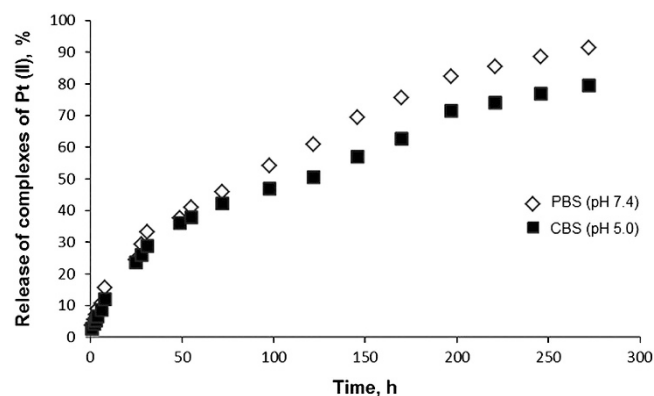


Figure 4 Release of platinum(II) complexes from drug-loaded stabilized SPAs-Pt micelles in phosphate-buffered saline (PBS; \diamond) and citrate buffer solution (CBS; \blacksquare).

conversion into PAA, as supported by the disappearance of the peak for the *tert*-butyl group at $\delta = 1.44$ p.p.m. (Supplementary Figure S2).

The SEC chromatogram of the *Pt*BA-PPO-*Pt*BA triblock copolymer showed a monomodal distribution and a rather low dispersity, indicating the high efficiency of the macroinitiators (Supplementary Figure S3). According to the NMR and SEC data, the polymerization of the *Pt*BA blocks was well controlled. Proton NMR and SEC analysis of PEO₁₉PPO₂₉PEO₁₉ confirmed that the second copolymer used for the preparation of mixed nanocarriers possesses defined composition and low dispersity as well (Table 1, Supplementary Figures S4 and S5).

Mixed polymeric aggregates were prepared by the co-assembly of PEO₁₉PPO₂₉PEO₁₉ and PAA₁₈PPO₃₅PAA₁₈ triblock copolymers in aqueous media at a molar ratio of 1.5:1.0. A slight excess of PEO₁₉PPO₂₉PEO₁₉ (PEO chains) was used to provide good colloidal stability of the system after complexation of the AA groups with cisplatin. The solution pH was adjusted to 9 to assure ionization of the PAA blocks. As a result, two highly hydrophilic chains, PEO and PAA, contributed to the steric stabilization of aggregates before the addition of cisplatin. Here, we used the term aggregates for self-assembled polymer structures possessing a hydrodynamic diameter larger than 100 nm.

The aggregates obtained were additionally stabilized by the cross-linking of PETA. PETA (15 wt% from the mass of polymers) was added to the colloid solution and was absorbed in the hydrophobic domains of the particles. The UV-assisted free radical polymerization of this tetrafunctional monomer resulted in the formation of an interpenetrating network of poly(PETA), in which the polymer

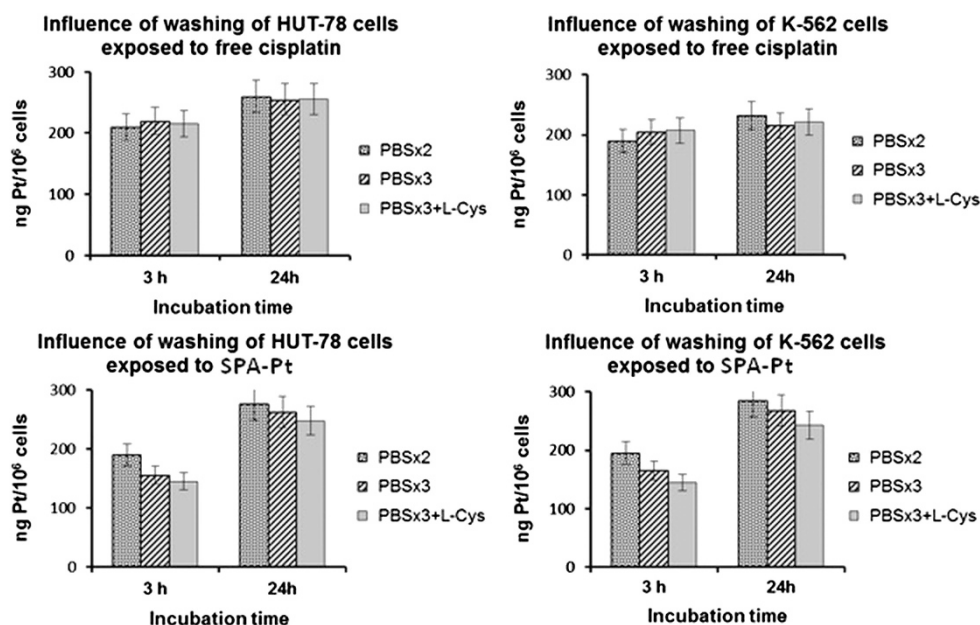


Figure 5 Intracellular levels of Pt in tumor cells measured after different washing procedures.

molecules were entrapped (Figure 1, left).^{32–34} Specifically, the poly (PETA) network is distributed in the form of small domains within the hydrophobic part of the aggregate and does not occupy the whole space.³² To remove the non-entrapped copolymer chains and acetone, after cross-linking, SPAs were dialyzed against water. The cross-linking efficiency gravimetrically determined was $78 \pm 4\%$. The composition of the stabilized aggregates was determined by ¹H NMR (Supplementary Figure S6) and was found to be very close to the composition of the original copolymer mixture, indicating that both copolymers equally participate in the aggregation process. Based on the known miscibility of PAA and PEO,³⁵ the two hydrophilic blocks were expected to be randomly dispersed within a mixed shell formed around an interior dominated by hydrophobic PPO chains (Figure 1). A DLS-analysis revealed a monomodal particle size distribution with a dispersity value of 0.29 ± 0.02 and a hydrodynamic diameter of 176.3 nm (Figure 2). The measured ξ -potential value of SPAs was -35 mV. Based on the DLS results and molar mass of the two copolymers, one may suggest that the blending of PEO₁₉PPO₂₉PEO₁₉ and PAA₁₈PPO₃₅PAA₁₈ copolymers in aqueous media and subsequent cross-linking of PETA lead to the formation of stabilized aggregates rather than stabilized micelles. The formation of such large aggregates can be attributed to the incomplete segregation of the hydrophilic and hydrophobic blocks at the experimental conditions reported. The crosslinking of PETA locked the structure formed and provided stability against fast dissociation of the aggregates upon dilution and changes in the media.

The SPAs studied in the present investigation are characterized by a combination of key features for cisplatin conjugation: nanosize, colloidal stability and hydrophilic shell with a high density of carboxylate groups that can reversibly exchange ligands with cisplatin and therefore regenerate the agent at physiological salt concentrations. Moreover, various biological experiments have revealed that SPAs do not have an adverse effect on neurogenesis, neurodevelopment and activation of the complement system.³⁶ All of the abovementioned advantages of the examined particles stimulated our further research that included testing of their loading capacity and release profile of

platinum(II) complexes, as well as biological assessment of cytotoxicity and cellular uptake of the cisplatin-loaded polymeric aggregates.

Loading of SPAs with cisplatin

The stabilized aggregates were loaded with cisplatin by adding the drug to the aqueous solution of SPAs under the following experimental conditions: cisplatin concentration = $2 \mu\text{g ml}^{-1}$, molar ratio [acrylate units]: [cisplatin] = 4:1, pH = 8, temperature = 22 °C and reaction time = 24 h. At the end of the loading, the solution was colorless and transparent. The UV spectrum of the loaded SPAs displayed disappearance of the absorption at 300 nm, at which wavelength the cisplatin solution showed a maximum.³⁷ Next, 48 h of dialysis was followed to remove the unbound drug. The loading of SPAs is schematically presented in Figure 1.

To increase the amount of loaded cisplatin, a molar ratio [acrylate units]: [cisplatin] = 2: 1 was applied. White sediment appeared in the solution, obviously due to cisplatin-induced hydrophobization and probably inter-particle crosslinking, leading to the formation of larger particles. Therefore, further experiments continued with cisplatin-loaded SPAs at a molar ratio [acrylate units]: [cisplatin] = 4:1.

A slight decrease from 176.3 to 170.6 nm of the hydrodynamic diameter and narrower size distribution (dispersity value 0.21 ± 0.09) of SPAs after their loading with the drug was monitored by DLS measurement (Figure 2). The decrease was attributed to cisplatin conjugation, leading to intramicellar crosslinking of the poly(acrylic acid) chains and increased hydrophobicity of the particles. Nevertheless, the observed size reduction was $<5\%$ due to the stabilizing effect of the tridimensional network of poly(PETA) and mixed shell of the particles. As expected, the drug conjugation resulted also in a decrease of the ξ -potential value of the loaded particles to -30 mV. ICP-OES analysis revealed that, under these experimental conditions, a drug-loading efficiency of 76% was achieved. The amount of the immobilized cisplatin was determined to be 20 wt.% of the mass of the particles. The aqueous solutions of the conjugates were stable in time, and no precipitation was observed for more than one year.

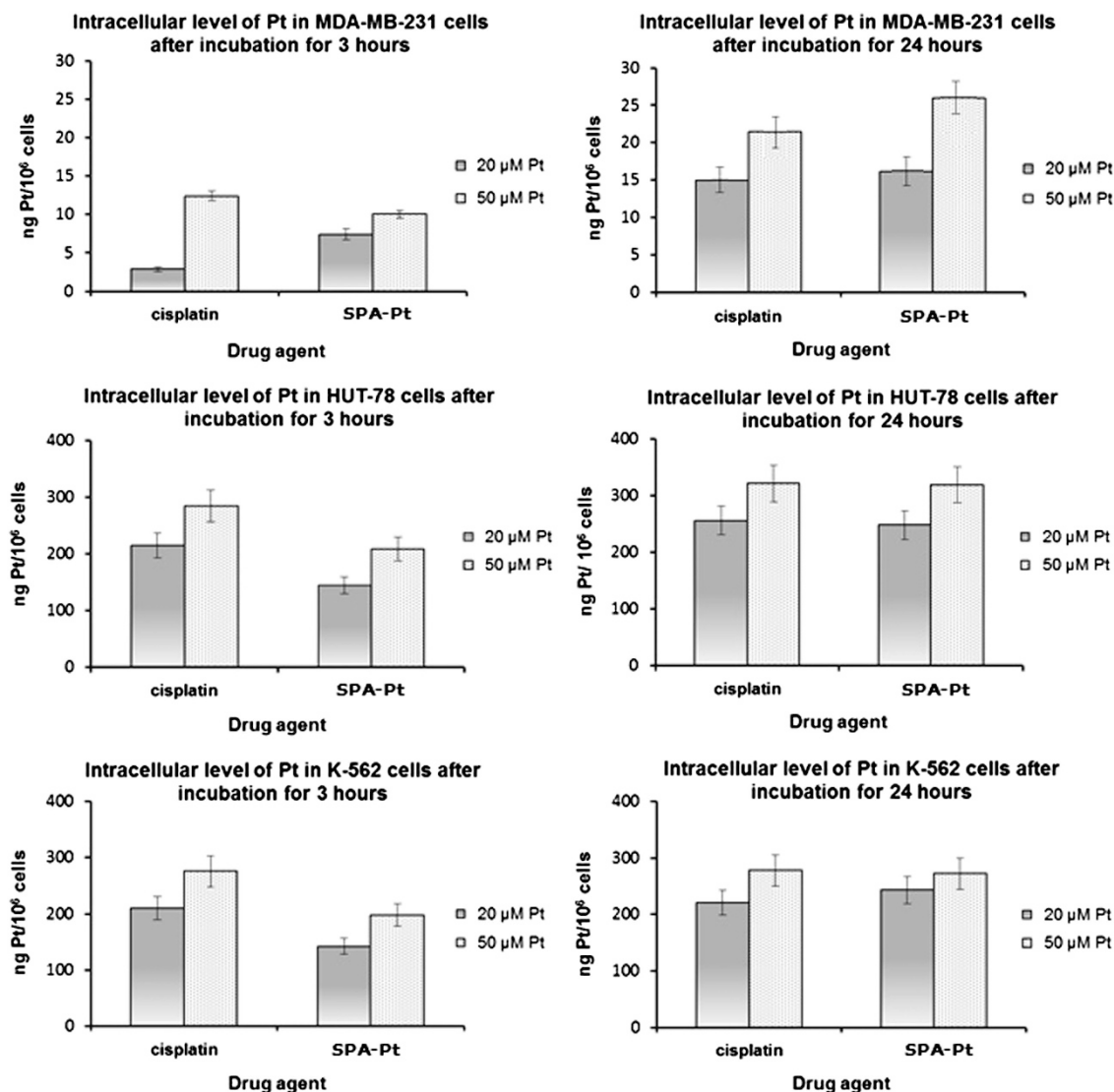


Figure 6 Intracellular concentration of platinum established after treatment of the tumor cells with free and immobilized cisplatin after exposure for 3 and 24 h.

The loaded SPAs were visualized by AFM. The AFM image of SPA-Pt (Figure 3) revealed spherically shaped particles with an average diameter of 85 nm. This diameter was smaller than the diameter measured by the method of DLS, which is attributed to the shrinkage of the SPAs after drying.

Release of platinum(II) complexes from SPAs

The release of platinum(II) complexes from the polymer carrier was studied by the dialysis method. Release of the complexes was investigated in two different buffer solutions—PBS (0.01 M PBS, pH 7.4, 0.14 M NaCl) and citrate buffer solution (0.1 M CBS, pH 5)—reflecting the conditions encountered in the plasma and intracellular compartments (lysosomes), respectively. In each of the two buffers, a dialysis bag containing a solution of loaded particles with a known drug content was immersed. The obtained release profiles in the two buffered media (shown in Figure 4) indicate a sustained manner of drug release from the polymer carrier. The observed sustained release of the platinum(II) complexes will be of a great advantage for the passive drug targeting to solid tumors because of the

prolonged time periods known to be required for macromolecular drugs to accumulate in solid tumors through the bloodstream.

In both buffer solutions, a high extent of the release of platinum(II) complexes was observed. Within 12 days, more than 90% of the loaded complexes were released in PBS and approximately 80% in CBS. No clearly expressed initial burst effect was noticed in both of the buffered media, possibly indicating that the whole amount of drug was complexed to the copolymer via ligand exchange. Thus, no platinum release was evident in distilled water on storage. Its replacement with a solution containing ligands to compete with the functional groups of the polymer carrier or affording conditions for complex hydrolysis was needed for the release process. It was earlier noted^{12,13,24} that the presence of chloride ions in the physiological solution was essential for the release process due to the inverse ligand substitution reaction. Formation of aqua or hydroxo platinum(II) complexes, much more reactive species, was also possible.³⁸ The analytical method (ICP-OES) used for the determination of the Pt content in the dialysates did not differentiate between the platinum complex species; therefore, the total release of platinum(II) complexes is presented in Figure 4.

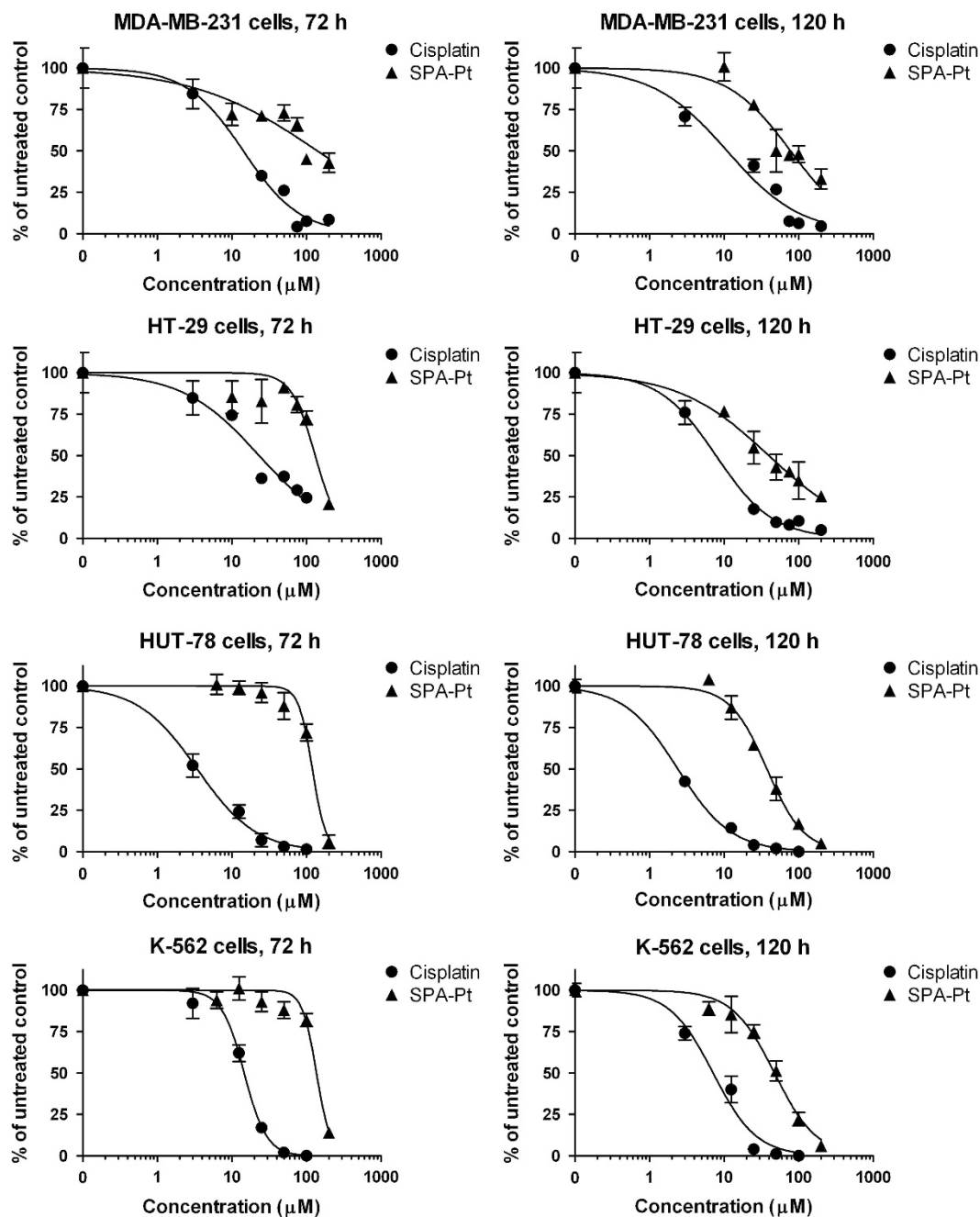


Figure 7 Cytotoxic effect of free (●) and immobilized cisplatin (▲).

Nevertheless, the combination of high-loading efficiency, excellent stability upon storage and a high degree of drug release in both PBS and CBS (contrary to the vehicles with high carboxylic group density) is the main advantage of SPA-Pt compared with the previously reported systems. Thus, SPAs-Pt could be regarded as a promising system for cisplatin delivery, providing predictability of the pharmacological properties.

Cellular uptake

To exert its cytotoxic activity, cisplatin has to reach its ultimate pharmacological target, DNA, in the cells. On this basis, attaining sufficient intracellular levels of the drug is a crucial prerequisite for its optimal antineoplastic activity. To define whether and to what extent the

investigated SPAs allows the intracellular accumulation of the immobilized drug, exponentially growing tumor cells (HUT-78, K-562 and MDA MB-231) were exposed to cisplatin as free drug and the nanoconjugate SPA-Pt at a concentration corresponding to $50 \mu\text{M}$ cisplatin for 3 and 24 h. After separating the cells from the medium, they were washed in three different ways: (1) twice with PBS, (2) three times with PBS and (3) three times with PBS plus once with L-cysteine. Thereafter, the intracellular concentration of platinum was determined using the ICP-MS technique. The results showed (Figure 5) that the procedure for the cells exposed to SPAs-Pt should include triple washing with PBS and a single washing with L-cysteine. However, after incubation with free cisplatin, twofold or threefold washing of the cells with PBS was sufficient to remove the extracellular drug, as reported elsewhere.^{39–41}

Table 2 IC₅₀ values for free and immobilized cisplatin

Compounds	IC ₅₀ (μmol l ⁻¹)							
	MDA-MB-231		HT-29		Hut-78		K-562	
	72 h	120 h	72 h	120 h	72 h	120 h	72 h	120 h
Cisplatin	14.25	10.96	23.24	7.79	3.42	2.39	14.64	7.14
SPA-Pt	140.6	77.82	131.20	39.82	120.10	37.55	136.5	47.40

The developed washing procedure was applied for the determination of the intracellular platinum concentration, and results were obtained for free cisplatin compared with those for the immobilized drug (Figure 6). It was noted that, for short periods of incubation, free cisplatin was more rapidly and completely accumulated in the three cell lines tested than the immobilized drug. This could be at least partly ascribed to the established negative zeta potential of the particles. Considering the natural negative surface polarization of living cells, the encountered charge of the prepared particles could condition some delay in the cellular uptake of the carrier and encapsulated cargo.

Nevertheless, the conjugated drug was also effectively internalized in MDA-MB-231 and K-562 tumor cell lines. In the case of the 24-h-long incubation, the levels of intracellular platinum were comparable to those attained after treatment with the free drug. It should be emphasized that the level of platinum uptake is not compatible with the observed relatively slow rate of drug release, especially considering the short incubation periods. Therefore, it can be inferred that the established cellular accumulation of cisplatin can be ascribed to the uptake of both pre-released complexes and up-taken drug conjugates. Although the negative zeta potential causes some delay in the cellular uptake of the particles, it is beneficial regarding biocompatibility, plasma stability and pharmacokinetics.

Cytotoxicity

To evaluate whether cisplatin loaded in SPAs retains its cytotoxic activity, an *in vitro* cell viability study against a panel of four human tumor cell lines [MDA-MB-231 (breast cancer), HT29 (colon cancer), HUT-78 (T-cell lymphoma) and K-562 (chronic myeloid leukemia)] was carried out. The cellular viability was assessed by the MTT-dye reduction assay, and the corresponding IC₅₀ values were calculated from the dose-response curves using non-linear regression analysis. From the data shown in Figure 7 and Table 2, it is evident that the immobilization of cisplatin in SPAs has led to an increase in the IC₅₀ values. The observed lower cytotoxicity of the entrapped drug is a consequence of the sustained drug release from the carrier. At the end of the second incubation period of the MTT assay (120 h of incubation), ~60% in PBS and 50% in CBS from the loaded drug were released. At the longer exposure period of 120 h, the differences between the equieffective levels of free vs immobilized platinum were less prominent.

It was reported that cisplatin-loaded micelles were effectively accumulated (20-fold higher than free cisplatin) in solid tumors (Lewis lung carcinoma cells) in a passive targeting manner.⁴² In a recent study, polymeric micelles derived from the styrene-maleic acid copolymer and cisplatin demonstrated a marked antitumor effect against S-180 solid tumors and less cytotoxicity than the free drug due to tumor-selective drug delivery based on the enhanced permeability and retention effect.⁴³ On that basis, considering the results obtained

for SPAs-Pt conjugates, it could be expected that the latter would exhibit prolonged blood circulation, protecting the drug from undesired deactivation and achieving selective accumulation in tumor tissue. To address this objective, *in vivo* experiments are envisaged.

CONCLUSION

A detailed study of SPAs comprising poly(acrylic acid) chains proved their applicability for cisplatin conjugation, leading to enhanced drug solubility. The nanocolloidal solution of the loaded SPAs displayed excellent storage stability. The functionalized shell containing PAA determines the high-loading efficiency, while the hydrophilic PEO chains contribute to the nanocolloid system stability and high percentage of released complexes of platinum(II) (above 80% from the loaded drug). It should be emphasized that platinum(II) complexes were released in PBS (pH 7.4) and in CBS (pH 5) in a sustained way. The established lower cytotoxicity for the immobilized drug than the free one is consistent with the sustained manner of drug release from the carrier. The cellular uptake of the conjugated drug was comparable to that of free cisplatin. Advantageous characteristics as a cisplatin delivery vehicle include the high drug payload, excellent stability upon storage and sustained drug release of the conjugated drug over 2 weeks. Thus, pharmacologically, the SPAs-Pt conjugate is a promising candidate for cisplatin delivery.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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