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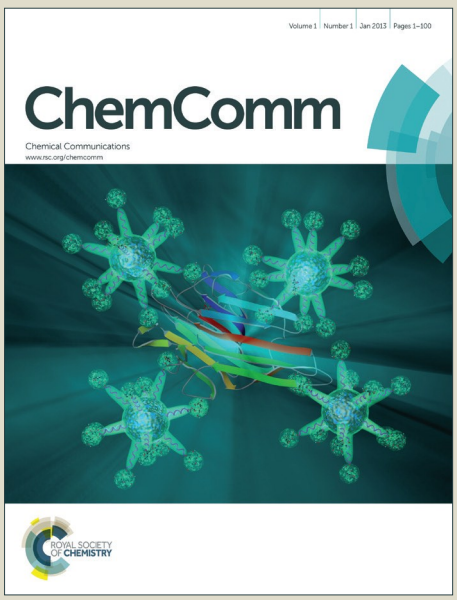

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High-Affinity Sequence-Selective DNA binding by Iridium(III) polypyridyl organometallopeptides

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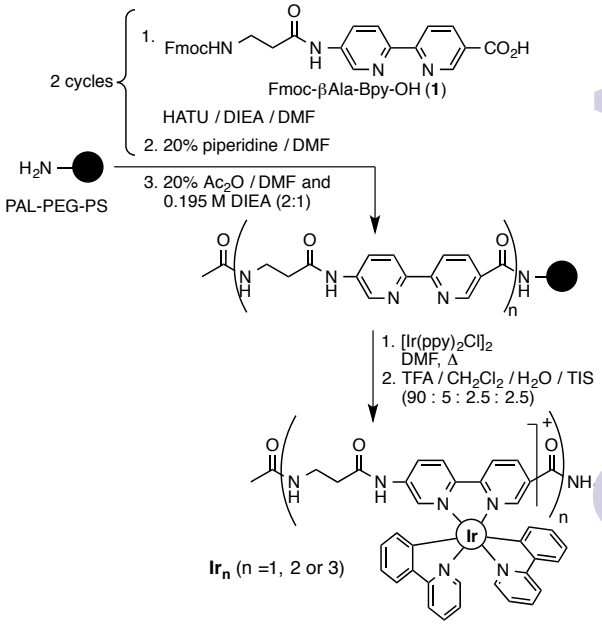
Ilaria Gamba,[a] Iria Salvadó,[a] Rosa F. Brissos,[b] Patrick Gamez,[b,c] José Brea,[c] Maria Isabel Loza,[d] M. Eugenio Vázquez[e,*] and Miguel Vázquez López[a,*]

We demonstrate the application of solid-phase peptide synthesis methods for the straightforward assembly of polynuclear Ir(III) organometallopeptides, and show that their oligoarginine derivatives exhibit high DNA binding affinity, sequence selectivity, and high cytotoxicicty towards a set of cancer cell lines.

DNA-binding drugs are the workhorse of current anticancer therapies.[1] However, despite their extensive use, many of them suffer from severe side-effects, which has fuelled the search of safer alternatives with improved pharmacological profiles.[2] In the past few years, there has been an increased interest in the application of coordination compounds as DNA-targeted probes, reactive agents and therapeutics,[3,4,5] Among them, Ru(II), Os(II) and Rh(III) polypyridyl mononuclear complexes have been exhaustively studied for their kinetic stability and convenient redox and optical properties.[6,7] In contrast, the potential of classical Werner and organometallic Ir(III) analogues as DNA-binding agents is still largely unexplored, despite being also kinetically inert and displaying excellent photochemical properties with tuneable excited states and long emission wavelengths for sensing and imaging.[6e,8] Indeed, current examples in the literature are practically limited to mononuclear intercalators,[9] with very few reports of di/poly-nuclear derivatives,[10,11] or groove-binding agents.[9]

We have recently reported the extension of standard solid-phase peptide synthesis procedures (SPPS) for the construction of polypyridyl dinuclear Ru(II) complexes.[5a] Intrigued by the potential of Ir(III) complexes as DNA binders, we decided to expand the scope of this methodology to the synthesis of Ir(III) organometallopeptides. Considering that the DNA binding properties of these complexes would be highly influenced by their nuclearity,[12] we also synthesized dinuclear and trinuclear derivatives in addition to the mononuclear complexes.[13] Finally, it is also worth noting that there are very few precedents of Ir(III)-peptide conjugates, and limited to examples in which the Ir(III) complexes are attached to the N-terminus,[14] or the side chains of the peptide chain, but never integrated in the peptide backbone structure.

Thus, based on our earlier studies with Ru(II) metallopeptides, we designed three peptidic ligands containing one, two or three βAla-bpy coordinating units (**1**, Scheme 1), to generate mono, di and trinuclear Ir(III) polypyridyl organometallopeptides. The peptidic ligands were synthesized following standard Fmoc/*t*Bu solid-phase protocols.[15] Once fully assembled, the peptides still attached to the solid support were reacted with $[Ir(ppy)_2Cl]_2$ (ppy: 2-phenylpyridine) to give the desired cyclometalated complexes **Ir**, **Ir$_2$** and **Ir$_3$** (Scheme 1). Acidic cleavage from the support, followed by reverse-phase HPLC purification, afforded the desired Ir(III) organometallopeptides as diasteromeric mixtures.



Scheme 1. Solid-phase peptide synthesis of the organometallopeptides **Ir**, **Ir$_2$** and **Ir$_3$**

Having at hand the desired Ir(III) organometallopeptides, we studied their DNA binding by exploiting the intrinsic environment-sensitive luminescence of the Ir(III) complexes. In contrast with the good DNA binding affinity of their Ru(II) analogs,[13] the fluorescence titrations experiments carried out with the **Ir**, **Ir$_2$** and **Ir$_3$** organometallopeptides did not show any significant affinity for short DNA oligonucleotides, so that addition of increasing amounts of hairpin oligonucleotides containing A/T or G/C-rich sequences did not induce any change in the emissive properties of the organometallopeptides (see ESI†).

A possible explanation for the low DNA affinity displayed by these Ir(III) metallopeptides is the reduced charge of $[Ir(ppy)_2bpy]^+$ units (+1 charge in each complex) in comparison to that of the $[Ru(bpy)_3]^{+2}$ DNA complexes (+2). We envisaged that the reduced electrostatic attraction for the negatively charged DNA might be compensated by introducing additional positively charged groups, and thus we decided to synthesize the octaarginine derivatives of **Ir**, **Ir$_2$** and **Ir$_3$** (named **Ir-R$_8$**, **Ir$_2$-R$_8$** and **Ir$_3$-R$_8$**, respectively). Indeed, it has been shown that tethering octaarginine domains to DNA binding agents results in conjugates that display increased DNA affinity,[16,17] as well as improved cell internalization and solubility.[18,19] We took advantage of the flexibility provided by the SPPS methodology to synthesize these oligocationic metallopeptides as described before, assembling the βAla-bpy coordinating units at the N-terminus of a previously synthesized R_8 peptide.

Once we synthesized the desired oligoarginine conjugates, we studied their DNA binding by fluorescence spectroscopy. Thus, incubation of 0.2 μM solutions of **Ir-R$_8$**, **Ir$_2$-R$_8$** and **Ir$_3$-R$_8$** with increasing concentrations of DNA hairpins resulted in a progressive increase of the 620 nm emission upon excitation at 320 nm (Fig 1, left). The corresponding titration profiles for each metallopeptide with different oligonucleotides could be fitted to the Bard model (Fig 1, right),[20] which allowed us to determine the affinity constants (*Ka*) for the different DNA sequences (Table 1). These results show that the binding affinity is dramatically increased with respect to the parent metallopeptides (**Ir**, **Ir$_2$** and **Ir$_3$**), and heavily dependent on the nuclearity of the metallopeptides. In fact, the *Ka* values are approximately in the order of 10^6, 10^7 and 10^8 M^{-1} for **Ir-R$_8$**, **Ir$_2$-R$_8$** and **Ir$_3$-R$_8$**, respectively. Besides, **Ir-R$_8$** shows a slight preference for for A/T-rich sequences, whereas **Ir$_2$-R$_8$** and **Ir$_3$-R$_8$** display a clear preference for hairpins with high G/C content, perhaps suggesting alternate binding preferences for the two metallopeptides.

Interestingly, the calculated association constants for the interaction of **Ir$_3$-R$_8$** for the DNA hairpin are 100 times higher than those typically reported for common mononuclear intercalating complexes (such as Ru(II)/dppz derivatives),[6c] and 1000 times stronger than those observed for other non-intercalating DNA-binding metal complexes,[5a] and in the order of the binding constants measured for widely used organic DNA minor-groove binders, like Hoechst 33258.[17b,21] Therefore, to the best of our knowledge, **Ir$_3$-R$_8$** displays the highest DNA affinity observed for a metal complex. In order to explore the role of the Arg_8 appendage we synthesized a scrambled trinuclear Ir(III) organometallopeptide in which the iridium centers are separated by groups of three arginine residues **Ir-R$_3$-Ir-R$_3$-Ir-R$_3$**. Interestingly, this analog displayed negligible affinity for duplex DNA (see figures S4 and S5 in the ESI†), thus suggesting an important role for the C-terminal R_8 domain beyond simple electrostatic stabilization of the complexes with the DNA.

Table 1. DNA association constants (K_a / 10^6 M^{-1}).[a]

	AAAATT	AAGCTT	GAAGGC	GGCCC
Ir-R$_8$	4.0 ± 0.3	3.9 ± 0.2	3.2 ± 0.2	2.7 ± 0.1
Ir$_2$-R$_8$	10.7 ± 0.8	10.4 ± 0.6	13.3 ± 0.7	18.3 ± 1.4
Ir$_3$-R$_8$	89.7 ± 9.2	76.8 ± 6.2	94.1 ± 9.2	158.0 ± 17.9

[a] Full sequences of the hairpin oligonucleotides used in this study (binding site underlined, central T_4 hairpin loop in italics): **AAAATT**: 5'-GGC AAAATTT CG *TTTT* CG AAATTTT GCC–3'; **AAGCTT**: 5'–GGC AAGCTT CGC *TTTT* GC AAGCTT GCC–3'; **GAAGGC**: 5'–GGC GAAGGC AGC *TTTT* GCT GCCTT GCC-3'; **GGCCC**: 5'–GGCA GGCCC AGC *TTTT* GCT GGGCCT GCC–3'. Titrations were performed adding increasing amounts of the corresponding hairpin oligos over 0.2 μM solutions of the peptides in 100 mM phosphate buffer, 100 mM NaCl, pH 6.8 at 25 ºC (see the ESI†).

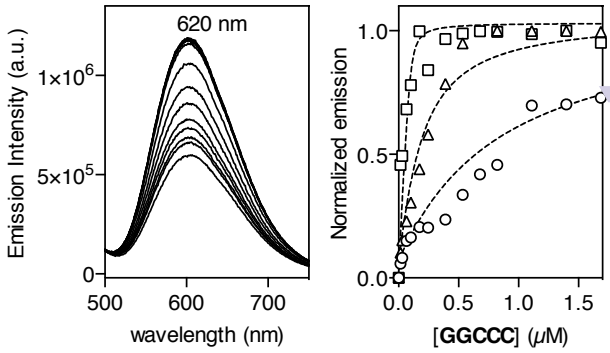


Fig 1. Left, Luminescence spectra of 0.2 μM solutions of **Ir$_2$-R$_8$** in phosphate buffer (100 mM), NaCl (100 mM), pH 6.8 (red line) and evolution upon addition of aliquots of a GGCCC hairpin oligonucleotide solution (10 μM) (black lines) until saturation (thick black line); right, titration profiles of **Ir-R$_8$** (black), **Ir$_2$-R$_8$** (red) and **Ir$_3$-R$_8$** (blue) with GGCCC oligonucleotides.

The interaction of **Ir$_2$** and **Ir$_2$-R$_8$** with DNA was investigated further using atomic-force microscopy (AFM). These studies were carried out with the relaxed pBR322 plasmid, which allows a direct observation of all possible interactions with the cyclometalated complexes.[22] The relaxed DNA molecules dispersed over a mica surface show some circular structures with a number of crossing points that are indicative of supercoiling initiation (Fig 2a, and Fig S1 in the ESI). Actually, some small supercoiled DNA fragments are observed (Fig S1 in the ESI). Incubation of **Ir$_2$** with the relaxed plasmid for 24 h affected the morphology of the DNA structure, so that significantly more crossing points are noticed, and longer supercoiled fragments are clearly formed (Figs 2b, and S2 in the ESI). In addition, several DNA molecules start to aggregate, which suggest strong interactions of **Ir$_2$** with the biomolecule. In agreement with the fluorescence titrations

incubation of pBR322 with **Ir_2-R_8** revealed even higher affinity for the DNA, with the induction of a major proportion of supercoiled DNA and of increased aggregation; the supercoiled fragments are significantly longer than those observed with **Ir_2**, and large open forms, such as those observed in Fig 2a, are not present anymore Furthermore, large complex-induced DNA aggregates are detected (see Fig 2c and Fig S3). Thus, the AFM results confirm the spectroscopic observations, and support the strong DNA-binding properties of **Ir_2-R_8**. Interestingly, this important effect of **Ir_2-R_8** on the degree of DNA coiling aggregation may be compared to proteins that pack the DNA into chromosomes.

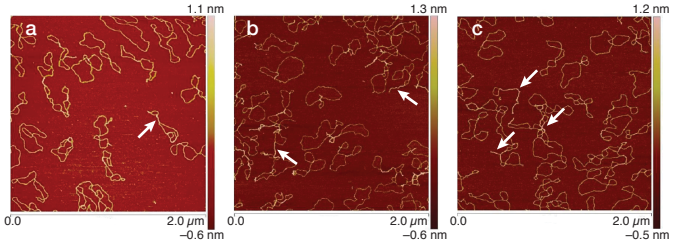


Figure 2. AFM images of (a) free relaxed pBR322 DNA (10 μM in base pairs) and plasmid incubated at 37 ºC for 24 h in HEPES with (b) complex **Ir_2** (25 μM) or (c) complex **Ir_2-R_8** (25 μM).

Following the in vitro characterization, we evaluated the cytotoxicity of **Ir_2** and **Ir_2-R_8** with a set of tumor cell lines including NCI-H460 (lung carcinoma), MCF-7 (breast cancer) and A2780 cis (ovarian carcinoma) cells using MTT method (detailed methods are reported in the ESI). In agreement with the DNA binding data, **Ir_2-R_8** displayed higher cytotoxic activity than **Ir_2**. Thus, **Ir_2** has negligible cytotoxic effects, but **Ir_2-R_8** exhibits significant effects on cell viability, so that the observed IC_{50} values are comparable to those obtained for cisplatin under the same experimental conditions (Table 2).[23] Furthermore, the cytotoxicity of the entire set of Ir(III) R_8-organometallopeptides was examined with the model doxorubicin-resistance NCI/ADR-RES ovarian cell line, which is particularly useful for the identification of compounds subjected to drug resistance. Interestingly, any of the three Ir(III) R_8-organometallopeptides caused up to 90 % inhibition of the cell viability of NCI/ADR-RES cells. The IC_{50} values estimated for **Ir-R_8**, **Ir_2-R_8** and **Ir_3-R_8** (IC_{50} = 32 μM, 50 μM, and 13 μM, respectively) are in the same range to that of cisplatin (IC_{50} = 14 μM).[24] The cytotoxicity of these compounds could be explained by the induction of highly supercoiled DNA—as observed in the AFM studies—and the resulting obstruction of processes requiring the access of proteins to the DNA (e.g., avoiding the formation of the replication fork, or the assembly of the transcriptional machinery).[25]

Table 2. IC_{50} (μM) and E_{max} (%) values of **Ir_2**, **Ir_2-R_8** and cisplatin for NCI-H460, A2780 cis and MCF-7 tumoral cell lines. The evaluation have been carried out using MTT method.

	NCI-H460	A2780 cis	MCF-7
Ir_2	>100.0, 34.0 ± 5	35.0 ± 1, 55.0 ± 3	> 100.0, 31.0 ± 2
Ir_2-R_8	15.0 ± 0.1, 91 ± 1	4.0 ± 0.2, 83 ± 1	12.0 ± 0.5, 90 ± 1
cisplatin	6.0 ± 0.3, 68 ± 2	6.8 ± 0.2, 91 ± 1	13.0 ± 0.3, 90 ± 1

Conclusions

In summary, we have applied a versatile solid phase peptide synthesis approach for the assembly of mono, di and trinuclear polypyridyl Ir(III) organometallopeptides and their octaarginine analogs, which display high DNA binding affinity and sequence selectivity. The DNA binding affinity of the trinuclear **Ir_3-R_8** metallopeptide is in the order of the best known organic DNA minor-groove binders, and, to the best of our knowledge, is one of the highest DNA affinity ever reported for a metal complex.[17b,26] Moreover, these Ir(III) R_8-derivatives are highly cytotoxic against diverse cell lines, including doxorubicin-resistance NCI/ADR-RES and display as much activity as cisplatin.

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Notes and references

[a] Departamento de Química Inorgánica and Centro Singular de Investigación en Química Biolóxica e Materiais Moleculares (CiQUS). Universidade de Santiago de Compostela. 15782 Santiago de Compostela, Spain. E-mail: miguel.vazquez.lopez@usc.es

[b] Departament de Química Inorgánica, QBI, Universitat de Barcelona, 08028 Barcelona (Spain).

[c] Catalan Institution for Research and Advanced Studies (ICREA) Passeig Lluís Companys 23, 08010 Barcelona (Spain)

[d] Grupo de Investigación BioFarma/Plataforma de Screening USEF. Centro de Investigación CIMUS. Universidade de Santiago de Compostela.

[e] Departamento de Química Orgánica and Centro Singular de Investigación en Química Biolóxica e Materiais Moleculares (CiQUS). Universidade de Santiago de Compostela. 15782 Santiago de Compostela, Spain. E-mail: eugenio.vazquez@usc.es

† Electronic Supplementary Information (ESI) available: [general remarks, synthesis of **1**, peptide synthesis and characterization, organometallopeptide synthesis and characterization, details of the DNA binding and cytotoxic studies]. See DOI: 10.1039/c[illegible]000

1 a) A. Ali, S. Bhattacharya, *Bioorg. Med. Chem.* **2014**, *22*, 4506–4521; b) V. T. DeVita, E. Chu, *Cancer Res.* **2008**, *68*, 8643–8653; K. Cheung-Ong, G. Giaever, C. Nislow, *Chem. Biol.* **2013**, *20*, 648–659.

2 a) G. S. Khan, A. Shah, Zia-ur-Rehman and D. Barker, *J. Photochem. Photobiol. B,* 2012, **115,** 105–118; b) K. Gurova, *Future Oncol.,* 2009, **5,** 1685–1704.

3 a) A. C. Komor, J. K. Barton, *Chem. Commun*. **2013**, *49*, 3617–3630; b) D. A. Richards, A. Rodger, *Chem. Soc. Rev*. **2007**, *36*, 471–483; c) B. M. Zeglis, V. C. Pierre, J. K. Barton, *Chem. Commun*. **2007**, 4565–4579; d) K. L. Haas, K. Franz, *Chem. Rev*. **2009**, *109*, 4921–4960; e) P. C. A. Bruijnincx, P. J. Sadler, *Curr. Opin. Chem. Biol*. **2008**, *12*, 197–206; f) A. Pyle, J. K. Barton, *Bioinorganic Chemistry*, Eds.: Lippard, S. J., John Wiley & Sons, Inc., New York, 1990, p. 413.

4 a) J. Reedijk, *Eur. J. Inorg. Chem*. **2009**, 1303-1312; b) L. Kelland, *Nat. Rev. Cancer* **2007**, *7*, 573–584; b) E. R. Jamieson, S. J. Lippard, *Chem. Rev*. **1999**, *99*, 2467–2498.

5 R. W.-Y. Sun, D.-L. Ma, E. L.-M. Wong, C.-M. Che, Dalton Trans. 2007, 4884–4892.

6 a) C. A. Puckett, J. K. Barton, J. Am. Chem. Soc. 2009, 131, 8738–8739; b) J. Brunner, J. K. Barton, Biochemistry 2006, 45, 12295–12302.

7 M. R: Gill, J. García-Lara, S. J. Foster, C. Smythe, G. Battaglia, J. A. Thomas, Nature Chem. 2009, 1, 662–667.

8 a) K. K.-W. Lo, M.-W. Louie, K. Y. Zhang, *Coord. Chem. Rev.*, **2010**, *254*, 2603–2622; b) Z. Liu, P. J. Sadler, *Acc. Chem. Res*., **2014**, *47*, 1174–1185; c) K. K-.W. Lo, S. P.-Y. Li, K. Y. Zhang, *New. J. Chem*., **2011**, *35*, 265–287.

9 S. Stimpson, D. R. Jenkinson, A. Sadler, M. Latham., A. Wragg, A. J. H. M. Meijer, J. A. Thomas, *Angew. Chem. Int. Ed*., **2015**, *54*, 1–5.

10 M. A. Nazif, R. Rubbiani, H. Alborzinia, I. Kitanovic, S. Wölfl, I. Ott, W. S. Sheldrick, *Dalton. Trans*., **2012**, *41*, 5587–.

11 K. Y. Zhang, H.W. L., T. T.-H. Fong, X.-G. Chen, K. K.-W. Lo, *Inorg. Chem*., **2010**, *49*, 5432–5443.

12 a) C. R. Brodie, J. R. Aldrich-Wright, *Eur. J. Inorg. Chem*., **2007**, 4781–4793; b) C. Metcalfe, J. A. Thomas, *Chem. Soc. Rev.*, **2003**, *32*, 215–224.

13 G. Rama, A. Ardá, J.-D. Maréchal, I. Gamba, H. Ishida, J. Jiménez-Barbero, M. E. Vázquez, M. Vázquez López, *Chem. Eur. J*., **2012**, *18*, 7030–7035.

14 a) C. Dolan, R. D. Moriarty, E. Lestini, M. Devocelle, R. J. Foster, T. E. Keyes, *J. Inorg. Biochem*., **2013**, *119*, 65–74; b) K. Koren, R. I. Dmitriev, S. M. Borisov, D. B. Papkovsky, I. Klimant, *ChemBioChem* **2012**, *13*, 1184–1190.

15 I. Coin, M. Beyermann and M. Bienert, *Nat. Protoc.,* 2007, **2,** 3247-3256.

16 (*a*) M. I. Sánchez, O. Vazquez, J. Martinez-Costas, M. E. Vazquez and J. L. Mascareñas, *Chem. Sci.,* 2012, **3,** 2383; (*b*) I. Gamba, I. Salvadó, G. Rama, M. Bertazzon, M. I. Sánchez, V. M. Sánchez-Pedregal, J. Martinez-Costas, R. F. Brissos, P. Gamez, J. L. Mascareñas, M. Vázquez López and M. E. Vázquez, *Chem. Eur. J.,* 2013, **19,** 13369; (*c*) J. Mosquera, M. I. Sánchez, J. Valero, J. Mendoza, M. E. Vázquez and J. L. Mascareñas, *Chem. Comm.*, 2015, **51,** 4811.

17 (*a*) C. Crane-Robinson, A. Dragan, and P. Privalov, *Trends Biochem. Sci.*, 2006, **31**, 547; (*b*) D. Mascotti and T. Lohman, *Biochemistry*, 1997, **36**, 7272; (c) D. E. Wetzler, M. J. Comin, K. Krajewski and M. Gallo, *PLoS One,* 2011, **6,** e22409.

18 (*a*) O. Vazquez, J. B. Blanco-Canosa, M. E. Vazquez, J. Martinez-Costas, L. Castedo and J. L. Mascareñas, *ChemBioChem,* 2008, **9,** 2822; (*b*) J. Brunner and J. K. Barton, *Biochemistry,* 2006, **45,** 12295.

19 Oligoarginines are known to be effective molecular transporters capable of carrying different cargoes across biological barriers: E. A. Goun, T. H. Pillow, L. R. Jones, J. B. Rothbard, and P. A. Wender, *Chembiochem,* 2006, **7**, 1439.

20 M. T. Carter, M. Rodriguez, A. J. Bard, *J. Am. Chem. Soc.,* **1989**, *111*, 8901–8911.

21 a) F. Han, N. Taulier, T. V. Chalikian, *Biochemistry*, **2005**, *44*, 9785–9794; b) J. B. Chaires, *Arch. Biochem. Biophys*., **2006**, *453*, 26–31.

22 G. B. Onoa, V. Moreno, *Int. J. Pharm*. **2002**, *245*, 55–65.

23 Conjugation to oligoarginines has been shown to modify the biological properties of metal complexes: (a) C. A. Puckett, J. K. Barton, *Bioorg. Med. Chem.* **2010**, *18*, 3564–3569; (b) C. A. Puckett and J. K. Barton, *J. Am. Chem. Soc.,* 2009, **131,** 8738–8739.

24 Previous reports clearly indicate that $(Arg)_8$ peptide produces substantially no obvious cytotoxic effects on HeLa cells. T. Suzuki, S. Futaki, M. Niwa, S. Tanaka, K. Ueda, Y. Sugiura. *J. Biol. Chem.* **2002**, *277*, 2437-2443.

25 a) M. J. Hannon, V. Moreno, M. J. Prieto, E. Moldrheim, E. Sletten, I. Meistermann, C. J. Isaac, K. J. Sanders, A. Rodger, *Angew. Chem. Int. Ed.* **2001**, *40*, 879–884; b) L. Childs, J. Malina, B. Rolfsnes, M. Pascu, M. Prieto, M. Broome, P. Rodger, E. M. Sletten, V. Moreno, A. Rodger, M. J. Hannon, *Chem. Eur. J*. **2006**, *12*, 4919–4927.

26 a) F. Han, N. Taulier, T. V. Chalikian, *Biochemistry*, **2005**, *44*, 9785–9794; b) J. B. Chaires, *Arch. Biochem. Biophys*., **2006**, *453*, 26–31.

