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Enhanced Cellular Uptake and Cytotoxicity Studies of Organometallic Bioconjugates of the NLS Peptide in Hep G2 Cells

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Intracellular delivery to biomolecular targets is still a major challenge in molecular and cell biology. We recently found that attaching an organometallic group, namely the cobaltocenium cation, to the SV 40 large T antigen nuclear localisation signal (NLS) greatly enhances cellular uptake of the conjugate (Noor et al., Angew. Chem. Int. Ed. ***2005***, 45, *2429). In addition, nuclear localisation of the conjugate was observed. In this work, we present a thorough investigation of this novel cellular delivery system with respect to the nature of the metal complex and the peptide sequence. A number of ferrocene ((Fe^{II}), neutral metal complex) and cobaltocenium ((Co^{III}), cationic metal complex) bioconjugates with both the NLS wild-type sequence PKKKRKV and a scrambled sequence (NLS$_{scr}$ KKVKPKR) were prepared by solid-phase peptide synthesis (SPPS). Cellular and nuclear uptake of these bioconjugates was studied by fluorescence microscopy on living Hep G2 cells. In addition, cytotoxicity screening on the conjugates was carried out, as the toxic effects of several simple metallocenes have been noted previously. Rapid cellular uptake as well as nuclear localisation was observed for the metal–NLS conjugates, but not for any dipeptide controls, the metal–NLS$_{scr}$ conjugates or any metal-free conjugates. It thus appears that the presence of a metallocene, but not its charge, and the correct NLS sequence is essential for cellular uptake. Fluorescence microscopy co-localisation studies did not reveal a significant endosomal entrapment of the conjugates. The metallocene not only provides a hydrophobic handle for membrane translocation but also facilitates the localisation and distribution of the conjugate in the cytoplasm. The NLS peptide on the other hand is responsible for the nuclear localisation of the bioconjugate. Finally, none of the conjugates were found to be toxic up to the highest concentrations that was tested (1 mM) against the Hep G2 cells that were used in this study. In conclusion, this work supports metallocene–NLS bioconjugates, in particular with the very robust cobaltocenium group, as a simple but potent, nontoxic system for cellular uptake and nuclear delivery. Concurrently, our finding is relevant to the still-unresolved question of cytotoxicity of metallocenes because it excludes binding and/or damage to the DNA as a mechanism of metallocene cytotoxicity. This finding is confirmed by a combined yeast cytotoxicity/genotoxicity assay, which also shows very little toxic effects for all organometal–NLS conjugates that were tested.*

Introduction

The passage of drugs and gene therapy agents (antisense DNA or plasmids) through biological barriers such as the cellular or nuclear membrane still remains a challenge, especially when a large population of cells is targeted. In gene therapy, the transport of the therapeutic DNA from the cytoplasm into the nucleus is an additional complication. Recent work has led to a better understanding of the mechanisms involved in the transport of proteins and RNA in and out of the nucleus.[1–3] This transport involves peptidic nuclear localisation signals (NLSs) or export signals that bind to nuclear transport proteins (namely the karyopherins), which mediate their movement in or out of the nucleus. The mechanism by which NLS delivers its cargo through the nuclear pore complex has been the subject of investigation.[4–8] NLS peptides have already been used as carriers to deliver cargo into the cellular nucleus,[9–11] especially as a nonviral DNA-transfection system. Thus, NLS peptides have been attached covalently[12–14] or noncovalently[15–23] to DNA. Conjugates of the NLS peptide with peptide nucleic acid oligomers (PNA) were shown to have enhanced antisense activity.[18] In another approach, acridine[24] or pyrene[25] conjugates were used as intercalating agents with improved nuclear delivery by the NLS peptide. In other studies, a radiolabelled monoclonal antibody was shown to be translocated into the

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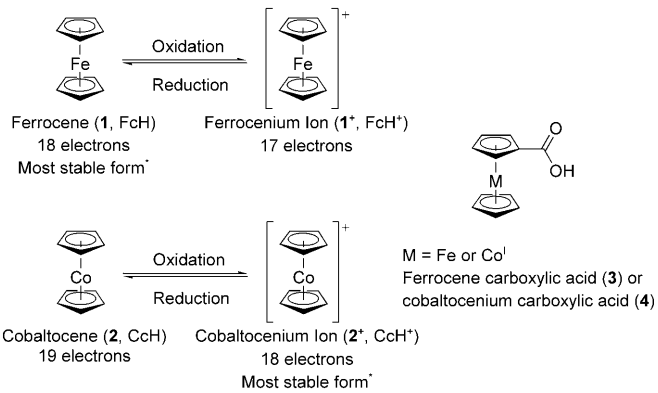
nuclei of acute myeloid leukaemia cells when conjugated with NLS,[26] and a Rh–intercalator complex with the NLS peptide was used by Brunner and Barton to target DNA mismatches.[27] A recent report from our group indeed shows high cellular uptake and nuclear localisation of PNA–NLS conjugates with Co complexes by atomic absorption spectroscopy.[28]

However, inefficient entry of NLS conjugates into cells[17,29] or their inability to escape endosomes[30,31] has been reported in live-cell studies and is a major hurdle for the applications that are described above. One solution to this problem and to enhance the cellular uptake of NLS conjugates was to increase the lipophilicity of the NLS peptide by conjugation to other peptides or fullerenes.[32] However, the fullerene conjugate was not localised in the nuclei, probably due to its inability to escape the endosomes.[31] We have recently suggested the use of a novel metal-containing NLS conjugate for cellular delivery. In this conjugate, an organometallic complex (a so-called metallocene), namely the cobaltocenium cation, was covalently linked to the NLS N terminus. We reasoned that the metallocene moiety might facilitate lipid anchoring and translocation through the cellular membrane, thereby increasing the cellular uptake of the conjugates.[33] This chemical strategy is intriguingly simple because conjugation of the metallocene to the NLS peptides requires only one single additional step in the chemical synthesis of the NLS peptide. To the best of our knowledge, this was the first demonstration of a simple construct of a hydrophobic metallocene with a cationic peptide that showed both cellular uptake and nuclear localisation after cytoplasmic distribution. The obvious question arises whether this was a unique finding or a general principle, and if so, which factors govern the uptake of metallocene–NLS conjugates. We have explored the influence of different metals and the charge of the metallocenes (Co in the cobaltocenium cation **2** and Fe in the uncharged ferrocene **1**, see Scheme 1) as well as changes in the NLS amino acid sequence on the cellular uptake and nuclear delivery properties of metallocene–NLS conjugates.

A generally applicable nuclear delivery system obviously cannot be toxic. Indeed, conflicting reports exist on the toxicity of the prototype metallocene, ferrocene and its derivatives. Moreover, even the mechanism of ferrocene cytotoxicity is presently unclear, and unspecific DNA binding or intercalation, DNA damage,[34] topoisomerase inhibition,[35] or immediate attack by ferrocene-generated hydroxide radicals ($OH^{\bullet}$) on the cell membranes have been suggested.[36] Contradictory to chemical intuition, the antiproliferative activity seems to be independent of the redox potentials of the tested compounds. On the other hand, Neuse and co-workers were able to greatly enhance the antiproliferative activity of ferrocene by loading polyamines such as polyaspartamide with ferrocenes.[37–39] These researchers attribute enhanced activity to improved aqueous solubility and do not consider the iron oxidation state (+II or +III) to be relevant for cytotoxicity.[40–42] Clearly, the question of possible cytotoxicity of metallocene–peptide conjugates needs to be addressed if their use in biological applications is considered. Furthermore, in trying to put all the pieces of the above-described puzzle together, we have developed an intriguing hypothesis. If DNA damage (direct binding or by $OH^{\bullet}$ radical generation) were the mechanism of cytotoxicity of the metallocenes, then it should be enhanced by direct delivery of the metallocene into the nucleus of a cell. We use ferrocene carboxylic acid and cobaltocenium carboxylic acids as metal moieties in this study. Although both metal complexes are similar in size and other properties (see Scheme 1), they differ significantly in two properties pertinent to this study. First, the stable cobaltocenium species is a cation, but ferrocene is a neutral molecule. Second, cobaltocenium compounds are inert and redox-inactive under physiological conditions. On the other hand, ferrocene, which contains iron in the +II oxidation state is readily oxidised to the ferrocenium cation, which contains iron in the +III oxidation state. This oxidation is reversible and resembles the well-known Fenton cycle for $OH^{\bullet}$ radical generation by using simple iron ions in aqueous solutions. If intracellular Fe^{II}/Fe^{III} redox activity is essential for cytotoxicity, then clearly the ferrocene conjugates should be active, but cobaltocenium cannot be. Thus, by comparing metallocenes with different charge and redox behaviour, more mechanistic insight should be gained not only for the cytotoxicity but also upon the cellular uptake and nuclear localisation of these metallocene–NLS bioconjugates.



Scheme 1. Metallocenes that were used in this study: Ferrocene (**1**) and cobaltocene (**2**) in their oxidised and reduced forms, respectively. For conjugation to peptides, the carboxylic acid derivatives **3** and **4** were used (see Scheme 2).

Results

Synthesis and characterisation

All bioconjugates were synthesised on solid-phase support by using the Fmoc strategy. In addition to metallocene conjugates with NLS or scrambled NLS peptides, conjugates that are devoid of the metallocenes as well as the metal-containing truncated peptides were prepared as controls. All of the compounds that were used in this study are compiled in Table 1. The peptide sequences (NLS or NLS_{scr}) were synthesised either on a computer-controlled peptide synthesiser or manually by syringe techniques.[43] Wang resin that was preloaded with Fmoc-Val-OH (0.63 mmol g^{-1}) was used for NLS and the truncated metal–dipeptides. For the NLS_{scr} sequence, Wang resin

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Table 1. Metallocene–peptide conjugates used in this study.

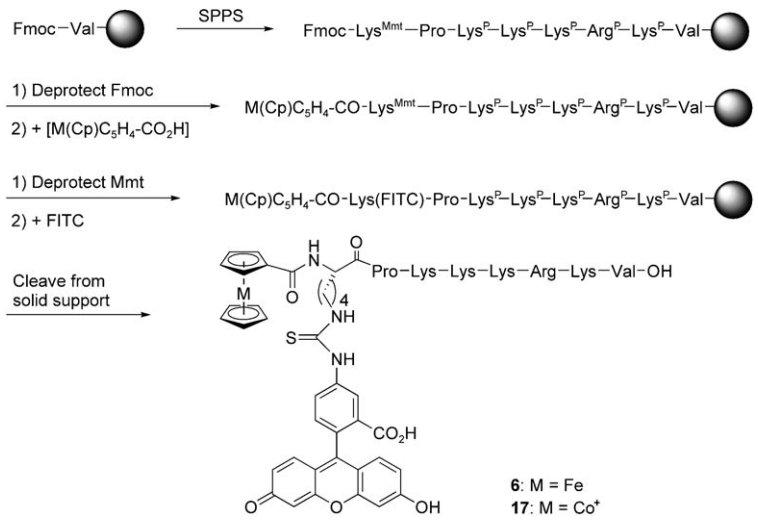
Compound	Sequence/compound	Abbreviation[a]	Mass [g mol^{-1}] calcd	exp.	composition
5	FcC(O)-KPKKKRKV	FcC(O)-Lys–NLS	1222.7	1223.5	$[M+H]^+$
				1246.6	$[M+Na]^+$
6	FcC(O)-K(FITC)PKKKRKV	FcC(O)-Lys (FITC)–NLS	1611.74	1614.2	$[M+2H]^+$
7	FcC(O)-K(FITC)V	FcC(O)-Lys (FITC)-Val	846.2	847.5	$[M+H]^+$
				869.5	$[M+Na]^+$
				885.5	$[M+K]^+$
8	KKKVKPKR	Lys–NLS$_{scr}$	1010.71	1011.8	$[M+H]^+$
				1033.8	$[M+Na]^+$
		Lys (FITC)–NLS$_{scr}$	1399.74	1402.2	$[M+2H]^+$
				1424.1	$[M+Na]^+$
10	FcC(O)-KKKVKPKR	FcC(O)-Lys–NLS$_{scr}$	1222.7	1224.1	$[M+H]^+$
				1246.6	$[M+Na]^+$
11	FcC(O)-K(FITC)KKVKPKR	FcC(O)-Lys (FITC)–NLS$_{scr}$	1611.74	1614.0	$[M+2H]^+$
12	CcC(O)-KKKVKPKR	CcC(O)-Lys–NLS$_{scr}$	1225.7	1226.9	$[M++H]^+$
				1248.9	$[M+Na]^+$
				1282.9	$[M+K]^+$
13	CcC(O)-K(FITC)KKVKPKR	CcC(O)-Lys (FITC)–NLS$_{scr}$	1614.73	1615.9	$[M+H]^+$
				1637.8	$[M+Na]^+$
14	KPKKKRKV[b]	Lys–NLS	1010.71	1011.9	$[M+H]^+$
15	CcC(O)-KPKKKRKV[b]	CcC(O)-Lys–NLS	1225.70	1226.5	$[M+H]^+$
16	CcC(O)-K(FITC)V[b]	CcC(O)-Lys (FITC)-V	849.20	849.3	$[M]^+$
17	CcC(O)-K(FITC)PKKKRKV[b]	CcC(O)-Lys (FITC)–NLS	1615.8	1616.3	$[M+H]^+$

[a] All free acids on C termini, free amino groups on N termini of nonmetallocene peptides. [b] Previously reported in ref. [33].

preloaded with Fmoc-Arg(Pbf)-OH (0.58 mmol g^{-1}) was used. In all cases, acid-labile linkers were used to generate carboxylic acid groups at the C termini of all bioconjugates upon acid cleavage. The complete synthetic scheme is exemplified for the FITC-labelled metallocene–NLS conjugates **6** and **17** in Scheme 2.

Ferrocene carboxylic acid (**3**) and cobaltocenium carboxylic acids (**4**) were used as metal labels. The metallocene carboxylic acids could be readily coupled to the peptides on the solid support. A fluorescent label, fluorescein isothiocyanate (FITC), was added manually in the last step of synthesis to minimise its exposure to other reagents during the synthesis. FITC was attached to an orthogonally protected Lys(Mtt) residue. HPLC chromatograms of most crude bioconjugates showed 98% purity. Therefore, both the labelling of the bioconjugates with FITC and the addition of metallocenes on solid-phase support is a fairly clean and easy-to-handle method.

All bioconjugates were comprehensively characterised by HPLC, mass spectrometry, NMR spectroscopic methods and electrochemistry. The mass of each compound was determined by MALDI-TOF mass spectrometry (Table 1). ^{1}H NMR spectra were also measured for all bioconjugates. A characteristic feature is the signals that arise from the cyclopentadienyl rings of the metallocenes around 4 ppm for ferrocene or 5 ppm for cobaltocenium conjugates, respectively. The FITC signals were identified in the aromatic region of the proton NMR spectra. Signal assignments were carried out with the help of 2D NMR spectra where necessary. All the metal-containing bioconjugates were electrochemically active, and their redox potentials were determined by square wave voltammetry.



Scheme 2. Synthesis of metallocene–peptide conjugates as exemplified for compounds **6** (M = Fe) and **17** (M = Co$^+$).

Cellular and nuclear uptake

Cellular uptake was studied on Hep G2 cells, which are an immortalised human liver cancer cell line. The uptake was monitored on live cells by fluorescence microscopy. The ferrocene–NLS bioconjugate **6**, at a concentration of 500 μM, was found to be localised in the nuclei (Figure 1 B). Fluorescence microscopy showed homogenous green fluorescence inside the cells that was much stronger in the nuclei. Some point-like fluorescence was also observed, which

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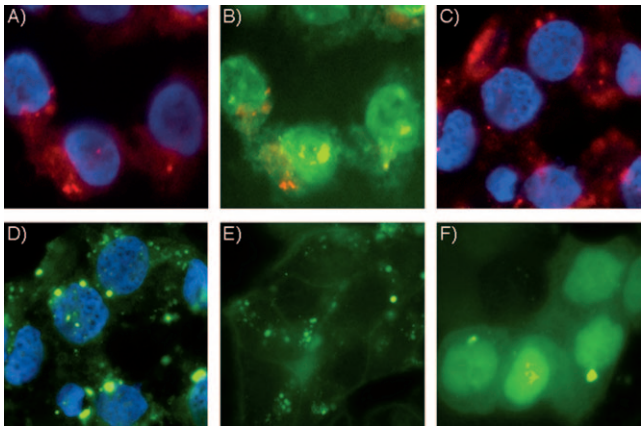

Figure 1. Uptake and distribution of metallocene–NLS bioconjugates. Hep G2 cells were incubated with test compounds for 24 h. The endosomal staining was carried out with the endosomal marker FM 4–64 (5 μM) for 15 min and the nuclei were stained with Hoechst 33342 dye for 1 min. A) FM 4–64 in live cells (red fluorescence) and nuclear staining by Hoechst 33342 dye (DAPI filter, blue) for bioconjugate **6** at a concentration of 0.5 mM. B) Distribution of bioconjugate **6** and the endosomal marker FM 4–64 (merged image). C) FM 4–64 in live cells (red fluorescence) and nuclear staining by Hoechst 33342 dye (DAPI filter, blue) for bioconjugate **7**. D) Distribution of bioconjugate **7** and Hoechst 33342 (merged image). E) Distribution of bioconjugate **16** in Hep G2 cells.[33] F) Distribution of bioconjugate **17** in Hep G2 cells.[33]

seemed to be mostly extracellular with possibly some small spot-like fluorescence located inside the cells. By using double fluorescence microscopy, the endosomal marker FM 4–64 revealed that this bioconjugate was not extensively co-localised in the endosomes (Figure 1 B) after 24 h of incubation. The same result was observed with the lower concentration (100 μM) of this compound, although less nuclear localisation was also observed; this indicates a concentration-dependent availability of the free bioconjugate in the cytosol for its nuclear translocation. In addition, the control compound **7**, FcC(O)-Lys(FITC)-Val-OH accumulated neither in the cytoplasm nor in the nuclei. This result is consistent with our previous study.[33] Most probably, the truncated dipeptide entered the cells to some extent by passive diffusion, but was not sufficiently accumulated to be visible inside the cells. In this case, bright dot-like fluorescence was observed outside the cells (Figure 1 D). The NLS peptide alone is unable to enter intact cells.[31,33]

Furthermore, we set out to determine the role of the NLS peptide upon cellular uptake and nuclear localisation. For this purpose, we used the scrambled sequence of the NLS (KKVKPKR) reported by Cutrona et al.,[44] which was shown to be unable to target the nuclei of the cells. The nuclear localisation function of the NLS is essentially dependent on the presence of Lys128.[45] Various scrambled sequences of NLS in which Lys128 is replaced by other amino acids have been shown to be unable to enter cells and translocate into their nuclei.[29,46,47]

Ferrocene (**11**) and cobaltocenium conjugates (**13**) of the scrambled NLS sequence, both labelled with FITC, were studied along with a metal-free, FITC-labelled scrambled peptide **9**. All three compounds **9**, **11** and **13** showed similar behaviour. They were not taken up by intact Hep G2 cells, nor were they distributed in the cytoplasm, and no nuclear localisation was observed in the concentrations that were tested (500 μM and 100 μM). Some bright green spots were seen, which is probably due to the tendency of these compounds to adhere to the cell membrane (Figure 2).

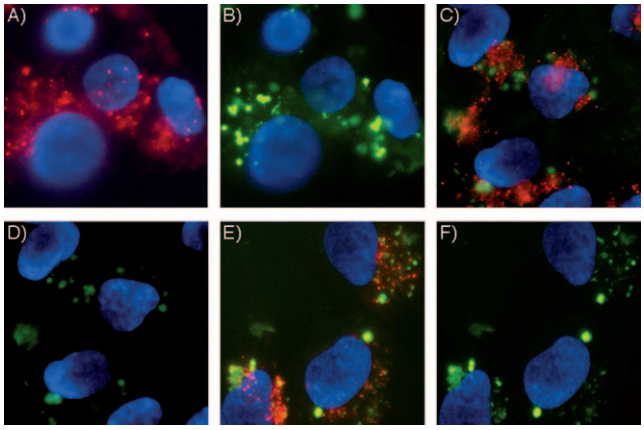

Figure 2. Uptake and distribution of metallocene–NLS_{src} bioconjugates. Hep G2 cells were incubated with test compounds for 24 h. Pictures shown for 0.5 mM concentration. The endosomal staining was carried out with the endosomal marker FM 4-64 (5 μM) for 15 min and the nuclei were stained with Hoechst 33342 for 1 min. A) Distribution of bioconjugate **9**, the endosomal marker FM 4-64 and Hoechst 33342 (merged image). B) Distribution of bioconjugate **9** and Hoechst 33342 (merged image). C) Distribution of bioconjugate **11**, the endosomal marker FM 4-64 and Hoechst 33342 (merged image). D) Distribution of bioconjugate **11** and Hoechst 33342 (merged image). E) FM 4–64 in live cells (red fluorescence) and nuclear staining with Hoechst 33342 (DAPI filter, blue) for bioconjugate **13.** F) Distribution of bioconjugate **13** and Hoechst 33342 (merged image).

Cytotoxicity

It should be noted that none the compounds that were tested for uptake studies appeared to have a toxic effect on the Hep G2 cells by visual inspection of cells during the above experiments. (For example, no irregular cell membrane or condensation of chromatin material by DAPI staining was observed). Cell integrity was further assured by incubating the cells with propidium iodide, which was not taken up by the cells in any of the experiments (data not shown). As an initial overview screening, a combined cytotoxicity–genotoxicity test on transformed yeast strains was carried out. Briefly, this test is based on yeast strains that carry reporter plasmids that express a green fluorescent protein (yEGFP) under the control of the promoters for the genes RNR2 and RAD54, which are induced in response to genotoxic DNA damage, and a control strain in which GFP is not expressed due to a frameshift mutation.[48,49] The tests were performed in black 96-well microplates for optimal fluorescence detection. All substances were tested in serial dilutions by starting from 2 mM stock solutions of the test compounds (in 4% (*v*/*v*) aqueous DMSO); the final concentration ranged from 1.0 mM to 0.002 mM. Measurements were carried out with a fluorescence/absorption reader and fluorescence measurements were corrected for optical density (for details see the Supporting Information). The results were classified as inactive, weak positive and strong positive. For the

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latter two, the lowest effective concentration (LEC) at which GFP fluorescence was detectable was given (Table S1). The cobaltocenium conjugate **15** was completely inactive in this assay, whereas the ferrocene conjugate **5** was weakly active on the RNR2 transformed strain (LEC = 1 mM). However, even the nonmetallated compound **14** showed weak cytotoxicity on both strains. From this preliminary screen, we conclude that there is no metal-based toxicity in these metal–NLS conjugates.

After this initial screening, cytotoxicity was determined on the Hep G2 cells that were used in this work for compounds **5**, **8**, **10**, **12**, **14** and **15**. For this purpose, we used the WST-1 assay as an easy, nonradioactive, spectrophotometric quantification of cell growth and viability.[50] At the same time, the crystal violet cell quantification assay[51] was performed after the WST-1 assay to determine the remnant number of adherent cells. A Hep G2 standard population curve was drawn by using different cell populations and their treatment with crystal violet to determine the number of cells that are required for this dual assay (Figure S1). Similarly, another curve was drawn to determine the optimal time of incubation with WST-1 for Hep G2 cells (Figure S2). In the cytotoxicity assay, cells were incubated with WST-1 reagent at 37 °C, and the absorbance was measured every 30 min for 4 h. In the end, the readings were further corrected by quantification of adherent cells as determined by the CV assay (Figure S3).

In the cases of K–NLS (**14**), Cc(CO)-K–NLS (**15**) and Fc(CO)-K–NLS (**5**), in all of the tested concentrations (0.1, 0.5 and 1 mM) no statistically significant difference was observed on the proliferation rate of the Hep G2 cells, as compared with the control (Figure S3). Similarly, in case of K–NLS_{scr} (**8**) and FcC(O)-K–NLS_{scr} (**10**), no statistically significant difference was found with respect to the control at concentrations of 0.1, 0.5 and 1 mM. However, in case of CcC(O)-K–NLS_{scr} (**12**) a slight increase in cell numbers was found in comparison with the control in concentrations of 0.5 and 1 mM; this indicates a stimulatory effect of this compound on the metabolic activity of the Hep G2 cells. No significant effect was observed at 0.1 mM concentration for this compound.

Discussion

For nuclear delivery of a given conjugate into intact living cells, two major obstacles need to be considered: 1) The cellular internalisation by direct translocation in an energy-independent manner or through endocytosis where escape from endosomes is essential for translocation into various cellular compartments and 2) passage through the nuclear envelope into the nuclei.

The cellular internalisation of cationic cell penetrating peptides (CPPs) is dependent on lipophilic interactions on the cell surface which lead to direct entry by diffusion or endocytosis. The exact mechanism by which the cationic NLS conjugates gain cellular entry is controversial. Recent work suggests, however, that artificial redistribution, at least on some if not all, cationic peptides might occur upon fixation.[52] Our studies were therefore entirely carried out on live cells. On the other hand, the practical utility of CPPs as transfection agents in general is hampered by their inability to escape the endosomes.[53] Release from endosomes is a complex process which depends on the physicochemical properties of the endocytosed material.[54] Facilitated endosomal release can be achieved by endosomolytic agents like chloroquine,[55] Ca^{II},[56] sucrose,[57] and polyion complex micelles[58] or laser illumination[59] and other photochemical internalisation treatments.[60] Once translocated across the cellular membrane and distributed in the cytoplasm, the nuclear delivery of NLS peptides such as the SV40 large T antigen NLS (PKKKRKV) that was used in this study occurs in an energy-dependent way. This transport is tightly controlled at multiple levels[8] and depends upon the permeability of the nuclear pore complexes at a given stage in cell cycle[61] as well as the ratio of these pore complexes available for entry.[62]

The present study significantly extends our previous work in which we reported that a cobaltocenium bioconjugate was showing enhanced cellular uptake and nuclear localisation.[33] Based on this observation, we analysed the effect of the metallocene as well as the peptide upon cellular uptake and nuclear localisation. Thus, conjugates with the cobaltocenium moiety were compared to conjugates in which the cobaltocenium group was replaced by ferrocene, which is geometrically very similar except that it lacks the positive charge. With the ferrocene conjugates of NLS we obtained similar results as for the cobaltocenium–NLS conjugate. At concentrations of 500 μM and 100 μM, the bioconjugates were not only taken up by intact live cells, but was also localised in the nuclei. Previous reports on live cells have suggested that the NLS does gain entry through endosomes.[30,63,64] After internalisation of our ferrocene–NLS bioconjugate, we could not observe any colocalisation of this compound in the endosomes after 24 h of incubation. Possibly the endosomes were permeable, the compound was quickly released from the endosomes, and then localised in the nuclei. It might also be possible that the internalisation occurred in an endocytosis-independent manner or by yet another mechanism in addition to the endocytotic pathway. To further characterise the role of the metallocenes, we tested the fluorescently-labelled metallocene dipeptides **7** and **16** as controls. These compounds are not observed inside the cells. In addition, they were not found to have nuclear localisation. This finding is highly relevant because dipeptide conjugates such as **7** and **16** are far more lipophilic than the K-rich NLS conjugates. Lipophilicity alone is therefore not an important factor for cellular uptake of metallocene–peptide conjugates.

The role of the NLS in nuclear localisation was further investigated by using a scrambled peptide sequence that was conjugated with the metallocenes. The scrambled sequence (KKVKPKR) was reported as nonfunctional because it is unable to reach the nucleus even when it is localised in the cytoplasm.[44,29,46,47] The bioconjugates of cobaltocenium and ferrocene with NLS_{scr} were totally different in their behaviour towards cellular uptake and nuclear localisation when compared with the wild-type NLS conjugates. These conjugates not only did not gain entry into the nuclei but were also unable to enter the intact cells. Rather, they showed a tendency to

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clump together and adhere to the outer membrane, which was seen as bright fluorescence outside the cells. These results are surprising because they imply that the correct NLS amino acid sequence is essential not only for nuclear distribution but also for the uptake of these compounds into the cells *in the first place*. The wild-type metal-free NLS sequence with an additional lysine residue at the N terminus was unable to enter the cells;[33] this is in line with the finding that NLS alone lacks the ability to cross the cell membrane.[31] We thus conclude that the combination of metallocene *and* the correct NLS sequence is necessary for the effects that we observed.

Naturally, all conjugates in this study were FITC-labelled for fluorescence microscopy. It is thus impossible to assess the influence of the fluorescence dye on cellular uptake. On the other hand, because FITC conjugates were used throughout, its presence is not pertinent to the metal or peptide-specific effects that are discussed herein.

In summary, the metal moiety helps the NLS to gain entry into the cells and the NLS is then responsible for shuttling inside the nuclei. The metallocene does play a role in the cellular uptake, and its charge does not lead to any drastic consequences upon the uptake. If endosomal uptake is involved, then the metallocene seems to play an important role in the rapid escape of these bioconjugates from the endosomes.

Although the antitumour activity of some ferrocene derivatives has been repeatedly reported in a number of studies, the site or mechanism of action still remains elusive. Another objective of our study was to obtain insight into the mechanism of action by directing the metallocene to one of the proposed sites of activity, namely the nuclear DNA. To this end, the first step was to confirm that the compounds do reach the nuclear target to validate the proposed mechanisms of direct DNA interaction or DNA damage by radical generation. Thus, fluorescently labelled conjugates were monitored for their uptake and nuclear localisation as described above. When the cells were visually inspected, they retained their smooth membrane structures and excluded propidium iodide. DAPI staining did not show condensation of the chromatin material, which would be an indication of DNA-mediated toxicity. Secondly, we screened nonfluorescent compounds in a WST/CV dual assay for cytotoxicity. None of our compounds was found to be cytotoxic in the tested concentrations or up to 1 mM. Compound **12** even had a slight stimulatory effect on the proliferation in concentrations of 0.5 mM and 1 mM. It can be concluded that the neutral ferrocene bioconjugate (Fe^{II}) as well as the cationic cobaltocenium bioconjugate (Co^{III}) are nontoxic when they are localised in the nuclei. We assume that ferrocene–NLS bioconjugate remains in the reduced state (Fe^{II}) during the experiments. A previous study showed that ferrocene–lipid conjugates in the reduced form were taken up better than the oxidised ferrocenium conjugates.[65]

With regard to the mechanism of cytotoxicity of the metallocenes that were investigated in this study, two conclusions can be drawn. First, the charge of the metallocene does not play a role; that is, the unspecific interaction of a positively charged cobaltocenium cation with the polyanionic DNA can probably be discounted. Second, the difference in redox properties does not play a role for cytotoxicity either. However, this second conclusion is, of course, strictly true only under the assumption that the metallocenes are placed *inside* cells, or more specifically inside the cellular nuclei. A recent suggestion puts forward the *extracellular* generation of $OH^{\bullet}$ radicals, followed by damage to the cell membrane lipids as a possible cause for the cytotoxicity of the ferrocenium ion. In line with this, earlier reports have claimed a lack of activity for neutral ferrocene derivatives.[36,66–71] Looking at the problem from a different angle, our present study supports these reports. No significant activity could be found in the combined yeast cytotoxicity–genotoxicity assay either.

The convenience of metallocene–NLS bioconjugates is that the metallocene moieties are robust and fairly stable to work with; this makes them worthy candidates in bioorganometallic chemistry. In addition, the ease of synthesis, the simplicity and nontoxicity of the molecules make these bioconjugates extremely interesting as vectors for drug delivery or as carriers in biotechnology. It may be noted that while both metal complexes show comparable results, the cobaltocenium conjugate might have an advantage due to its greater robustness, redox inactivity and a clean record for nontoxicity. The potential of metallocenes as organometallic labels to biomolecules remains far from being completely exploited. Further studies on the use of metallocene–peptide bioconjugates are underway in our laboratory.

Experimental Section

General methods and materials: The peptides were synthesised by using routine methods of solid-phase peptide synthesis on an Eppendorf automatic synthesiser. DMF for peptide synthesis (amine-free) was purchased from Roth Chemicals (Karlsruhe, Germany). All other solvents were reagent grade unless otherwise specified. All amino acids and resins were purchased from Novabiochem (Laufelfingen, Switzerland), and pure L-amino acids were used throughout. Other reagents or chemicals were purchased from Sigma–Aldrich and used as received. FITC was purchased from Fluka Chemicals. The synthesis and full characterisation of compounds **14**–**17** was described previously.[33]

Mass spectra (MALDI-TOF) were measured on a Bruker Biflex Instrument in the mass spectrometry laboratory at the Institute of Organic Chemistry at the University of Heidelberg. NMR spectra were measured at room temperature on Bruker DRX 300 (^1H at 300.13 MHz) and Varian Unity 400 (^1H at 399.90 MHz) instruments. Square wave voltammograms were obtained with a three-electrode cell on a Princeton Applied Research Basic Electrochemical System Potentiostat (Princeton, USA). An Ag/AgCl (0.01 M AgCl in MeCN) reference electrode, a glassy carbon disk working electrode with 2 mm diameter and a Pt wire auxiliary electrode in MeCN that contained 0.1 M tetrabutylammonium hexafluorophosphosphate ($TBAPF_6$; Sigma–Aldrích) as a supporting electrolyte were used. Ferrocene was used as an internal standard.

Analytical HPLC was carried out on a Varian Prostar Instrument by using a RP Varian Dynamax analytical column (C18 microsorb 60 Å, diameter 4.5 mm, 250 mm length) by using either method A or method B. Method A: Linear gradient; 5–95% MeCN in 20 min, eluents: H_2O and MeCN both containing 0.1% TFA). Method B: Linear

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gradient; 5–95% MeOH in 25 min, eluents: H_2O containing 0.1% TFA and MeOH).

Purifications were done on a preparative column (C18 microsorb 60 Å, diameter 21.4 mm, 250 mm length).

Solid-phase peptide synthesis: All the bioconjugates were synthesised by using standard SPPS methods, by using the Fmoc strategy. Wang resin that was preloaded with Fmoc-Val-OH (0.63 mmol g^{-1}) was used for NLS bioconjugates and for the truncated peptide. In the case of NLS_{scr} sequence, Wang resin that was preloaded with Fmoc-Arg(Pbf)-OH (0.58 mmol g^{-1}) was used. The resin was swelled in DMF for 1 h. Fmoc groups were cleaved by using 20% piperidine in DMF. Amino acids (3 equiv) along with *O*-(Benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU; 3 equiv) and 1-hydroxy-benzotriazole (HOBt) as coupling reagents were used to synthesise the peptide part of the bioconjugates. *N*,*N*-Diisopropylethylamine (Sigma–Aldrích) was used as a base, and the activation time was one minute. After the NLS (PKKKRKV) and NLS_{scr} (KKVKPKR) sequence, another lysine (Fmoc-Lys(Mtt)-OH) was used as a linker and carrier of the fluorescent label for compounds **6**, **7**, **9**, **11** and **13**. The side-chain of the linker lysine was used for labelling with FITC. The terminal amino group was used to carry the metal moiety, which was coupled by using the standard method on the solid-phase support (by using 3 equiv of cobaltocenium carboxylic acid or ferrocene carboxylic acid, 3 equiv of TBTU and 3 equiv of HOBt). Upon successful coupling of the metal complex (**3** or **4**) to the peptide, the resin changed colour from off-white to orange or yellow, respectively.

For metal-containing compounds, the metallocene was coupled to the peptide chain on the resin prior to labelling with FITC. The resin was washed with DMF to remove impurities and after washing with CH_2Cl_2, it was shrunk by using MeOH for efficient removal of the Mtt group. The side-chain Mtt group was removed with 1% TFA in CH_2Cl_2 (by using 5% TIS in the cleavage mixture). The resin was treated with the cleavage mixture (2 mL) for 2 min, and this step was repeated (5×). The resin was washed with CH_2Cl_2 and again swelled for 30 min in DMF. The resin was then shaken with a solution of FITC in DMF and iPr_2EtN at room temperature for 4 h.

At the end of the synthesis, the resin was washed repeatedly with DMF, CH_2Cl_2 and finally shrunken with MeOH. The resin was thoroughly dried over KOH under vacuum overnight prior to cleavage of the bioconjugate. Cleavage from the resin was achieved with 95% TFA, 2.5% TIS and 2.5% H_2O for 3 h in case of nonmetal and cobaltocenium-containing bioconjugates. Due to the sensitivity of ferrocene to harsh acidic conditions the cleavage mixture for ferrocene-containing compounds consisted of 10% phenol and 5% H_2O in TFA. The cleaved bioconjugates were filtered and precipitated from the filtrate upon addition of cold Et_2O. The precipitated product was dried and lyophilised. The crude yield was quantitative in all cases. Purification was achieved by preparative HPLC. Compounds **14**–**17** were reported earlier.[33]

Uptake studies, materials: An immortalised human liver cancer cell line (Hep G2) was used in this study, which was purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany). All materials for cell culture were purchased from Invitrogen Corporation (Karlsruhe, Germany) unless otherwise specified. The culture medium was supplemented with 10% fetal bovine serum (FBS, not heat inactivated, which was purchased from PAA Lab., Cölbe, Germany) penicillin (100 U mL^{-1}), streptomycin (100 μg mL^{-1}), and glutamine (2 mM) and will be designated as medium A from henceforth. The RPMI 1640 medium without FBS was supplemented with penicillin (100 U mL^{-1}), streptomycin (100 μg mL^{-1}), and glutamine (2 mM) and will be referred as medium B in later descriptions. Dubecco's phosphate buffered saline for washing and trypsin–EDTA solution for trypsinisation were obtained from Sigma–Aldrich. Trypan blue for cell counting and viability checking was purchased from Serva Feinbiochemika, (Heidelberg, Germany). Gelatine for coating of plates and coverslips was from Riedel–de-Haën AG (Seelze, Germany).

Cell culture and microscopy for uptake: Hep G2 cells were maintained in medium A at 37 °C in a humidified incubator in an atmosphere that contained 5% CO_2. For experiments and microscopy, cells were seeded at 7.5×10^4 cells/well on cover slips that were coated with 0.2% gelatine for 6 h. The cells were incubated overnight in medium A. Before incubation with the test compounds, the cells were starved for 30 min in medium B. Hep G2 cells were then incubated with compounds **6**, **7**, **9**, **11** and **13** in concentrations of 0.5 mM and 0.1 mM for 24 h at 37 °C in a humidified incubator (5% CO_2). After treatment, cells were washed with medium A (2×) and with 10 mM phosphate-buffered saline (PBS, 2×). For nuclear staining, Hoechst 33342 dye (25 μg mL^{-1} from Molecular Probes, Göttingen, Germany) was used for 1 min on the cells, and the cells were again washed with PBS (3×). In the case of the endosome marker, the cells were incubated with FM 4–64 (10 mM, Molecular Probes, Göttingen, Germany) in medium B for 15 min. Afterwards, the cells were again washed with PBS (3×). The endosomal staining was carried out prior to the nuclear staining with Hoechst dye. Fluorescence was observed by a fluorescent microscope (Axioplan-2, Zeiss, Jena, Germany) that was equipped with FITC, DAPI and red filters and photographed.

Cytotoxicity, materials: The yeast assay is described in detail in the Supporting Information. The same Hep G2 cell line was used for this study as for the uptake studies. All the culture mediums and the buffered solutions were the same as were used in the study above. Phenol red-free RPMI 1640 (medium C, Gibco, Invitrogen Corporation) was used without FBS supplement. WST-1 reagent (4-[3-(4-iodopheny)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate) was from Roche Diagnostics. Paraformaldehyde was purchased from Riedel-de-Haën (Seezle, Germany). Crystal Violet was purchased from Merck. Sodium-*n*-dodecyl sulfate was purchased from Boehringer–Mannheim. For the absorbance measurement, a Bio-Rad Microplate reader 550 (Tokyo, Japan) was used.

WST-1 assay: 96-Well plates were coated with gelatine (0.2%) for 6 h at 37 °C. After one wash with PBS, 2.0×10^4 cells in medium A were seeded in each well. The plates were incubated for 12 h. Because the doubling time for Hep G2 cells was reported to be 50–60 h,[72,73] the cells were incubated for 48 h with test compounds. The test compounds were introduced in concentrations of 0.1, 0.5 and 1 mM and incubated in a humidified incubator. The 0 h plate was used as a control to determine the background absorbance in the case of both the WST-1 assay and the crystal violet assay. After treatment, the cells were washed twice with medium A then once with medium C. Medium C (100 μL) along with WST-1 reagent (10 μL) was added to each well, and the plates were incubated at 37 °C. The reduction of WST-1 to formazan dye by metabolically active cells at 0, 60 and 90 min was quantified by scanning the plates at 415 nm against a reference of wavelength 655 nm in a microtitre plate reader.[50]

Crystal violet cell quantification assay: After the WST-1 assay, the cells were fixed with 4% paraformaldehyde for 5 min at room temperature and later washed with PBS (3×) and with 0.1% Triton ×

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100 buffer (Serva Feinbiochemika, Heidelberg, Germany) in PBS (3×). Crystal violet [100 μL, 0.04% crystal violet in 4% (*v*/*v*) EtOH] was then added to each well, and the plates were shaken for 40 min at room temperature. After treatment with crystal violet, the cells had to be washed with distilled water (7×), with two minutes shaking. Finally, SDS (sodium-*n*-dodecyl sulfate, 1%, 100 μL) was added to each well, and the plates were incubated at room temperature with vigorous shaking for 1 h. At the end, the absorbance was measured at 570 nm against a reference wavelength of 655 nm.

Analytical data

FcC(O)-Lys-NLS (5): Orange-yellow solid, soluble in H_2O and EtOH. HPLC: t_R=9.7 min (method A); ^{1}H NMR (400 MHz, D_2O): δ=4.50 (s, 2H; substituted Cp), 4.45–4.35 (m, 1H; H_α Val), 4.15 (brs, 9H; unsubstituted Cp, H_α), 3.99–3.94 (m, 2H; H_α), 3.92–3.88 (m, 1H; H_α Lys terminal), 3.79–3.50 (m, 2H; H_δ Pro), 3.15 (pseudo t, 2H; H_δ Arg), 2.99–2.88 (brs, 10H; H_ε Lys), 2.30–2.24 (m, 1H; H_β Val), 2.10–1.95 (m, 3H; $H_{\beta,\gamma}$ Pro), 1.90–1.60 (m, 25H; H_γ Pro, $H_{\beta\ \delta}$ Lys, $H_{\beta,\gamma}$ Arg), 1.50–1.30 (m, 10H; H_γ Lys), 0.85 (two overlapping d, J=6 Hz, 6H; 2×CH_3 Val); MALDI-TOF: *m*/*z* calcd for $C_{57}H_{98}FeN_{16}O_{10}$: 1222.7; found: 1223.5 [*M*+H]$^+$ 1246.6 [*M*+Na]$^+$.); SWV: $E_{1/2}$=239 vs. FcH/FcH$^+$;

FcC(O)-Lys(FITC)-NLS (6): Bright orange solid, soluble in H_2O and EtOH. HPLC: t_R=12.2 min (method A); ^{1}H NMR (400 MHz, CD_3OD): δ=8.29 (s, 1H; FITC), 7.75 (d, J=8.6 Hz, 1H; FITC), 7.39–7.31 (m, 1H; FITC), 7.22–7.17 (m, 1H; FITC), 6.76–6.68 (m, 3H; FITC), 6.58 (dd, J=6.2 Hz, 2H; FITC), 4.77–4.69 (pseudo t, 1H; H_α, Val), 4.49–4.43 (m, 3H; H_α), 4.42 (s, 2H; substituted Cp), 4.38–4.31 (m, 4H; H_α), 4.29 (s, 2H; substituted Cp), 4.24 (s, 5H; unsubstituted Cp), 4.04–3.98 (m, 1H; H_δ Pro), 3.85–3.76 (m, 1H; H_δ Pro), 3.26–3.16 (m, 2H; H_δ Arg), 3.05–2.89 (m, 10H; H_ε Lys), 2.34–2.26 (m, 1H; H_β Val), 2.23–2.18 (m, 1H; H_γ Pro), 2.06–1.98 (m, 2H; $H_{\beta,\gamma}$ Pro), 1.96–1.65 (m, 25H; H_β Pro, $H_{\beta,\delta}$ Lys, $H_{\beta,\gamma}$ Arg), 1.60–1.45 (m, 10H; H_γ Lys), 1.00 (d, J=6.2 Hz, 6H; 2×CH_3 Val); MALDI-TOF: *m*/*z* calcd for $C_{78}H_{109}FeN_{17}O_{15}S$: 1611.74; found: 1614.2 [*M*+2H]$^+$; SWV: $E_{1/2}$=166 mV vs. FcH/FcH$^+$.

FcC(O)-Lys(FITC)-Val-OH (7): Dark-orange solid, soluble in DMSO and MeOH, sparingly soluble in H_2O; HPLC: t_R=21.8 min (method B); ^{1}H NMR (400 MHz, CD_3OD): δ=8.18 (s, 1H; FITC), 7.79 (d, J=8.2 Hz, 1H; FITC), 7.17 (d, J=8.2 Hz, 1H; FITC), 6.83–6.75 (m, 4H; FITC), 6.64 (d, J=8.2 Hz, 2H; FITC), 4.67 ((pseudo t, 1H; H_α Val), 4.45–4.39 (m, 3H; substituted Cp, H_α Lys), 4.23 (s, 5H; unsubstituted Cp), 3.70 (brs, 2H; H_ε Lys), 2.29–2.19 (m, 1H; H_β Val), 2.01–1.89 (m, 2H; H_β Lys), 1.81–1.75 (m, 2H; H_δ Lys), 1.66–1.51 (m, 2H; H_γ Lys), 1.03 (d, J=6.6 Hz, 6H; 2×CH_3 Val); MALDI-TOF: *m*/*z* calcd for $C_{43}H_{42}FeN_4O_9S$: 846.2; found: 847.5 [*M*+H]$^+$ 869.5 [*M*+Na]$^+$; SWV: $E_{1/2}$=258 mV vs. FcH/FcH$^+$.

H-Lys-NLS$_{scr}$(8): White solid, soluble in H_2O and EtOH. HPLC: t_R=6.7 min (method A); ^{1}H NMR (300 MHz, D_2O): δ=4.63–4.56 (m, 1H; H_α Val), 4.47–4.41 (m, 1H; H_α Lys), 4.37–4.29 (m, 2H; H_α, Lys), 4.28–4.22 (m, 2H; H_α), 4.14 (d, J=7.3 Hz, 1H; H_α), 4.06–3.92 (pseudo t, 1H; H_α), 3.92–3.83 (m, 1H; H_δ Pro), 3.71–3.62 (m, 1H; H_δ Pro), 3.21 (t, J=7 Hz, 2H; H_δ Arg), 3.05–2.93 (m, 10H; H_ε Lys), 2.36–2.24 (m, 1H; H_β Val), 2.08–1.98 (m, 2H; $H_{\beta,\gamma}$ Pro), 1.93–1.58 (m, 26H; $H_{\gamma,\beta}$ Pro, $H_{\beta,\delta}$ Lys, $H_{\beta,\gamma}$ Arg), 1.53–1.33 (m, 10H; H_γ Lys), 0.92 (dd, J=6.2 Hz, 6H; 2×CH_3 Val); MALDI-TOF: *m*/*z* calcd for $C_{46}H_{90}N_{16}O_9$: 1010.71; found: 1011.8 [*M*+H]$^+$.

H-Lys(FITC)-NLS$_{scr}$ (9): Bright-yellow solid, soluble in H_2O and EtOH; HPLC: t_R=10.1 min (method A); ^{1}H NMR (400 MHz, D_2O): δ=8.06 (s. 1H; FITC), 7.69 (d, J=8.9 Hz, 1H; FITC), 7.42 (pseudo t, 1H; FITC), 7.37–7.27 (m, 1H; FITC), 7.20 (pseudo t, 1H; FITC), 7.01 (s, 2H; FITC), 6.86 (d, J=8.9 Hz, 2H; FITC), 4.40–4.25 (m, 7H; $H_{\alpha)}$, 4.15–4.05 (m, 1H; H_α), 3.90–3.82 (m, 1H; H_δ Pro), 3.70–3.65 (m, 1H; H_δ Pro), 3.58–3.51 (m, 2H; $H_{\delta,}$ Arg), 3.05–2.90 (m, 10H; $H_{\varepsilon,}$ Lys), 2.35–2.21 (m, 1H; $H_{\beta,\ Val}$), 1.95–1.86 (m, 3H; $H_{\beta,\ \gamma,\ Pro}$), 1.80–1.55 (m, 25H; H_β Pro, $H_{\beta,\delta}$ Lys, $H_{\beta,\gamma}$ Arg), 1.49–1.31 (m, 10H; H_γ Lys), 0.91 (d, J=6.6 Hz, 6H; 2×CH_3 Val); MALDI-TOF: *m*/*z* calcd for $C_{67}H_{101}N_{17}O_{14}S^+$: 1399.74; found: 1402.2 [*M*+2H]$^+$, 1424.1 [*M*+Na]$^+$;

FcC(O)-Lys-NLS$_{scr}$ (10): Yellow solid, soluble in H_2O and EtOH. HPLC: t_R=9.1 min (method A); ^{1}H NMR (300 MHz, D_2O): δ=4.53–4.60 (m, 2H; H_α), 4.34–4.38 (m, 2H; H_α), 4.17–4.30 (m, 7H; H_α unsubstituted Cp), 4.05 (d, J=7.7 Hz, 2H; H_α), 3.87–3.76 (m, 1H; H_δ Pro), 3.64–3.55 (m, 1H; H_δ Pro), 3.15 (t, J=6.7 Hz, 2H; H_δ Arg), 3.00–2.80 (m, 10H; H_ε Lys), 2.30–2.20 (m, 1H; H_β Val), 2.08–1.92 (m, 4H; $H_{\beta,\gamma}$ Pro), 1.90–1.52 (m, 24H; $H_{\beta,\delta}$ Lys, $H_{\beta,\gamma}$ Arg), 1.50–1.30 (m, 10H; H_γ Lys), 0.91 (d, J=6.2 Hz, 6H; 2×CH_3 Val); MALDI-TOF: *m*/*z* calcd for $C_{57}H_{98}FeN_{16}O_{10}$: 1222.7; found: 1224.1 [*M*+2H]$^+$, 1246.6 [*M*+Na]$^+$; SWV: $E_{1/2}$=279 mV vs. FcH/FcH$^+$.

FcC(O)-Lys(FITC)-NLS$_{scr}$ (11): Bright-orange solid, soluble in H_2O and EtOH. HPLC: t_R=12.0 min (method A); ^{1}H NMR (400 MHz, CD_3OD): δ=8.29 (s, 1H; FITC), 7.70 (d, J=8.2 Hz, 1H; FITC), 7.39–7.30 (m, 1H; FITC), 7.17 (d, J=8.2 Hz, 1H; FITC), 6.75–6.66 (m, 3H; FITC), 6.57 (dd, J=6.4 Hz, 2H; FITC), 4.62 (pseudo t, 1H; H_α val), 4.50–4.34 (m, 10H; substituted Cp, H_α), 4.24 (s, 5H; unsubstituted Cp), 4.20 (d, J=7.4 Hz, 1H; H_α), 3.95–3.85 (m, 1H; H_δ Pro), 3.71–3.65 (m, 1H; H_δ Pro), 3.27–3.19 (m, 2H; H_δ Arg), 2.98 (brs, 10H; H_ε Lys), 2.32–2.21 (m, 1H; H_β Val), 2.11–1.94 (m, 3H; $H_{\beta,\gamma}$ Pro), 1.85–1.65 (m, 25H; H_β Pro, $H_{\beta,\delta}$ Lys, $H_{\beta,\gamma}$ Arg), 1.62–1.45 (m, 10H; H_γ Lys), 0.98 (dd, J=6.2 Hz, 6H; 2×CH_3 Val); MALDI-TOF: *m*/*z* calcd for $C_{78}H_{109}FeN_{17}O_{15}S$: 1611.74; found: 1614.0 [*M*+2H]$^+$; SWV: $E_{1/2}$=279 mV vs. FcH/FcH$^+$.

CcC(O)-Lys-NLS$_{scr}$ (12): Pale-yellow solid, soluble in H_2O and EtOH. HPLC: t_R=7.4 min (method A); ^{1}H NMR (400 MHz, D_2O): δ=6.20 (s, 1H; substituted Cp), 6.18 (s, 1H; substituted Cp), 5.80 (s, 2H; substituted Cp), 5.70 (s, 5H; unsubstituted Cp), 4.51–4.44 (pseudo t, 1H; H_α, Val), 4.35–4.28 (m, 2H; H_α), 4.26–4.19 (m, 3H; H_α), 4.18–4.13 (m, 1H; H_α), 3.99 (d, J=7.8 Hz, 1H; H_α), 3.80–3.78 (m, 1H; H_δ Pro), 3.61–3.58 (m, 1H; H_δ Pro), 3.09 (pseudo t, 2H; H_δ Arg), 2.93–2.83 (m, 10H; H_ε Lys), 2.23–2.18 (m, 1H; H_β Val), 1.98–1.90 (m, 3H; $H_{\beta,\gamma}$ Pro), 1.70–1.45 (m, 25H; H_β Pro $H_{\beta,\gamma}$, Lys, $H_{\beta,\gamma}$ Arg), 1.38–1.18 (m, 10H; H_γ Lys), 0.93 (d, J=7 Hz, 6H; 2×CH_3 Val). MALDI-TOF: *m*/*z* calcd for $C_{57}H_{98}Co^+N_{16}O_{10}$: 1225.7; found: 1226.9 [*M*+H]$^+$ 1248.9 [*M*+Na]$^+$ 1282.9 [*M*+K]$^+$; SWV: $E_{1/2}$=−1.38 V vs. FcH/FcH$^+$.

CcC(O)-Lys(FITC)-NLS$_{scr}$ (13): Bright-yellow solid, soluble in H_2O and EtOH. HPLC: t_R=10.7 min (method A); ^{1}H NMR (300 MHz, D_2O): δ=7.93 (s, 1H; FITC), 7.60–7.53 (d, J=8.1, 1H; FITC), 7.32 (d, J=8 Hz, 1H; FITC), 7.22–7.16 (m, 2H; FITC), 6.95 (s, 2H; FITC), 6.80 (dd, J=8.4 Hz, 2H; FITC), 6.20–6.15 (m, 2H; substituted Cp), 5.79 (pseudo t, 2H; substituted Cp), 5.72 (s, 5H; unsubstituted Cp), 4.50–4.00 (m, 7H; H_α), 3.80–3.37 (m, 2H; H_δ, Pro), 3.15–3.04 (pseudo t, 2H; H_δ, Arg), 3.00–2.89 (m, 10H; H_ε Lys), 2.00–1.85 (m, 9H; $H_{\beta,\gamma}$, Pro, H_β Val, $H_{\beta,\gamma}$ Arg), 1.84–1.56 (m, 20H; $H_{\beta,\delta,}$ Lys), 1.50–1.38 (m, 10H; H_γ Lys), 0.85 (d, J=6.9 Hz, 6H; 2×CH_3 Val); MALDI-TOF: calcd *m*/*z* for $C_{78}H_{109}Co^+N_{17}O_{15}S$: 1614.73; found: 1615.9 [*M*+H]$^+$ 1637.8 [*M*+Na]$^+$; SWV: $E_{1/2}$=−1.35 mV vs. FcH/FcH$^+$.

Abbreviations:Boc: *tert*-butyloxycarbonyl, Cc: cobaltocene, CcC(O): cobaltocenium carboxylic acid fragment in peptides, CV: crystal violet, DMF: dimethylformamide, Fc: ferrocene, FcC(O): ferrocene carboxylic acid fragment in peptides, FITC: fluorescein isothiocyanate, Fmoc: 9-fluorenylmethyloxycarbonyl, HOBt: *N*-hydroxybenzotriazole·H_2O, Mtt: 4-methyltrityl, NLS: nuclear localisation signal,

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NLS_{src}: scrambled nuclear localisation signal, Pbf: 2,2,4,6,7-pentamethyldihydrobenzofurane-5-sulfonyl, SPPS: solid-phase peptide synthesis, SWV: square wave voltammetry, TBTU: 2-(1*H*-benzotriazole-1-yl)-1,3,3-tetramethyluronium tetrafluoroborate, TFA: trifluoroacetic acid, TIS: triisopropylsilane, WST-1 reagent: (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate).

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