

Preparation, Characterisation, and Measurement of the *in vitro* Cytotoxicity of Mesoporous Silica Nanoparticles Loaded with Cytotoxic Pt(II) Oxadiazoline Complexes

G. Wagner, R. Herrmann

Abstract—Cytotoxic platinum compounds play a major role in the chemotherapy of a large number of human cancers. However, due to the severe side effects for the patient and other problems associated with their use, there is a need for the development of more efficient drugs and new methods for their selective delivery to the tumours. One way to achieve the latter could be in the use of nanoparticulate substrates that can adsorb or chemically bind the drug. In the cell, the drug is supposed to be slowly released, either by physical desorption or by dissolution of the particle framework. Ideally, the cytotoxic properties of the platinum drug unfold only then, in the cancer cell and over a longer period of time due to the gradual release. In this paper, we report on our first steps in this direction. The binding properties of a series of cytotoxic Pt(II) oxadiazoline compounds to mesoporous silica particles has been studied by NMR and UV/vis spectroscopy. High loadings were achieved when the Pt(II) compound was relatively polar, and has been dissolved in a relatively nonpolar solvent before the silica was added. Typically, 6-10 hours were required for complete equilibration, suggesting the adsorption did not only occur to the outer surface but also to the interior of the pores. The untreated and Pt(II) loaded particles were characterised by C, H, N combustion analysis, BET/BJH nitrogen sorption, electron microscopy (REM and TEM) and EDX. With the latter methods we were able to demonstrate the homogenous distribution of the Pt(II) compound on and in the silica particles, and no Pt(II) bulk precipitate had formed. The *in vitro* cytotoxicity in a human cancer cell line (HeLa) has been determined for one of the new platinum compounds adsorbed to mesoporous silica particles of different size, and compared with the corresponding compound in solution. The IC₅₀ data are similar in all cases, suggesting that the release of the Pt(II) compound was relatively fast and possibly occurred before the particles reached the cells. Overall, the platinum drug is chemically stable on silica and retained its activity upon prolonged storage.

Keywords—Cytotoxicity, mesoporous silica, nanoparticles platinum compounds.

I. INTRODUCTION

MESOPOROUS silica nanoparticles have been studied in much detail and proposed as vehicles for the targeted delivery of drugs [1]. Among the promising properties of this

material are the straightforward synthesis from easily accessible starting materials and the fact that both particle size and pore size can be tailored according to the reaction conditions applied. Additionally, the favorable adsorption characteristics for organic compounds allow for high loading and slow release in many cases, and the relatively high chemical inertness is important to avoid drug decomposition. Silica particles generally exhibit a high degree of biocompatibility, and their surface can be modified with biomarkers, to allow for targeting of cells with specific pathogenic characteristics.

Pioneering studies targeted the drug delivery of the anti-psoriatic Alendronate bound to propylamine-modified MCM-41 [2], the antibiotic Vancomycin adsorbed to CdS capped MCM-41 nanoparticles and its release through the action of reducing agents [3], or the photoresponsive release of the anti-cancer drug Paclitaxel from MCM-41 capped with Au nanoparticles [4]. In cancer chemotherapy, platinum complexes such as cisplatin, carboplatin and oxaliplatin play a major role, and targeted delivery to the cancer cells appears to be a promising approach to enhance drug efficiency and reduce toxicity connected with the adverse side effects experienced by the patient. Thus, it has been found that mesoporous silica microparticles delay and enhance the cytotoxicity of cisplatin and transplatin to leukemia cells [5], and delayed release of cisplatin from hybrid mesoporous silica particles has also been observed in another study [6]. The cisplatin delivery and anticancer effect was also examined using Pt(IV) cisplatin prodrugs attached to silica particles. In the reductive cellular environment, a Pt(II) species is formed and released [7]. Carboxyl-modified silica particles have been used to adsorb Pt drugs with high efficiency, leading to a higher loading [8], and this strategy has also been employed for the synthesis of mesoporous silica nanoparticle-oxaliplatin conjugates [9]. The adsorption kinetics of carboplatin to MCM-41 has been studied, but in this case the overall uptake seems to be fairly low [10].

In our previous work, we developed a type of cytotoxic platinum compounds bearing oxadiazoline ligands [11]. These compounds are of particular interest because of the absence of cross-resistance with cisplatin and carboplatin in *in vitro* experiments, offering a chance for complementary treatment of patients with an acquired resistance to the established drugs. Unfortunately, these compounds are relatively poorly

G. Wagner is with the Department of Natural Sciences, University of Chester, Thornton Science Park, Pool Lane, Ince, Chester, CH2 4NU, UK (corresponding author; phone: +49 (0)1244 512427; fax: +49 (0)1244 511300; e-mail: g.wagner@chester.ac.uk).

R. Herrmann is with the Institute of Physics, University of Augsburg, Universitätsstr. 1, D-86135 Augsburg, Germany (e-mail: rudolf.herrmann@physik.uni-augsburg.de).

soluble in aqueous media, making their administration difficult. There is also no active mechanism at work that would restrict uptake and activity to cancer cells. With the present work we attempt to address and overcome some of these problems.

II. RESULTS AND DISCUSSION

A. The Platinum Compounds

The platinum compounds used in this work were synthesized and characterized as described previously [12]–[14]. Their structures are shown in Fig. 1, together with an outline of the synthesis. All compounds have a *trans*-configured PtCl₂(oxa-diazoline) moiety in common, but the residual ligand varies from benzonitrile (in 2) to oxadiazoline (in 3), pyridine (in 4) and 7-nitro-1,3,5-triazadamantane (in 5 and 6).

B. The Mesoporous Silica Particles

MCM-41-type mesoporous silica nanoparticles have been synthesized as described in the literature [15], starting from CTAB and TEOS in aqueous methanol solution. The ratio MeOH:H₂O determines the particle size, whereas the pore size is defined by the nature of the surfactant used in the formation of the template micelles. The general synthetic strategy is shown in Fig. 2. Three samples, A, B and C, have been made, using MeOH:H₂O ratios of 0.81, 0.67 and 0.54, under otherwise identical conditions. The samples were characterized by TEM, BET/BJH and C,H,N elemental analysis, and the results are summarized in Table I.

Fig. 3 shows the result of a typical TEM experiment. The spherical particles are well dispersed but show a considerable size distribution. The program *ImageJ* [16] was used to determine the average size and the standard deviation over a representative number of particles, typically 50 to 200. The smaller particles thereby showed larger standard deviations and also had a slight tendency to agglomerate.

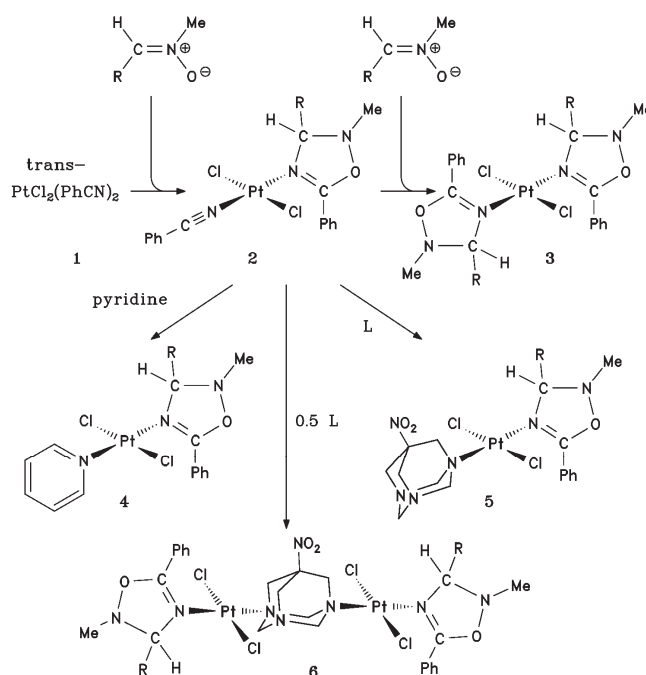


Fig. 1 Synthesis of the platinum compounds used in this work [12]–[14]; R = 2-methoxyphenyl; L = 7-nitro-1,3,5-tri-azaadamantane

TABLE I
CHARACTERISATION OF THE SILICA PARTICLES A, B AND C

Quantity [unit]	Method	A	B	C
Average size [nm]	TEM	229	120	36
Stand. deviation [nm]	TEM	23,3	42.8	25.2
Surface area [m ² g ⁻¹]	BET	603.53	746.47	802.98
Pore diameter [nm]	BJH (adsorption)	2.618	2.627	2.095
Pore volume [cm ³ g ⁻¹]	BJH (adsorption)	0.652	0.731	0.974
% C	microanalysis	0.04	0.06	0.16
% H	microanalysis	0.03	0.04	0.07
% N	microanalysis	0	0	0

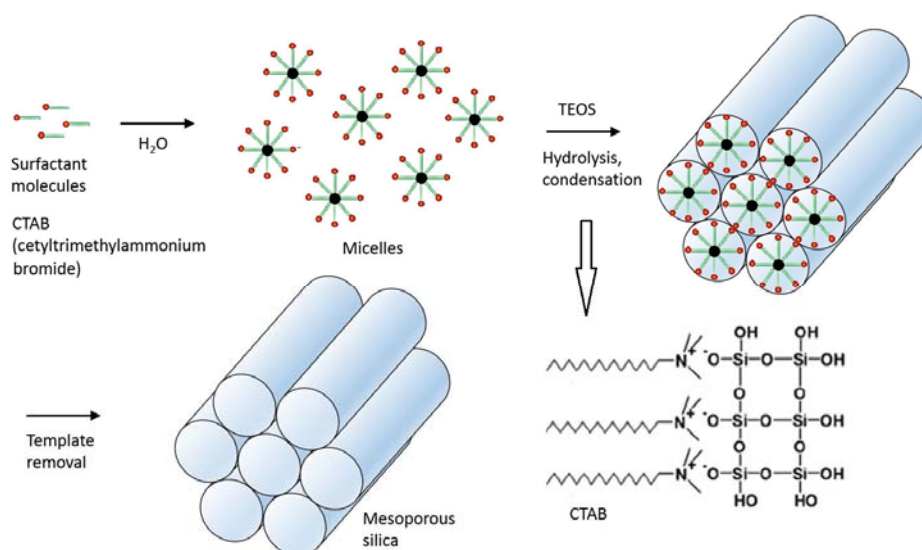


Fig. 2 Strategy for the synthesis of the MCM-41 type mesoporous silica nanoparticles

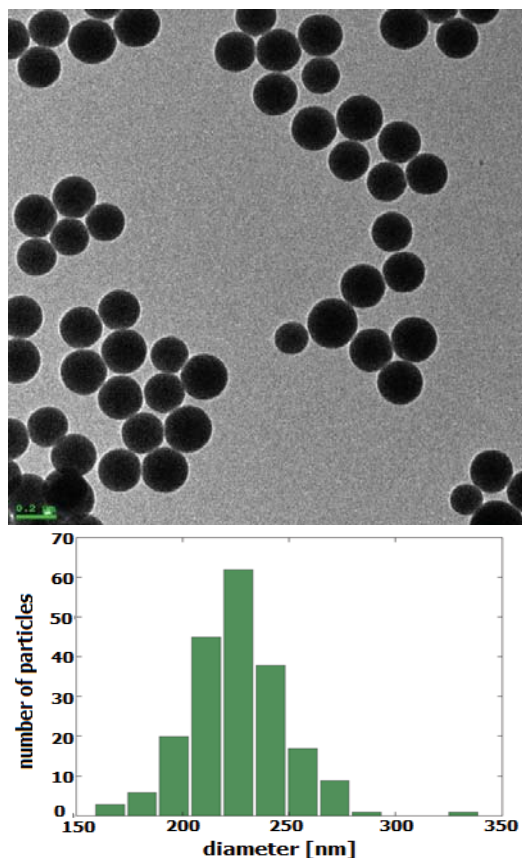


Fig. 3 TEM of the mesoporous silica particles **A** (top), with scale bar = 0.2 μm. Size distribution measured over a total of 202 particles (bottom); average size = 229 nm, standard deviation = 23.3 nm

BET/BJH provide consistent data about the surface area and pore diameter and volume. All values are in a range one would expect for MCM-41 type particles, due to the use of the same surfactant (CTAB), the pore diameter is constant, apart from sample **C**, where the pores seem to be a bit smaller. From the C,H,N microanalysis it can be concluded that the surfactant has been completely removed from the pores of the particles **A** and **B**. **C** seems to contain a small amount of residual CTAB, although the same extraction procedure was employed. This

could account for the slightly smaller pore size observed for this sample.

C. Loading of the Particles with the Pt Compounds

¹H NMR was used to follow the uptake of the Pt compounds by the silica particles. At first, the adsorption kinetics were addressed. Compound **5** (3.05 mg, 4.5 mmol) was dissolved in 0.6 ml of CDCl₃ and a ¹H spectrum was taken. The intensity ratio between the residual CHCl₃ signal of the solvent and the aromatic signal of **5** at 8.95 ppm was used as a reference = 100%. Then, 50 mg of **A** was added to the sample and ¹H spectra were measured in regular time intervals over a period of 24 hours. Between the experiments, the sample was shaken and then left for sedimentation of the particles. Over time, the relative signal intensity, showing the amount of dissolved compound, decreases exponentially to disappear below the detection limit after about 6 h. Compounds **2**, **3**, **4** and **6** show fairly similar adsorption rates, and uptake is complete after 6 – 10 h. During the experiments, we did not observe other signals than those of the platinum compound, suggesting sufficient stability in the presence of the silica particles.

Next, we studied the loading capacity of the silica materials with respect to the individual Pt compounds, using a similar technique. Again, 3.05 mg of **5** was dissolved in 0.6 ml of CDCl₃ and a ¹H spectrum was taken to obtain the relative signal intensity for 100% dissolved compound. The silica material **A** was added in 5 mg portions. After each addition, the sample was left at room temperature for 24 h, and then a ¹H NMR was recorded. This was repeated until the signals of **5** could be no longer detected. The results for **5** and the other Pt compounds is shown in Fig. 4. Thus, 3.05 mg of **5** can be adsorbed onto 30 mg of **A**, suggesting a loading of approx. 10 weight %. The other compounds are less polar than **5**, and the loading, expectedly, is a little lower, in a range of 8-9 weight %.

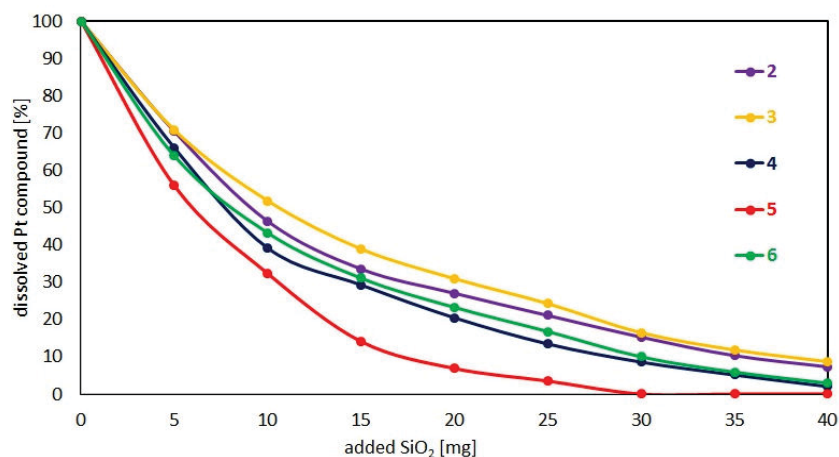


Fig. 4 Loading capacity of silica particles **A** for the platinum compounds **2** – **6**, as determined by ¹H NMR spectroscopy

To determine the concentration of the platinum compounds left in solution, the particles were removed by centrifugation and the supernatant solution examined by UV/vis spectroscopy at 278 nm. Typical concentrations in a range of 2×10^{-5} (for **5**) to 4×10^{-5} mol/l (for **3**) were found, corresponding to 0.4 to 0.9 % of the initially applied amount of the Pt compounds.

D. Characterisation of the Platinum-Loaded Particles

The Pt loaded particles thus prepared were isolated by centrifugation and dried at 45 °C in a vacuum of 12 mm (membrane pump) for 6 h. The results from C, H, N analysis, shown in Table II, is consistent with an approximate 10 weight % loading for compound **5**, and slightly lower loadings in a range of 9 weight % for the other compounds.

TABLE II

C, H, N MICROANALYSIS DATA OF THE SILICA PARTICLES **A**, **B** AND **C**, LOADED WITH PLATINUM COMPOUNDS **3** AND **5**

Quantity [unit]	A2	B2	C2	A3	B3	C3
% C	4.28	4.19	4.36	4.56	4.62	4.67
% H	0.36	0.47	0.57	0.35	0.48	0.59
% N	0.58	0.60	0.51	0.62	0.66	0.59
Loading [weight%]	9.3	9.6	8.9	9.2	9.5	9.1
Quantity [unit]	A4	B4	C4	A5	B5	C5
% C	4.02	4.10	4.18	3.92	3.88	3.75
% H	0.38	0.40	0.56	0.47	0.52	0.51
% N	0.61	0.65	0.63	1.25	1.21	1.18
Loading [weight%]	9.4	9.7	9.6	10.4	10.2	9.8
Quantity [unit]	A6	B6	C6			
% C	3.62	3.59	3.71			
% H	0.31	0.34	0.53			
% N	0.89	0.80	0.82			
Loading [weight%]	9.8	9.3	9.5			

The Loading was calculated from the C and N analytical data and averaged.

The weight % loadings were determined from the C and N microanalysis data only and averaged over these two elements. H was not used because it was always found too high relative to C and N, and this can be attributed to residual moisture adsorbed to the particles. At higher drying temperatures, however, the Pt compounds noticeably decomposed and a color change from pale yellow to brownish could be observed. Also the C, H, N analysis taken afterwards gave results inconsistent with the Pt compound used.

TEM experiments confirmed that the particle size and distribution has not changed during adsorption of the platinum compounds, although a slightly higher tendency towards agglomeration could be observed. This was particularly pronounced with platinum loaded particles derived from silica particles **C**, which could not be re-suspended by prolonged sonication and are thus unsuitable for cytotoxicity studies.

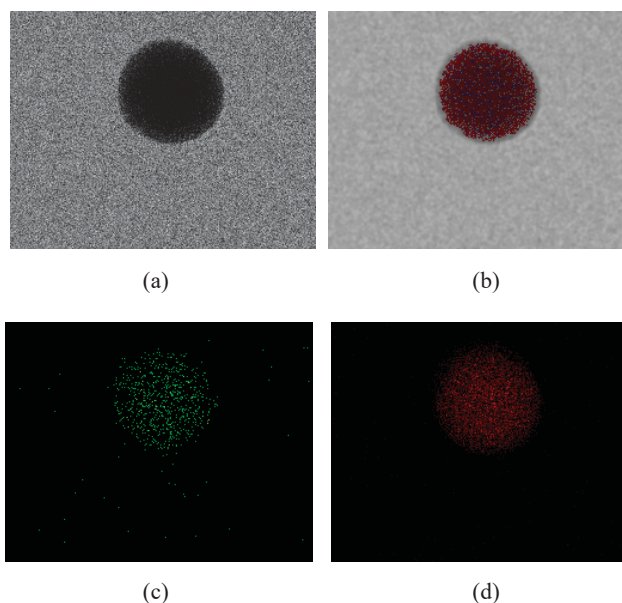


Fig. 5 Elemental distribution measured by EDX mapping. (a) TEM image of one representative particle of **A5**; (b) TEM image overlaid with Si-L and Pt-M EDX scans; (c) Pt-M EDX scan; (d) Si-L EDX scan

The elemental distribution, in particular the distribution of Pt across the silica particles was determined from EDX scans, using the Si-K and Pt-M line. As can be seen in Fig. 5 for a representative particle of **A5**, there is a clear co-localization of platinum and silicon, and the homogenous distribution of Pt across the silica particles is indicative of molecular adsorption (rather than nucleation and co-crystallisation).

E. In vitro Cytotoxicity

The *in vitro* cytotoxicity of **A5**, **B5** and free **5** in solution in the epithelial human cancer cell line HeLa [17] was determined by means of the CellTiter-Glo[®] luminescent cell viability assay [18], as described in the experimental part. HeLa cervical cancer cells are known to respond to cisplatin with an IC₅₀ of 1.1 to 1.3 μM [19], and compound **5**, with an IC₅₀ of about 4.8 μM, is only slightly less potent. For carboplatin, an IC₅₀ of 10.0 μM is reported [20].

The Pt loaded silica materials **A5** and **B5** were applied to HeLa cells as aqueous sonicated suspension, at Pt levels equal to those used before for free **5**. For comparison, pure **A** and **B** were also tested, to demonstrate the biocompatibility of the material in a concentration range used in the other experiments. Overall, the cell viability profile of **A5** and **B5** strongly resemble that of the free drug and also the IC₅₀ data are practically the same. This suggests that the release of the Pt(II) compound was relatively fast and possibly occurred before the particles reached the cells. Overall, however, the platinum drug is chemically stable on silica and retained its activity even upon prolonged storage of several months.

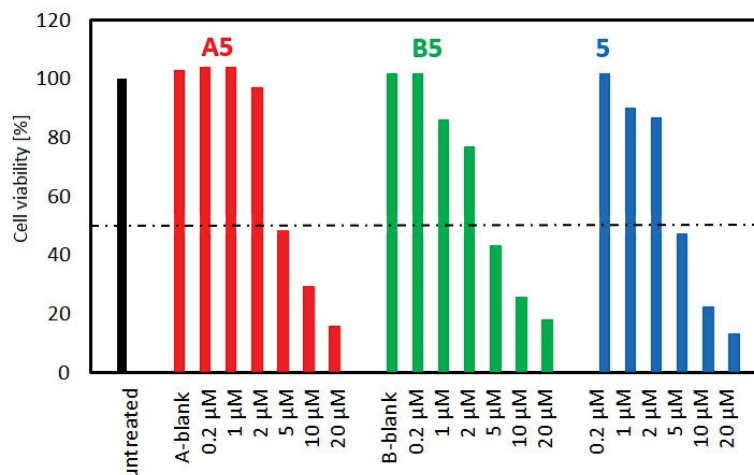


Fig. 6 Cell viability after 24 h of incubation in the presence of the free Pt compound **5** and the silica-bound analogues **A5** and **B5**, as well as the Pt-free silica nanoparticles A-blank and B-blank. Data are mean values over three experiments and given relative to untreated cells = 100 %. The dashed line indicates a cell viability of 50 %

III. CONCLUSION

In summary, we showed in this work that a series of novel platinum compounds, which had been identified as promising candidates for cancer treatment before, can be adsorbed to mesoporous silica nanoparticles as drug delivery systems. Depending on the polarity of the compounds, loadings of 9 – 10 weight % can be achieved, which is in a practicable range for drug delivery systems, in particular when the drug is highly active. The distribution over the particles is fairly homogenous, as shown by EDX experiments, suggesting that binding takes place by molecular adsorption (rather than co-crystallisation). The resulting materials exhibit a good shelf life and general stability, and the handling and dosage of aqueous suspensions is far easier than the use of solutions of the poorly water soluble free platinum compounds. *In vitro* experiments with one of the platinum compounds (**5**) demonstrated a cytotoxicity similar to cisplatin in HeLa cervical cancer cells, and the activity is fully retained when the silica bound materials **A5** and **B5** were used. However, the drug release seems to occur pretty fast and most likely before the particles enter the cells.

Controllable drug release has not been achieved yet but this will be addressed in our future work. Among the possible strategies, one can envisage covalent binding of the Pt compound via an enzymatically hydrolysable functional group, so that cleavage from the silica particle only takes place after the particle has entered the cell. Another option is the secondary modification of the Pt-loaded silica particles, where a bio-degradable material is used to block the pore exits of the Pt loaded particles and prevent premature loss of the active drug. Once drug release can be actively controlled, improvements with respect to the delivery will be aimed for. So far, only some degree of passive targeting can be expected to occur as a result of the leaky vasculature and impaired lymphatic function of tumor tissues. More efficient active targeted delivery to cancer cells should be feasible by modifying the particles with a relevant biomarker. Work in

this direction is in progress.

IV. EXPERIMENTAL PART

Instrumentation: ^1H NMR spectra were recorded on a Bruker 400 MHz spectrometer in CDCl_3 solution. C,H,N elemental analysis data were obtained from a Elemental Vario Micro Cube instrument. N_2 adsorption-desorption isotherms were recorded on a Quadrachrome QuadraSorb SI automated sorption analyzer. The samples were degassed at 60°C for 5 h. Specific surface areas were calculated from the adsorption data in the low pressure range using the BET model and pore size was determined using the BJH method. TEM micrographs and EDX mappings were performed on a JEM 2100 F instrument (JEOL, Japan), operating at 200 kV. Dispersions of the particles in ethanol were applied onto carbon-coated copper grids (Plano, Formvar/coal-film on a 200mesh net).

Particle preparation: CTAB (3.94 g) was dissolved in MeOH (360 g for **A**, 320 g for **B** and 280 g for **C**), and water (440 g for **A**, 480 g for **B** and 520 g for **C**) and 2.28 ml 1M NaOH were added under stirring at 400 rpm. After 10 min, TEOS (1.3 ml) was added dropwise, and stirring was continued for at least 8 h. After addition of NH_4NO_3 (30 g) the particles were isolated by centrifugation (10 min, 10000 rpm) and washed with EtOH (50 ml). The particles were re-suspended into acidic EtOH (250 ml containing 1 g conc. HCl) by ultrasound treatment for 1 h, and then centrifuged. This process was repeated three times. After a final wash with pure EtOH the particles were dried at 160°C and a vacuum of 12 mm overnight.

Cytotoxicity studies: HeLa cells were obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen) and cultured as attached monolayers in Dulbecco's Modified Eagle's Medium (DMEM, high glucose 4.5 g/L) with 10% fetal bovine serum, 1% MEM non-essential amino acids and 1% Penicillin/Streptomycin supplements. Cytotoxicity was determined by means of the luminescent cell viability assay CellTiter-Glo[®] [18], obtained from Promega, which measures

the ATP content of metabolically active cells (as a measure for the number of living cells), based on a luciferase reaction. Cultured cell monolayers were converted into single cell suspension by treatment with trypsin-EDTA solution, and then seeded into 96-well tissue culture plates at a density of 1×10^5 cells per 100 μl . Cells were allowed to settle under standard culture incubation conditions for 24 h and then treated with freshly prepared sonicated suspensions of the platinum-loaded particles, or solutions of the Pt compound, at Pt concentrations in the cell medium of 2.5 μM and 10 μM , respectively. After 24 h incubation under standard culture conditions cells were lysed for 10 minutes with the CellTiter-Glo[®] reagent solution and the luminescence signal was read using a multiwell plate luminometer. The quantity of live cells was expressed relative to untreated control cells ("untreated"). Cell viability data given are mean values over three experiments.

ACKNOWLEDGMENT

The authors thank Prof. Tanja Weil (Organic Chemistry III, University of Ulm, Germany) for access to cell lab facilities and Magdalene Zimmermann for measuring cell viabilities. Prof. Mika Lindén (Inorganic Chemistry II, University of Ulm, Germany) is acknowledged for general support of this work.

REFERENCES

- [1] P. Yang S. Gai and J. Lin, „Functionalized mesoporous silica materials for controlled drug delivery“, *Chem. Soc. Rev.*, 41, 2012, pp. 3679-3698. See also references cited therein.
- [2] F. Balas, M. Manzano, P. Horcajada and M. Vallet-Regí, „Confinement and controlled release of bisphosphonates on ordered mesoporous silica-based materials“ *J. Am. Chem. Soc.*, 128, 2006, pp. 8116-8117.
- [3] C.-Y. Lai, B. G. Trewyn, D. M. Jeftinija, K. Jeftinija, S. Xu, S. Jeftinija and V. S.-Y. Lin, „A mesoporous silica nanosphere-based carrier system with chemically removable CdS nanoparticle caps for stimuli-responsive controlled release of neurotransmitters and drug molecules“, *J. Am. Chem. Soc.*, 125, 2003, pp. 4451-4459.
- [4] J. L. Vivero-Escoto, I. I. Slowing, C.-W. Wu and V. S. Y. Lin, „Photo-induced Intracellular Controlled Release Drug Delivery in Human Cells by Gold-Capped Mesoporous Silica Nanosphere“, *J. Am. Chem. Soc.*, 131, 2009, pp. 3462-3463.
- [5] Z. M. Tao, B. Toms, J. Goodisman and T. Asefa, „Mesoporous silica micro-particles enhance the cytotoxicity of anticancer platinum drugs“ *ACS Nano*, 4, 2010, pp. 789-794.
- [6] L. Pasqua, F. Testa, R. Aiello, S. Cundari and J. B. Nagy, „Preparation of bifunctional hybrid mesoporous silica potentially useful for drug targeting“ *Micropor. Mesopor. Mater.* 103, 2007, pp. 166-173.
- [7] B. Ahn, J. Park, K. Singha, H. Park and W. J. Kim, „Mesoporous silica nanoparticle-based cisplatin prodrug delivery and anticancer effect under reductive cellular environment“ *J. Mater. Chem. B*, 1, 2013, 2829-2836.
- [8] J. L. Gu, S. S. Su, Y. S. Li, Q. J. He, F. Y. Zhong, J. L. Shi, „Surface Modification-Complexation Strategy for Cisplatin Loading in Mesoporous Nanoparticles“ *J. Phys. Chem. Lett.* 1, 2010, pp. 3446-3450.
- [9] H. He, H. Xiao, H. Kuanga, Z. Xie, X. Chen, X. Jing, Y. Huang, „Synthesis of mesoporous silica nanoparticle-oxaliplatin conjugates for improved anticancer drug delivery“ *Colloids and Surfaces B: Biointerfaces*, 117, 2014, pp. 75-81.
- [10] A. J. Di Pasqua, S. Wallner, D. J. Kerwood, and J. C. Dabrowiak, „Adsorption of the PtIII Anticancer Drug Carboplatin by Mesoporous Silica“ *Chemistry & Biodiversity*, 6, 2009, pp. 1343-1349.
- [11] H. M. Coley, J. Sarju and G. Wagner, „Synthesis and Characterization of Platinum(II) Oxadiazoline Complexes and their *In Vitro* Antitumor Activity in Platinum Sensitive and Resistant Cancer Cell Lines“ *J. Med. Chem.*, 51, 2008, pp. 135-141.
- [12] B. Desai, T. N. Danks and G. Wagner, „Ligand Discrimination in the Reaction of Nitrones with $(\text{PtCl}_2(\text{PhCN})_2)$. Selective Formation of Mono-oxadiazoline and Mixed Bis-oxadiazoline Complexes under Thermal and Microwave Conditions.“ *Dalton Trans.*, 2004, pp. 166-171.
- [13] J. Sarju, J. Arbour, J. Sayer, B. Rohrmoser, W. Scherer and G. Wagner, „Synthesis and Characterisation of Mixed Ligand Pt(II) and Pt(IV) Oxadiazoline Complexes.“ *Dalton Trans.*, 2008, pp. 5302-5312.
- [14] G. Wagner, A. Marchant and J. Sayer, „Design, Synthesis, Characterisation and Chemical Reactivity of Mixed-ligand Platinum(II) Oxadiazoline Complexes with Potential Cytotoxic Properties.“ *Dalton Trans.*, 39, 2010, pp. 7747-7759.
- [15] J. M. Rosenholm, A. Meinander, E. Peuhu, R. Neimi, J. E. Ericsson, C. Sahlgren and M. Lindén, „Targeting of porous hybrid silica nanoparticles to cancer cells“ *ACS Nano, Nanoscale*, 3, 2009, pp. 197-208.
- [16] ImageJ program, see: <http://rsb.info.nih.gov/ij/download.html>
- [17] J. R. Masters, „HeLa cells 50 years on: the good, the bad and the ugly.“ *Nat. Rev. Cancer*, 2, 2002, pp. 315-19.
- [18] Promega Technical Bulletin TB288, Revised 12/2012, Promega Corporation, 2800 Woods Hollow Road, Madison, WI 53711-5399 USA.
- [19] Y. Minagawa, J. Kigawa, H. Ishihara, H. Itamachi and N. Terakawa, „Synergistic enhancement of cisplatin cytotoxicity by SN-38, an active metabolite of CPT-11, for cisplatin resistant HeLa cells.“ *Jpn. J. Cancer. Res.* 85, 1994, pp. 966-971.
- [20] K. Takamura, T. Sakaeda, T. Yagami, H. Kobayashi, N. Ohmoto, M. Horinouchi, K. Nishiguchi and K. Okumura, „Cytotoxic effect of 27 anticancer drugs in HeLa and MDR-1 overexpressing derivative cell lines.“ *Biol. Pharm. Bull.* 25, 2002, pp 771-778.