Journal of Organometallic Chemistry 796 (2015) 17–25

Contents lists available at ScienceDirect

Journal of Organometallic Chemistry

journal homepage: www.elsevier.com/locate/jorganchem

Arene ruthenium dithiolato–carborane complexes for boron neutron capture therapy (BNCT)

Isolda Romero-Canelón [a], Ben Phoenix [b], Anaïs Pitto-Barry [a], Johanna Tran [a], Joan J. Soldevila-Barreda [a], Nigel Kirby [c], Stuart Green [b, *], Peter J. Sadler [a, *], Nicolas P.E. Barry [a, *]

[a] Department of Chemistry, University of Warwick, Gibbet Hill Road, Coventry CV4 7AL, UK
[b] School of Physics and Astronomy, University of Birmingham, Birmingham B15 2TT, UK
[c] Australian Synchrotron, 800 Blackburn Road, Clayton, Victoria 3168, Australia

ARTICLE INFO

Article history:
Received 6 March 2015
Received in revised form
7 May 2015
Accepted 8 May 2015
Available online 18 May 2015

Dedicated to Professor Georg Süss-Fink on the occasion of his 65th birthday

Keywords:
Arene ruthenium
Carborane
Pluronic
Micelles
Boron neutron capture therapy

ABSTRACT

We report the effect of low-energy thermal neutron irradiation on the antiproliferative activities of a highly hydrophobic organometallic arene ruthenium dithiolato–carborane complex [Ru(*p*-cymene) (1,2-dicarba-*closo*-dodecarborane-1,2-dithiolato)] (**1**), and of its formulation in Pluronic® triblock copolymer P123 core–shell micelles (RuMs). Complex **1** was highly active, with and without neutron irradiation, towards human ovarian cancer cells (A2780; IC_{50} 0.14 μM and 0.17 μM, respectively) and cisplatin-resistant human ovarian cancer cells (A2780cisR; IC_{50} 0.05 and 0.13 μM, respectively). Complex **1** was particularly sensitive to neutron irradiation in A2780cisR cells (2.6 × more potent after irradiation compared to non-irradiation). Although less potent, the encapsulated complex **1** as RuMs nanoparticles resulted in higher cellular accumulation (2.5×), and was sensitive to neutron irradiation in A2780 cells (1.4× more potent upon irradiation compared to non-irradiation).

Introduction

Boron neutron capture therapy (BNCT) has raised considerable interest for the treatment of high-grade gliomas and either cutaneous primaries or cerebral metastases of melanoma [1]. This binary method consists of the nuclear reaction of nontoxic and nonradioactive ^{10}B atoms and low-energy thermal neutrons that produces high-energy $^{4}He^{2+}$ α-particles and $^{7}Li^{3+}$ ions. The dissipation of the high kinetic energy of these particles is achieved in a small distance (less than one cell diameter), which allows accurate destruction of the targeted cells [2].

Dicarba-*closo*-dodecarboranes are a class of boron-rich compounds with globular structure and diameter of *ca.* 1 nm (diameter of a rotating phenyl) that possess unusual properties, including high symmetry and remarkable stability [3]. These clusters contain ten boron atoms; they possess a rather low cytotoxicity and are extremely stable in biological media. They are well suited to boron neutron capture therapy [4,5], but also have potential in other fields of drug discovery, molecular imaging, and targeted radionuclide therapy [6]. However, effective delivery of boron agents is still a critical issue which impairs their further clinical development [7]. We have recently discussed how the combination of arene ruthenium(II) complexes and carboranes has unexplored potential in medicine [8]. Such complexes also exhibit unusual chemistry: coordination of the bulky, electron-deficient carborane ligand 1,2-dicarba-*closo*-dodecarborane-1,2-dithiolato to an arene-Ru metal center leads to the isolation of a stable 16-electron complex [Ru(*p*-cymene) (1,2-dicarba-*closo*-dodecarborane-1,2-dithiolato)] (**1**) [9]. However, since this complex is highly hydrophobic, exploration of its biological applications is hampered by the lack of solubility in water [10]. To exploit the chemistry of carborane-containing arene ruthenium complexes in aqueous solution, and to take advantage of their unique properties, we have encapsulated the 16-electron complex **1** in Pluronic® triblock copolymer P123 micelles (Fig. 1). We have recently shown that although entrapment of the 16-

* Corresponding authors.
E-mail addresses: Stuart.Green@uhb.nhs.uk (S. Green), P.J.Sadler@warwick.ac.uk (P.J. Sadler), N.Barry@warwick.ac.uk (N.P.E. Barry).

http://dx.doi.org/10.1016/j.jorganchem.2015.05.011

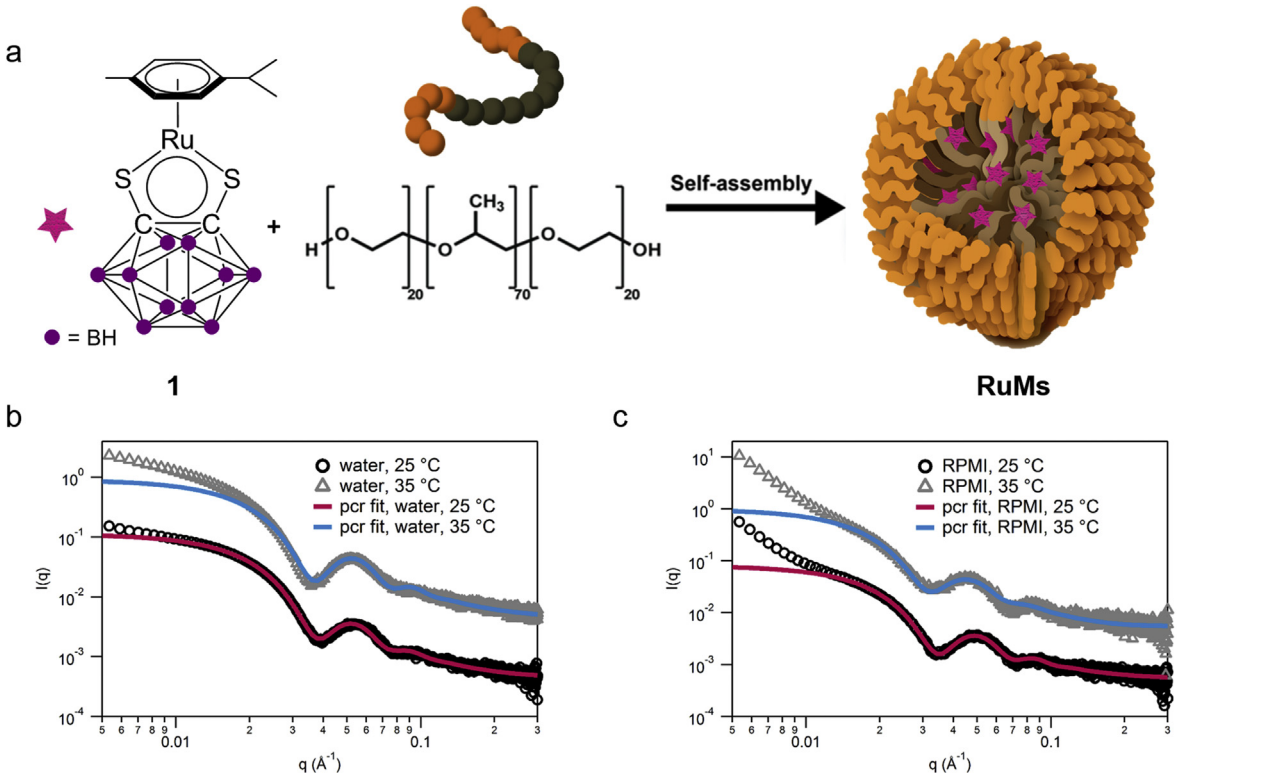

Fig. 1. (a) Self-assembly formation of **RuMs** (purple dots in **1** are B–H vertices). (b) and (c) Small-angle X-ray scattering (SAXS) experimental profiles and fitting with spherical core–shell micelle model of micelles **RuMs** at 25 °C and 35 °C in water and at 25 °C and 35 °C in RPMI, respectively; 5 mg/mL aqueous solutions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

electron complex **1** in Pluronic® micelles (**RuMs**) leads to a reduction in its anticancer potency towards ovarian cancer cells A2780, the micelles exhibit enhanced selectivity towards cancer cells compared to normal cells (up to a factor 8) [11]. This formulation was fully characterised by using a combination of analytical techniques, including synchrotron small-angle X-ray scattering, high-resolution transmission electron microscopy, and light scattering methods [11]. Polymer encapsulation of metal carborane complexes provides the potential for delivering high amounts of boron to cells which is of interest for BNCT [12]. We report here the effect of low-energy thermal neutron irradiation on the antiproliferative activity of both complex **1** and **RuMs** particles in the A2780 ovarian cancer cell line, and in A2780cisR cisplatin-resistant cancer cell line.

Results

Synthesis and characterisation

The organometallic half-sandwich Ru^{II} arene complex [Ru(*p*-cymene) (1,2-dicarba-*closo*-dodecarborane-1,2-dithiolate)] (**1**) was synthesised as reported previously [13]. This complex has a *pseudo*-octahedral structure, with a π-bonded arene occupying 3 coordination sites, a S-bound chelated dithiolato dicarba-*closo*-dodecarborane ligand, and a vacant 6th site (Fig. 1). It is a 16-electron complex and therefore electron-deficient at the metal [14]. Complex **1** is highly hydrophobic and insoluble in water [15]. To achieve dispersion in water [16], we encapsulated complex **1** in the water-soluble amphiphilic triblock copolymer P123 (poly(ethylene glycol)-*block*-poly(propylene glycol)-*block*-poly(ethylene glycol)) (PEO-*b*-PPO-*b*-PEO), according to a previously reported procedure (Fig. 1) [11].

To gain further insight into the structure of **RuMs** in RPMI cell culture medium, and to compare the sizes of the assembly in RPMI *versus* water at ambient temperature and at 35 °C, solutions of **RuMs** were analysed by synchrotron small-angle X-ray scattering (SAXS; Fig. 1). The experimental profiles were fitted using IgorPro software [17] to a core–shell spherical micelle model PolyCoreShellRatio [18] (PCR) according to a previous procedure for similar micelles [19]. Some aggregation was observed for all the samples (high turn at low q values), however the PCR model fitted excellently for all micellar solutions from 0.2 Å^{-1} with very low dispersity parameters (between 0.13 and 0.16, 0 being an ideal mono-disperse system; Table 1).

Cell testing

We studied the time-dependence of the antiproliferative activity of complex **1** and micelles **RuMs** and P123Ms (micelles made of Pluronic® copolymers without complex **1**) in A2780 human ovarian cancer cells (Table 2). Cells were exposed for variable times (1, 4, 16, 24, 48 and 72 h) to complex **1** (dissolved in 5% dimethyl sulfoxide (dmso)/95% saline:RPMI and further diluted in cell culture medium until working concentrations were achieved) or to **RuMs** micelles (dissolved in 100% saline:RPMI, further diluted with cell culture medium to working solutions). After this, drugs were removed and cells were washed and placed in fresh growth medium for a further 72 h as a recovery period. Cell viability was then assessed using the sulforhodamine B (SRB) colorimetric assay. Complex **1** was found to be highly potent towards A2780 cells (Table 2), particularly after 24 h of drug exposure (IC_{50} 170 nM), and it is also 39 × more potent than **RuMs** micelles, which still exhibit good (micromolar) activity towards cancer cells.

Since the optimum time for drug exposure was 24 h, we determined the IC_{50} values of complex **1** and micelles **RuMs** in A2780cisR cells after 24 h of drug exposure. Complex **1** was found

Table 1
Physical characteristics of **RuMs** determined by SAXS at 5 mg/mL in water and RPMI, at 25 °C and 35 °C.

Parameter	Water (25 °C)	Water (35 °C)	RPMI (25 °C)	RPMI (37 °C)
Radius core (nm)	7.53 ± 0.02	7.32 ± 0.02	7.93 ± 0.20	7.55 ± 0.06
Thickness shell (nm)	0.80 ± 0.04	0.60 ± 0.03	0.90 ± 0.39	1.81 ± 0.10
Total radius (nm)	8.33 ± 0.06	7.92 ± 0.05	8.83 ± 0.59	9.36 ± 0.16
Dispersity	0.14 ± 0.01	0.13 ± 0.01	0.13 ± 0.02	0.16 ± 0.02

Table 2
Time-dependence of IC_{50} values for complex **1** and **RuMS** in A2780 ovarian cancer cells. All experiments included 48 h of pre-incubation time, 72 h of recovery time in drug-free medium and variable drug exposure times.

Treatment	IC_{50} (μM)					
Exposure time	1 h	4 h	16 h	24 h	48 h	72 h
Complex **1**	>50	>50	20.4 ± 0.8	0.17 ± 0.02	0.16 ± 0.08	0.16 ± 0.05
RuMs	>50	>50	18 ± 2	6.7 ± 0.3	5.4 ± 0.3	5.2 ± 0.5

to be more active towards the cisplatin resistant cancer cell line (A2780cisR), as noted in Table 3, whilst micelles **RuMs** exhibit similar cytotoxicity in both cell lines (resistance factor 1.1).

We then investigated the cellular accumulation of ruthenium from A2780 cells exposed to complex **1** or to the **RuMs** using equimolar Ru concentrations of 0.5 μM. For this experiment, cells were exposed for 24 h and no recovery time was allowed. The metal content was determined by inductively coupled plasma mass spectrometry (ICP-MS) after cells had been digested overnight in concentrated nitric acid. Intracellular ruthenium in samples with **RuMs** was 2.5 × higher than that from complex **1** (15.6 ± 0.3 ng of Ru × 10^6 cells for **RuMs** *versus* 6.2 ± 0.4 ng Ru for complex **1**).

Finally, we investigated whether complex **1** and **RuMs** micelles induced apoptosis in A2780 cells. For this experiment, we used 24 h of drug exposure time and no-recovery time. Fig. 2 shows there is no significant apoptosis after the first 24 h, highlighting the importance of the recovery time in the mechanism of action of the ruthenium compounds.

Boron neutron capture experiments

We then studied the antiproliferative activity of complex **1** and **RuMs** micelles in A2780 and cisplatin-resistant A2780cisR human ovarian cancer cells, after thermal neutron irradiation. The measurements were performed in the thermal neutron field available at the Dynamitron accelerator in Birmingham, United Kingdom. This field has a large epithermal neutron fluence and a low gamma-dose rate contamination, approximately 1 Gy/h at an accelerator current of 1 mA. The plates containing the cancer cells incubated with complex **1** and **RuMs** micelles were sealed in a plastic bag containing culture medium, and the bag was clipped on a holder submerged in a tank of water (Fig. 3). This water tank provides further neutron moderation, which results in a peak in the thermal neutron flux at several centimetres depth. On-line monitoring of the neutron beam was provided by a pair of fission chambers (Centronic FC05A/500/U235) embedded in the beam shaping assembly near to the beam port. The relationship between the count rate in these chambers and the thermal neutron flux at depth in the water phantom has previously been well characterised *via* foil activation measurements [20]. At a proton beam current of 1 mA, the thermal neutron flux at 20 mm depth in water is 3.60 × 10^8 cm^{-2} s^{-1}.

The water tank was positioned in front of the beam, and the cells were irradiated for *ca.* 90 min at a nominal beam current of 600 μA. Exact beam-on time in the cell irradiations was controlled based on the chamber counts to ensure that each irradiation provided an equal cumulative thermal neutron flux. The integrated thermal neutron flux was 1.38 × 10^{12} cm^{-2} at the position of the cells. We also incubated cells with boric acid (1 mM concentration) as a positive control.

After 90 min of neutron irradiation, drugs were removed and cells were washed without recovery or placed in fresh growth medium for a further 72 h as a recovery period. Cell viability was then assessed using the SRB colorimetric assay. Fig. 4 shows the concentration dependence of A2780 and A2780cisR cell-survival upon incubation of complex **1** and **RuMs** micelles with and without neutron irradiation, and with and without recovery (Table 4). Since 72 h recovery time offered the best conditions for A2780 cells, we investigated the antiproliferative activity of complex **1** and **RuMs** micelles in cisplatin-resistant A2780cisR cells only after 72 h recovery.

Discussion

*Design and stability of complex **1** and of the ruthenium micellar system*

Inorganic compounds offer different mechanisms of drug action depending on the metal used, their structures and redox properties [21–61]. Thus, they can be utilised for the design of novel drugs in the treatment of a broad range of diseases [62]. Ruthenium complexes have been recognised as particularly promising drug candidates for the treatment of cancer since the beginning of the 1990s [63]. In 1992 [64], Tocher and co-workers observed an increase of the hypoxic cell cytotoxicity of metronidazole [1-β-(hydroxyethyl)-2-methyl-5-nitro-imidazole] after coordination to a benzene ruthenium dichlorido fragment [65]. Since then several groups have explored the anticancer activity of air-stable and water-soluble arene ruthenium(II) complexes [66,67]. The combination of the remarkable properties of half-sandwich complexes with the unique features of dicarba-*closo*-dodecarborane clusters results in

Table 3
IC_{50} values (μM), resistance factors ((IC_{50}(A2780cisR)/IC_{50}(A2780)), and cellular accumulation of complex **1** and micelles P123Ms and **RuMs** for A2780 human ovarian cancer cells, and A2780cisR cisplatin-resistant human ovarian cancer cells after 24 h of drug exposure.

Compound	IC_{50} (μM)			Cellular accumulation (ng of Ru × 10^6 cells)
	A2780	A2780cisR	Resistance factor	A2780
P123Ms	>100	>100	–	–
1	0.17 ± 0.02	0.130 ± 0.008	0.76	6.2 ± 0.4
RuMs	6.7 ± 0.3	7.93 ± 0.04	1.1	15.6 ± 0.3
cisplatin	1.2 ± 0.1	12.4 ± 0.3	10.3	–

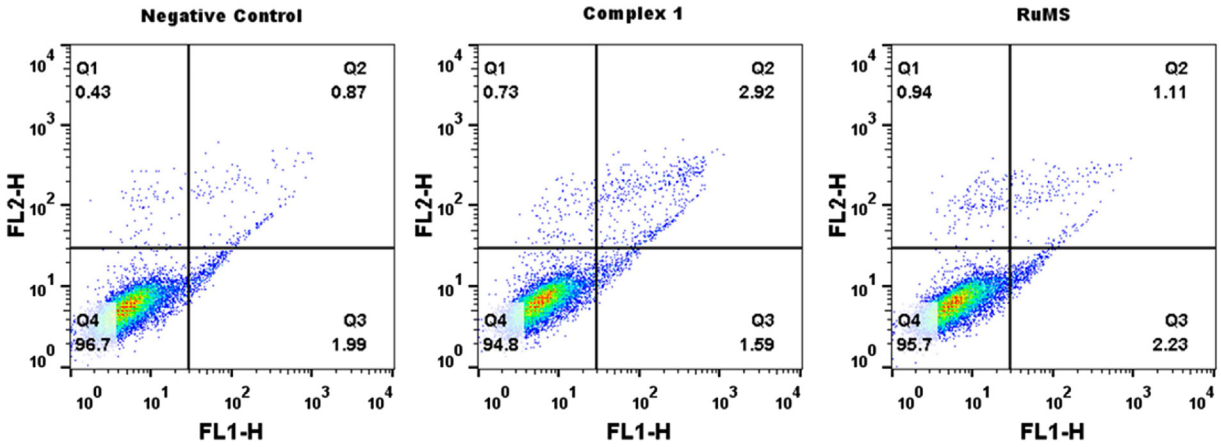

Fig. 2. Flow cytometry analysis of A2780 cells exposed to complex **1** or **RuMs** micelles compared to a negative control. FL1 reads Annexin fluorescence and FL2 reads propidium iodide fluorescence.

interesting new molecules [8]. Applications of these in organometallic synthesis, catalysis, or bioinorganic chemistry, for example, can be envisaged, although their high hydrophobicity impairs their further development as anticancer drug candidates.

We have recently shown that the combination of nanotechnology tools with medicinal inorganic chemistry has the potential to offer several advantages for drug formulation and delivery [68]. Control of drug solubility by increasing the aqueous solubility of highly lipophilic complexes or decreasing the solubility of complexes which might otherwise be rapidly excreted provides a 'slow-release' strategy that may engender less toxicity and improve the therapeutic response than a burst release. Modulation of drug distribution may also be achieved. The uptake of drugs encapsulated in nanoparticles is likely to depend on the shape, size and surface recognition of the nanoparticles by cells rather than on the characteristics of the drug. The nanoparticle might be designed so that it has vectors on its surface which can target specific cell receptors as well as having the capacity to encapsulate the drug, so reducing side effects and limiting attack to target cells or organelles only. Also nanomedicines may provide multidrug delivery and theranostic compounds since more than one drug can be encapsulated for combination therapy and reporter groups can be conjugated onto particles [16].

Synthetic polymer therapeutics are of particular interest in medicine, due to their synthetic versatility, as well as their tunable properties [69]. A number of biologically-active polymer–drug conjugates and polymeric formulations, such as micelles, hydrogels and polymer-coated nanoparticles, are currently in clinical development [70]. Among the most commonly used polymers for applications in medicine, the ABA Pluronic® triblock block copolymers are particularly suitable for the design of bio-inspired, bioengineered and biomimetic polymer nanoparticles [10]. The utilisation of Pluronic® block copolymers as drug delivery systems [71–76], biological response modifiers [77–81], pharmaceutical

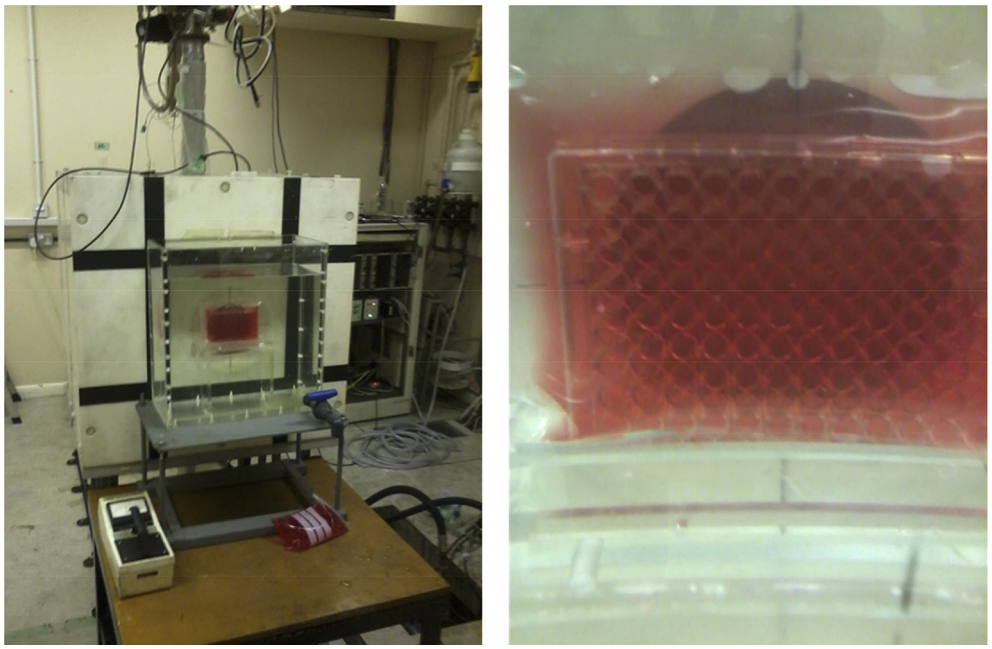

Fig. 3. Experimental set-up used for the neutron beam irradiation of complex **1** and **RuMs** micelles and P123Ms in cells. Right: Irradiation chamber; Left: Cell plate in the water tank positioned in front of the neutron beam.

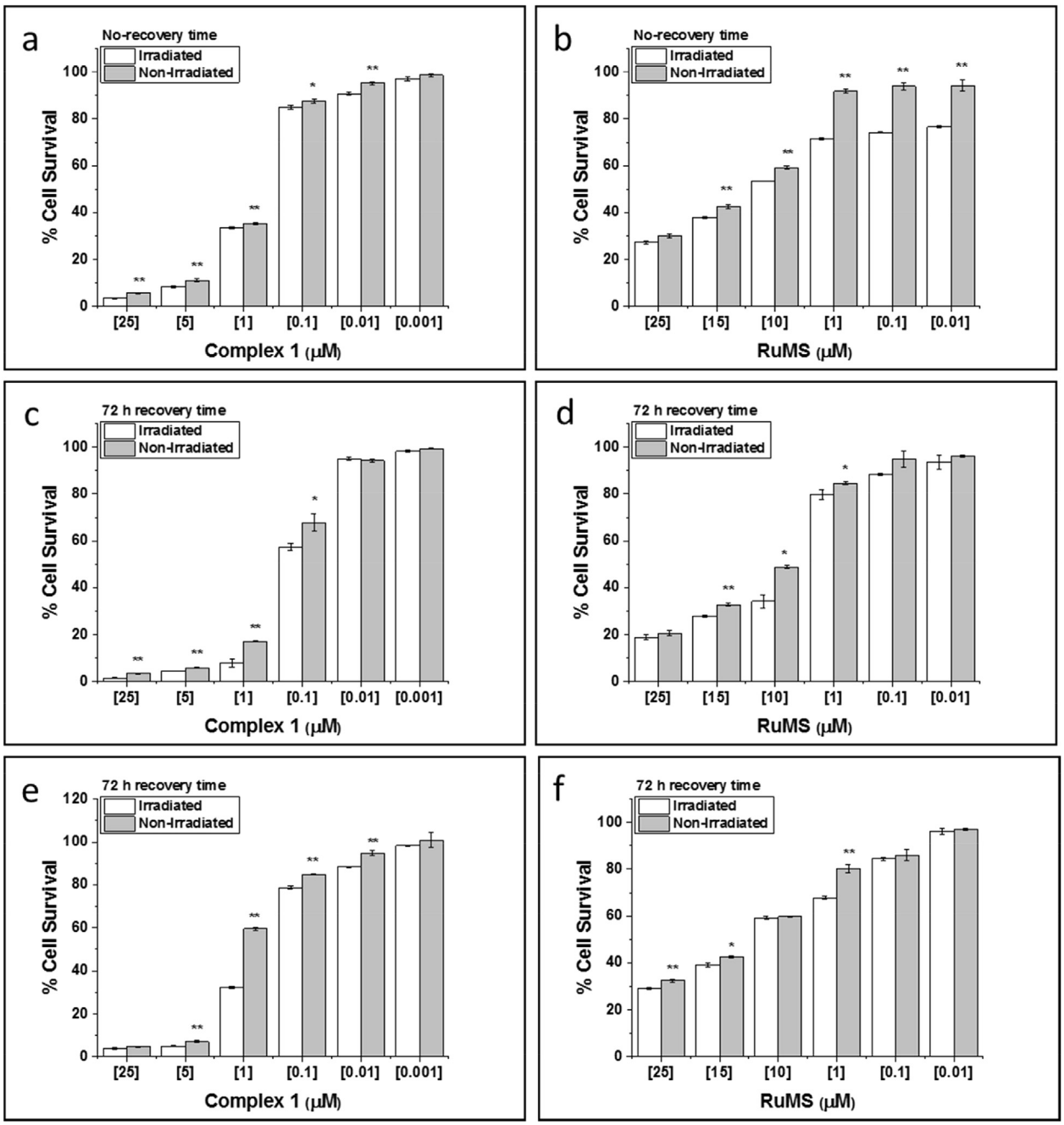

Fig. 4. Concentration dependence of A2780 cell survival upon incubation of complex **1** (a) without and (c) with 72 h recovery, and of **RuMs** micelles (b) without and (d) with 72 h recovery. Concentration dependence of A2780cisR cell survival upon incubation (e) with complex **1** and (f) of **RuMs** micelles, with 72 h recovery.

Table 4
IC_{50} values (μM), resistance factors ((IC_{50}(A2780)/IC_{50}(A2780cisR)) of complex **1** and micelles P123Ms and **RuMs** towards A2780 human ovarian cancer cells, and A2780cisR cisplatin-resistant human ovarian cancer cells after neutron irradiation. Ratio of IC_{50} values (non-irradiated *versus* irradiated) for **1** and **RuMs** in both cell lines.

Recovery time (h)	Compound	IC_{50} (μM)				IC_{50} ratio (−/+ Irrad)	
		A2780		A2780cisR		A2780	A2780cisR
		Non-irradiated	Irradiated	Non-irradiated	Irradiated		
0	**1**	0.60 ± 0.08	0.55 ± 0.04	–	–	1.1	–
	RuMs	8.12 ± 0.05	9.70 ± 0.03	–	–	0.8	–
72	**1**	0.177 ± 0.002	0.140 ± 0.003	0.130 ± 0.008	0.050 ± 0.004	1.3	2.6
	RuMs	6.7 ± 0.3	4.8 ± 0.2	7.93 ± 0.04	9.33 ± 0.09	1.4	0.8

ingredients [72,82,83], and steric stabilisers to lyotropic liquid crystalline particles [84–86], has led to recent advances in biochemistry [10].

The SAXS analyses demonstrated that **RuMs** self-assembly is the same in water and in RPMI media and leads to core-shell micelles with a core radius of around 7.7 nm, and a shell thickness of around 1.1 nm. Although the thickness of the shell is very thin, the same model used with no shell did not provide an acceptable fit, which thus confirms the presence of a shell. It is also anticipated that the core seen by SAXS is not only composed of the entire core of the micelle, but also of the part of the PEO corona which is poorly hydrated. The shell seen by SAXS is more likely to reflect the part of the corona which is fully hydrated. Interestingly, the diameters of **RuMs** micelles in RPMI and water are similar at ambient temperature and at 35 °C.

Antiproliferative activity of the ruthenium systems without neutron irradiation

We have recently shown that not only does the entrapment of the 16-electron complex **1** in Pluronic® micelles lead to a retention of the anticancer activity of **1**, but also that a certain selectivity between cancer and healthy cells is achieved by the utilization of a nanocarrier (selectivity factor of 8 for the micelles, compared to 2 for the complex alone) [11]. This might be due to a passive targeting of cancer cells *via* the "enhanced permeation and retention" (EPR) effect [87]. This effect is widely used in oncology since the discovery made by Maeda *et al.*, who in the 1980's demonstrated the principle of passive targeting of colloidal particles to tumours [88–90]. EPR is most effective for colloidal material of molecular weight above 40 kDa and can occur even in the absence of targeting ligands on nanoparticles [91]. **RuMs** are made of 66 ± 4 P123 monomers of individual average weight 5800 g/mol, and 59 ± 14 complexes **1** of molecular weight 441 g/mol [11], so the molecular weight of **RuMs** is about tenfold greater than this threshold. Nonetheless, the size of the micelles is relatively small (*ca.* 19 nm in diameter), which may impair the passive targeting of the micelles *via* the EPR effect. However, the class of Pluronic® copolymers offers a pool of more than 50 materials with various molar mass ratio between the PEO and PPO blocks, and there is a wide scope for adapting this combination of organometallic complexes and Pluronic® copolymers for designing bigger particles and for increasing the selectivity factor.

Importantly, the IC_{50} value of complex **1** remains unchanged after 24 h of drug exposure (Table 2), while the value for the **RuMs** micelles further improves at 72 h. We hypothesise that this could be related to the release of the complex from the micelles and into the cancer cells. Furthermore, according to the cellular uptake studies, the ruthenium accumulation is more efficient with **RuMs**, since at the same administered Ru concentration there is a two-fold greater accumulation of Ru for the micelles compared to the complex alone (15.6 ± 0.3 ng of ruthenium × 10^6 cells for **RuMs** *versus* 6.2 ± 0.4 ng for complex **1**). Finally, formulating complex **1** in polymer micelles also allows their dispersion in water in a manner suitable for administration to cancer cells (without the need to add dmso).

Here, we also showed that complex **1** is highly potent towards A2780 cells (Table 2), but is more active towards cisplatin-resistant A2780cisR cells than towards the parent A2780 cells (0.13 ± 0.02 *versus* 0.17 ± 0.01 μM; resistance factor 0.76), whilst **RuMs** micelles exhibit similar cytotoxicity towards both cell lines (6.7 ± 0.3 *versus* 7.93 ± 0.04; resistance factor 1.1). These results suggest that both complex **1** and **RuMs** micelles have a different mode of action from that of cisplatin, a tendency observed previously for arene ruthenium metal-based drugs [92,93].

Antiproliferative activity of the ruthenium systems after neutron irradiation

Boron neutron capture therapy is the traditional area for application of dicarba-*closo*-dodecarborane molecules in medicine. This binary method consists of the nuclear reaction of nontoxic and nonradioactive ^{10}B atoms and low-energy thermal neutrons that produces high-energy $^4He^{2+}$ α-particles and $^7Li^{3+}$ ions. The dissipation of the high kinetic energy of these particles is achieved in a small distance (less than one cell diameter), which allows accurate destruction of the targeted cells. Therefore, the efficiency of this therapy depends on the number of boron atoms delivered to cancer cells, while the selectivity strongly depends on the preferential accumulation of boron in tumour tissues rather than in normal tissues [94]. Dicarba-*closo*-dodecarboranes contain ten boron atoms; they possess a rather low cytotoxicity and these clusters are extremely stable in biological media. These characteristics explain why dicarba-*closo*-dodecarborane clusters have the potential to be efficient BNCT agents. However, dicarba-*closo*-dodecarborane clusters on their own do not possess the ability to target cancer cells selectively.

To increase the selectivity of dicarba-*closo*-dodecarboranes towards cancer cells and therefore to increase the clinical feasibility of boron neutron capture therapy, various approaches have been developed. A first strategy is to attach borane clusters to cellular building blocks. Indeed, most solid tumours are known to possess a hypervasculature, a defective vascular architecture, and an impaired lymphatic drainage [95]. Thus, while the normal endothelial layer surrounding the blood vessels feeding healthy cells restricts the amount of constituents (amino acids and nucleic acid precursors for example) necessary for cell replication, the endothelial layer of blood vessels in diseased tissues allows an elevated quantity of such nutrients to enter the cells [6]. Another strategy is to attach the borane cluster to tumour antibodies that can target specific cell types [96]. A third approach is to use nano-containers such as lipoproteins and liposomes [97]. Encapsulation of hydrophilic borane compounds in aqueous cores of liposomes, or incorporation of boron-containing lipids in liposome bilayers can lead to a selective delivery of BNCT therapeutics to tumours.

Here, we have studied the polymer encapsulation of a metal carborane complex, its antiproliferative activity alone and in polymer micelles, with and without activation by neutrons to assess its potential for BNCT. Complex **1** is highly potent towards cisplatin-resistant A2780cisR cancer cells, with IC_{50} values in the nanomolar range. When irradiated for 60 min with low-energy thermal neutrons, the antiproliferative effect of complex **1** was 2.6 × higher. This dramatic enhancement of the cytotoxicity of **1** after neutron irradiation unambiguously demonstrates the potential of this organometallic compound for BNCT, in particular for treating tumours having developed resistance mechanisms toward cisplatin, one of the most-used drugs in cancer chemotherapy. It is also apparent from Fig. 4 that the optimum conditions for achieving enhanced effects from neutron capture activation of the ruthenium compound is to allow the cells to recover for a period of 72 h after irradiation. It is known that the antiproliferative effects of neutron irradiation are not immediate [98], and our results seem to confirm this delayed effect. Although the IC_{50} values with and without neutron irradiation of **RuMs** micelles are in a similar range, in all cases the effect of the neutron irradiation on the antiproliferative activity of **RuMs** micelles is the strongest at 1 μM concentrations of ruthenium. A dramatic effect of the neutron irradiation on the potency of the **RuMs** micelles is observed for the two cell lines (up to 32% difference in cell survival). Interestingly, this effect is less important in the antiproliferative activity of complex **1**, which

might be related to the more efficient accumulation of Ru observed with **RuMs** compared with complex **1**.

Conclusions

BNCT has been investigated as an alternative anticancer therapy in clinical trials in Japan, Europe, and the United States using especially sodium borocaptate ($Na_2B_{12}H_{11}SH$). However, selective and effective delivery of boron agents is still a critical issue. For this reason it is of central importance to explore new concepts able to take advantage of these unique pharmacophores. We showed that the combination of dicarba-*closo*-dodecarboranes with half-sandwich complexes of ruthenium formulated in micelles made of polymers can provide new agents which are potentially useful in boron neutron capture therapy. Our results demonstrate that the formulation of complex **1** in micelles leads to a two-fold increase in accumulation of metal complex in cells, and that the effect of neutron irradiation on the antiproliferative activity of the micelles is dramatic at a micromolar concentration in ruthenium. Our strategy is highly versatile, with choice of the metal complex and polymer used for the self-assembly of the micelles. The natural abundance of the activate isotope ^{10}B is only 20% and it will be interesting in future work to investigate the neutron capture ability of ^{10}B-enriched carborane-containing complexes in micelles.

Materials and methods

Materials

The preparation of the complexes [Ru(*p*-cym)(1,2-dicarba-*closo*-dodecaborane-1,2-dithiolato)] (**1**) was based on a published procedure [13]. The purity of complex **1** was assessed by ^1H NMR spectroscopy in $CDCl_3$ and was in accordance with previous reports [9,11,14]. The preparation of the **RuMs** micelles was carried out as previously described [11].

Instrumentation

Inductively coupled plasma-mass spectrometry: Ruthenium content was determined using an ICP-MS Agilent technologies 7500 series instrument. Calibration curves were prepared using Ru standard solutions in double-deionised water (ddw) with 3% nitric acid, ranging between 50 and 0.5 ppb (9 points). Samples were freshly prepared in ddw with 3% nitric acid. Readings were made in no-gas mode with a detection limit of 1 ppt.

Small-angle X-ray scattering (SAXS): Measurements were carried out on the SAXS/WAXS beamline at the Australian Synchrotron facility at a photon energy of 11 keV. The samples in solution were in 1.5 mm diameter quartz capillaries. The data were collected at a sample-to-detector distance of 3.252 m to give a q range of 0.004–0.2 $Å^{-1}$, where q is the scattering vector and is related to the scattering angle (θ) and the photon wavelength (λ) by the following Equation (1):

$$q = \frac{4\pi \sin(\theta)}{\lambda} \quad (1)$$

The scattering from a blank solution (H_2O or RPMI) was measured in the same location as sample collection and was subtracted for each measurement. Data were normalised for total transmitted flux using a quantitative beamstop detector and absolute-scaled using water as an absolute intensity standard. The two-dimensional SAXS images were converted in one-dimensional SAXS profiles ($I(q)$ *versus* q) by circular averaging, where $I(q)$ is the scattering intensity. Functions were used from the NCNR package. Scattering length densities were calculated using the "Scattering Length Density Calculator" provided by NIST Center for Neutron Research.

Cell culture

A2780 human ovarian carcinoma cells and its cisplatin-resistant derived cell line A2780cisR were obtained from the European Collection of Cell Cultures (ECACC). Both cell lines were grown in Roswell Park Memorial Institute medium (RPMI-1640) supplemented with 10% of fetal calf serum, 1% of 2 mM glutamine and 1% penicillin/streptomycin. All cells were grown as adherent monolayers at 310 K in a 5% CO_2 humidified atmosphere and passaged at *ca.* 70–80% confluency.

In vitro growth inhibition assays.

A) Assays including 72 h recovery time. The antiproliferative activities of complex **1** and **RuMs** were determined for A2780 and A2780cisR human ovarian cancer cells. Briefly, 96-well plates were used to seed 5000 cells per well. The plates were left to pre-incubate with drug-free medium at 310 K for 48 h before adding different concentrations of the compounds to be tested. A drug exposure period of 24 h was allowed. After this, supernatants were removed by suction and each well was washed with PBS. A further 72 h was allowed for the cells to recover in drug-free medium at 310 K. The SRB assay was used to determine cell viability. IC_{50} values, as the concentration which causes 50% cell death, were determined as duplicates of triplicates in two independent sets of experiments and their standard deviations were calculated.

B) Assays with no recovery time. Experiments were carried out as described above, with the following modification. After the 24 h exposure time, drugs were removed by suction, each of the wells was washed with PBS and the SRB assay was run immediately. In both cases (assays with and without recovery time) stock solutions of complex **1** were prepared by dissolving the solid in a mixture of dmso (5% v/v) and 1:1 saline:RPMI-1640 (95% v/v), working solutions were achieved by dilution of the stock with cell culture medium. Stock solutions of **RuMs** were prepared similarly but without dmso. Exact metal concentrations for all stock solutions were determined using ICP-MS.

BNCT experiments

The *in vitro* growth inhibition assays were carried out as described above using A2780 and A2780cisR ovarian cancer cells with the following experimental modifications. After drug exposure the 96-well plate was sealed in a plastic bag containing media, and the bag was clipped on a holder submerged in a tank of water which acts as a neutron moderator (Fig. 2). The water tank was positioned in front of the beam, and the cells were irradiated for 60 min.

Ruthenium accumulation in cancer cells

Briefly, 1.5×10^6 cells/well were seeded on a 6-well plate. After 24 h of pre-incubation, complex **1** and separately **RuMs** were added to give final concentrations equal to 0.5 μM Ru and a further 24 h of drug exposure was allowed. After this time, cells were washed, treated with trypsin-EDTA, counted, and cell pellets were collected. Each pellet was digested overnight in concentrated nitric acid (73%) at 353 K; the resulting solutions were diluted using double-distilled water to a final concentration of 5% HNO_3 and the amount of Ru

taken up by the cells was determined by ICP-MS. These experiments did not include any cell recovery time in drug-free media; they were all carried out as duplicates of triplicates and the standard deviations were calculated.

Inductively coupled plasma-mass spectrometry (ICP-MS)

Cellular ruthenium content was determined using an ICP-MS Agilent technologies 7500 series instrument. Calibration curves were prepared using Ru standard solutions in double deionised water (ddw) with 3% nitric acid. Samples were freshly prepared after nitric acid digestion in ddw to 3% nitric acid dilution.

Induction of apoptosis

Flow cytometry analysis of apoptosis in A2780 cells caused by exposure to complex **1** and **RuMs**, was carried out using the Annexin V-FITC Apoptosis Detection Kit (Sigma Aldrich) according to the manufacturer's instructions. Briefly, A2780 cells were seeded in 6-well plates (1.0×10^6 cells per well), pre-incubated for 24 h in drug-free medium at 310 K, after which they were exposed to either complex **1** or **RuMs** (concentration equal to IC_{50}). Cells were harvested using trypsin and stained using PI/Annexin V-FITC. After staining, cell pellets were analysed in a Becton Dickinson FACScan Flow Cytometer. For positive-apoptosis controls, A2780 cells were exposed for 2 h to staurosporine (1 μg/mL). Cells for apoptosis studies were used with no previous fixing procedure as to avoid non-specific binding of the annexin V-FITC conjugate.

Acknowledgments

We thank the Leverhulme Trust (Early Career Fellowship No. ECF-2013-414 to NPEB), the University of Warwick (Grant No. RD14102 to NPEB), the University of Birmingham/EPSRC Follow-on-Fund (Grant No UOBFOF026 to BP), the ERC (Grant No. 247450 to PJS), EPSRC (EP/F034210/1 to PJS). We also thank COST Action CM1105 for stimulating discussions, Dr Magdalena Moss for technical assistance with the cell culture, the Australian Synchrotron and the University of Monash for allocation of beamtime on the SAXS/WAXS beamline and funding.

References

[1] S.-V. Martha, G. Maria da, Boron Science, CRC Press, 2011, pp. 209–242.
[2] V.I. Bregadze, I.B. Sivaev, S.A. Glazun, Anticancer Agents Med. Chem. 6 (2006) 75–109.
[3] Z.J. Lesnikowski, Collect. Czech. Chem. Commun. 72 (2007) 1646–1658.
[4] W. Tjarks, R. Tiwari, Y. Byun, S. Narayanasamy, R.F. Barth, Chem. Commun. (2007) 4978–4991.
[5] V.I. Bregadze, I.B. Sivaev, Polyhedral Boron Compounds for BNCT in Boron Science: New Technologies and Applications, CRC Press, 2011.
[6] A.F. Armstrong, J.F. Valliant, Dalton Trans. (2007) 4240–4251.
[7] S. Korbe, P.J. Schreiber, J. Michl, Chem. Rev. 106 (2006) 5208–5249.
[8] N.P.E. Barry, P.J. Sadler, Chem. Soc. Rev. 41 (2012) 3264–3279.
[9] N.P.E. Barry, R.J. Deeth, G.J. Clarkson, I. Prokes, P.J. Sadler, Dalton Trans. 42 (2013) 2580–2587.
[10] A. Pitto-Barry, N.P.E. Barry, Polym. Chem. 5 (2014) 3291–3297.
[11] N.P.E. Barry, A. Pitto-Barry, I. Romero-Canelon, J. Tran, J.J. Soldevila-Barreda, I. Hands-Portman, C.J. Smith, N. Kirby, A.P. Dove, R.K. O'Reilly, P.J. Sadler, Faraday Discuss. 175 (2014) 229–240.
[12] Z. Yinghuai, J.A. Maguire, N.S. Hosmane, Recent Developments in the Boron Neutron Capture Therapy Driven by Nanotechnology in Boron for Living: Boron Neutron Capture Therapy, CRC Press, 2011.
[13] M. Herberhold, H. Yan, W. Milius, J. Organomet. Chem. 598 (2000) 142–149.
[14] N.P.E. Barry, T.F. Kemp, P.J. Sadler, J.V. Hanna, Dalton Trans. 43 (2014) 4945–4949.
[15] M. Scholz, E. Hey-Hawkins, Chem. Rev. 111 (2011) 7035–7062.
[16] N.P.E. Barry, P.J. Sadler, ACS Nano 7 (2013) 5654–5659.
[17] S. Kline, J. Appl. Crystallogr. 39 (2006) 895–900.
[18] J.B. Hayter, in: V. DeGiorgio, M. Corti (Eds.), Physics of Amphiphiles–micelles, Vesicles, and Microemulsions, North-Holland Publishing Company, 1983, pp. 59–93.
[19] N.P.E. Barry, A. Pitto-Barry, A.M. Sanchez, A.P. Dove, R.J. Procter, J.J. Soldevila-Barreda, N. Kirby, I. Hands-Portman, C.J. Smith, R.K. O'Reilly, R. Beanland, P.J. Sadler, Nat. Commun. 5 (2014) 3851.
[20] Z. Ghani, The Physics, Dosimetry and Microdosimetry of Boron Neutron Capture Therapy, Diss, University of Birmingham, 2013.
[21] Z. Liu, P.J. Sadler, Acc. Chem. Res. 47 (2014) 1174–1185.
[22] Y. Zhao, J.A. Woods, N.J. Farrer, K.S. Robinson, J. Pracharova, J. Kasparkova, O. Novakova, H. Li, L. Salassa, A.M. Pizarro, G.J. Clarkson, L. Song, V. Brabec, P.J. Sadler, Chem. Eur. J. 19 (2013) 9578–9591.
[23] N.A. Smith, P.J. Sadler, Phil. Trans. R. Soc. A 371 (2013) 1995.
[24] I. Romero-Canelón, L. Salassa, P.J. Sadler, J. Med. Chem. 56 (2013) 1291–1300.
[25] I. Romero-Canelón, P.J. Sadler, Inorg. Chem. 52 (2013) 12276–12291.
[26] A.M. Pizarro, N.P.E. Barry, P.J. Sadler, in: J. Reedijk (Ed.), Comprehensive Inorganic Chemistry II, vol. 3, Elsevier, 2013, pp. 752–784.
[27] Z. Liu, I. Romero-Canelón, B. Qamar, J.M. Hearn, A. Habtemariam, N.P.E. Barry, A.M. Pizarro, G.J. Clarkson, P.J. Sadler, Angew. Chem. Int. Ed. 53 (2014) 3941–3946.
[28] Y. Fu, R. Soni, M.J. Romero, A.M. Pizarro, L. Salassa, G.J. Clarkson, J.M. Hearn, A. Habtemariam, M. Wills, P.J. Sadler, Chem. Eur. J. 19 (2013) 15199–15209.
[29] J. Schulz, J. Tauchman, I. Císařová, T. Riedel, P.J. Dyson, P. Štěpnička, J. Organomet. Chem. 751 (2014) 604–609.
[30] A.A. Nazarov, C.G. Hartinger, P.J. Dyson, J. Organomet. Chem. 751 (2014) 251–260.
[31] K.J. Kilpin, S. Crot, T. Riedel, J.A. Kitchen, P.J. Dyson, Dalton Trans. 43 (2014) 1443–1448.
[32] C.M. Clavel, E. Paunescu, P. Nowak-Sliwinska, P.J. Dyson, Chem. Sci. 5 (2014) 1097–1101.
[33] K.J. Kilpin, P.J. Dyson, Chem. Sci. 4 (2013) 1410–1419.
[34] G. Gupta, A. Garci, B.S. Murray, P.J. Dyson, G. Fabre, P. Trouillas, F. Giannini, J. Furrer, G. Suss-Fink, B. Therrien, Dalton Trans. 42 (2013) 15457–15463.
[35] F. Giannini, J. Furrer, G. Süss-Fink, C.M. Clavel, P.J. Dyson, J. Organomet. Chem. 744 (2013) 41–48.
[36] A. Leonidova, V. Pierroz, R. Rubbiani, J. Heier, S. Ferrari, G. Gasser, Dalton Trans. 43 (2014) 4287–4294.
[37] T. Joshi, V. Pierroz, C. Mari, L. Gemperle, S. Ferrari, G. Gasser, Angew. Chem. Int. Ed. 53 (2014) 2960–2963.
[38] M. Patra, T. Joshi, V. Pierroz, K. Ingram, M. Kaiser, S. Ferrari, B. Spingler, J. Keiser, G. Gasser, Chem. Eur. J. 19 (2013) 14768–14772.
[39] M. Patra, K. Ingram, A. Leonidova, V. Pierroz, S. Ferrari, M.N. Robertson, M.H. Todd, J. Keiser, G. Gasser, J. Med. Chem. 56 (2013) 9192–9198.
[40] M. Patra, J. Hess, S. Konatschnig, B. Spingler, G. Gasser, Organometallics 32 (2013) 6098–6105.
[41] P.K. Sasmal, C.N. Streu, E. Meggers, Chem. Commun. 49 (2013) 1581–1587.
[42] Y. Xiang, C. Fu, T. Breiding, P.K. Sasmal, H. Liu, Q. Shen, K. Harms, L. Zhang, E. Meggers, Chem. Commun. 48 (2012) 7131–7133.
[43] A.L. Merkel, E. Meggers, M. Ocker, Expert Opin. Investig. Drugs 21 (2012) 425–436.
[44] A. Kastl, A. Wilbuer, A.L. Merkel, L. Feng, P. Di Fazio, M. Ocker, E. Meggers, Chem. Commun. 48 (2012) 1863–1865.
[45] E. Meggers, Angew. Chem. Int. Ed. 50 (2011) 2442–2448.
[46] K.D. Mjos, C. Orvig, Chem. Rev. 114 (2014) 4540–4563.
[47] M.A. Telpoukhovskaia, C. Orvig, Chem. Soc. Rev. 42 (2013) 1836–1846.
[48] Y.L.K. Tan, P. Pigeon, S. Top, E. Labbe, O. Buriez, E.A. Hillard, A. Vessieres, C. Amatore, W.K. Leong, G. Jaouen, Dalton Trans. 41 (2012) 7537–7549.
[49] G. Sava, G. Jaouen, E.A. Hillard, A. Bergamo, Dalton Trans. 41 (2012) 8226–8234.
[50] A. Vessières, C. Corbet, J.M. Heldt, N. Lories, N. Jouy, I. Laïos, G. Leclercq, G. Jaouen, R.-A. Toillon, J. Inorg. Biochem. 104 (2010) 503–511.
[51] J.W. Yi, N.P.E. Barry, M.A. Furrer, O. Zava, P.J. Dyson, B. Therrien, B.H. Kim, Bioconjugate Chem. 23 (2012) 461–471.
[52] A. Pitto-Barry, O. Zava, P.J. Dyson, R. Deschenaux, B. Therrien, Inorg. Chem. 51 (2012) 7119–7124.
[53] N.P.E. Barry, O. Zava, W. Wu, J. Zhao, B. Therrien, Inorg. Chem. Comm. 18 (2012) 25–28.
[54] N.P.E. Barry, O. Zava, P.J. Dyson, B. Therrien, J. Organomet. Chem. 705 (2012) 1–6.
[55] G.S. Smith, B. Therrien, Dalton Trans. 40 (2011) 10793–10800.
[56] A. Pitto-Barry, N.P.E. Barry, O. Zava, R. Deschenaux, B. Therrien, Chem. Asian J. 6 (2011) 1595–1603.
[57] A. Pitto-Barry, N.P.E. Barry, O. Zava, R. Deschenaux, P.J. Dyson, B. Therrien, Chem. Eur. J. 17 (2011) 1966–1971.
[58] L.E.H. Paul, B. Therrien, J. Furrer, Inorg. Chem. 51 (2011) 1057–1067.
[59] N.P.E. Barry, O. Zava, J. Furrer, P.J. Dyson, B. Therrien, Dalton Trans. 39 (2010) 5272–5277.
[60] N.P.E. Barry, O. Zava, P.J. Dyson, B. Therrien, Aust. J. Chem. 63 (2010) 1529–1537.
[61] N.P.E. Barry, F. Edafe, P.J. Dyson, B. Therrien, Dalton Trans. 39 (2010) 2816–2820.
[62] N.P.E. Barry, P.J. Sadler, Chem. Commun. 49 (2013) 5106–5131.
[63] F. Wang, H. Chen, S. Parsons, I.D.H. Oswald, J.E. Davidson, P.J. Sadler, Chem. Eur. J. 9 (2003) 5810–5820.

[64] L.D. Dale, J.H. Tocher, T.M. Dyson, D.I. Edwards, D.A. Tocher, Anti-Cancer Drug Des. 7 (1992) 3–14.
[65] G. Süss-Fink, Dalton Trans. 39 (2010) 1673–1688.
[66] C.S. Allardyce, P.J. Dyson, D.J. Ellis, S.L. Heath, Chem. Commun. (2001) 1396–1397.
[67] R.E. Morris, R.E. Aird, P.d.S. Murdoch, H. Chen, J. Cummings, N.D. Hughes, S. Parsons, A. Parkin, G. Boyd, D.I. Jodrell, P.J. Sadler, J. Med. Chem. 44 (2001) 3616–3621.
[68] T.C. Johnstone, N. Kulak, E.M. Pridgen, O.C. Farokhzad, R. Langer, S.J. Lippard, ACS Nano 7 (2013) 5675–5683.
[69] A.R. Kirtane, J. Panyam, Nat. Nanotechnol. 8 (2013) 805–806.
[70] O.C. Steinbach, Ther. Deliv. 5 (2014) 113–118.
[71] D.Y. Alakhova, Y. Zhao, S. Li, A.V. Kabanov, PLoS One 8 (2013) e72238.
[72] E.V. Batrakova, S. Li, A.M. Brynskikh, A.K. Sharma, Y. Li, M. Boska, N. Gong, R.L. Mosley, V.Y. Alakhov, H.E. Gendelman, A.V. Kabanov, J. Control. Release 143 (2010) 290–301.
[73] E.V. Batrakova, A.V. Kabanov, J. Control. Release 130 (2008) 98–106.
[74] T. Minko, E.V. Batrakova, S. Li, Y. Li, R.I. Pakunlu, V.Y. Alakhov, A.V. Kabanov, J. Control. Release 105 (2005) 269–278.
[75] A.V. Kabanov, E.V. Batrakova, V.Y. Alakhov, J. Control. Release 82 (2002) 189–212.
[76] E.V. Batrakova, S. Li, W.F. Elmquist, D.W. Miller, V.Y. Alakhov, A.V. Kabanov, Br. J. Cancer 85 (2001) 1987–1997.
[77] D.Y. Alakhova, N.Y. Rapoport, E.V. Batrakova, A.A. Timoshin, S. Li, D. Nicholls, V.Y. Alakhov, A.V. Kabanov, J. Control. Release 142 (2010) 89–100.
[78] E.V. Batrakova, S. Li, V.Y. Alakhov, W.F. Elmquist, D.W. Miller, A.V. Kabanov, Pharm. Res. 20 (2003) 1581–1590.
[79] E. Batrakova, S. Li, V. Alakhov, D. Miller, A. Kabanov, J. Pharmacol. Exp. Ther. 304 (2003) 845–854.
[80] A. Kabanov, E. Batrakova, V. Alakhov, Adv. Drug Deliv. Rev. 54 (2002) 759–779.
[81] A. Venne, S. Li, R. Mandeville, A. Kabanov, V. Alakhov, Cancer Res. 56 (1996) 3626–3629.
[82] E.R. Gariepy, J.C. Leroux, Eur. J. Pharm. Biopharm. 58 (2004) 409–426.
[83] I.R. Schmolka, Poloxamers in the Pharmaceutical Industry, CRC Press, Boca Ratin FL, 1991.
[84] J.Y.T. Chong, X. Mulet, L.J. Waddington, B.J. Boyd, C.J. Drummond, Soft Matter 7 (2011) 4768–4777.
[85] K.W. Lee, T.H. Nguyen, T. Hanley, B.J. Boyd, Int. J. Pharm. 365 (2009) 190–199.
[86] B.J. Boyd, Y.D. Dong, T. Rades, J. Liposome Res. 19 (2009) 12–28.
[87] N. Kamaly, Z. Xiao, P.M. Valencia, A.F. Radovic-Moreno, O.C. Farokhzad, Chem. Soc. Rev. 41 (2012) 2971–3010.
[88] H. Maeda, Adv. Enzyme Regul. 41 (2001) 189–207.
[89] Y. Matsumura, H. Maeda, Cancer. Res. 46 (1986) 6387–6392.
[90] T. Konno, H. Maeda, K. Iwai, S. Tashiro, S. Maki, T. Morinaga, M. Mochinaga, T. Hiraoka, I. Yokoyama, Eur. J. Cancer Clin. Oncol. 19 (1983) 1053–1065.
[91] H. Maeda, Bioconjugate Chem. 21 (2010) 797–802.
[92] O. Zava, J. Mattsson, B. Therrien, P.J. Dyson, Chem. Eur. J. 16 (2010) 1428–1431.
[93] P. Chellan, K.M. Land, A. Shokar, A. Au, S.H. An, D. Taylor, P.J. Smith, T. Riedel, P.J. Dyson, K. Chibale, G.S. Smith, Dalton Trans. 43 (2014) 513–526.
[94] I.B. Sivaev, V.V. Bregadze, Eur. J. Inorg. Chem. (2009) 1433–1450.
[95] H. Maeda, T. Maeda, K. Mizuno, K. Fujimoto, H. Shimizu, M. Inouye, Chem. Eur. J. 12 (2006) 824–831.
[96] R.N. Grimes, J. Chem. Educ. 81 (2004) 658–672.
[97] S.B. Kahl, Tetrahedron Lett. 31 (1990) 1517–1520.
[98] J.F. Valliant, K.J. Guenther, A.S. King, P. Morel, P. Schaffer, O.O. Sogbein, K.A. Stephenson, Coord. Chem. Rev. 232 (2002) 173–230.