Cellular Uptake

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Cellular Uptake Quantification of Metalated Peptide and Peptide Nucleic Acid Bioconjugates by Atomic Absorption Spectroscopy**

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Advances in molecular biology have allowed the design of targeted biopolymers such as proteins and antibodies to become a reality in drug discovery. Research into cell uptake and tissue selectivity of such macromolecules is one of the most important areas in drug development, for instance in the design of tumor-specific antiproliferative agents. Interestingly, the principles observed for cell uptake also apply to the intracellular distribution of exogenous compounds.[1] For example, a compound that is designed to interfere with DNA or DNA-processing enzymes should ideally be located exclusively in the nucleus of a cell. When properly localized, undesired side effects that might result from interaction with other targets in the cell are minimized. Sophisticated techniques are therefore warranted to allow insight into the intracellular localization of drugs. The uptake and transport of metal ions in nature is tightly controlled and regulated by so-called metallochaperones.[2] The trafficking of copper ions, which is associated with a number of malfunctions such as Menke's and Wilson's disease, is particularly well-investigated.[3] With the exception of antitumor-active platinum complexes, the intracellular localization of exogenous metal complexes has hardly been explored. Indeed, the design of artificial metallochaperones for the directed intracellular delivery of metal complexes has only recently been addressed.[4,5] Since DNA, which codes for the genetic information of the cell, is located in the cellular nucleus, it would be a particularly sensitive target for toxic metal ions.

The nucleus of eukaryotic cells is protected by the nuclear membrane. However, the pores in this membrane are large enough to allow the passive diffusion of small molecules. Alberto and co-workers were able to demonstrate the accumulation of ^{99m}Tc ions, which may cause DNA cleavage by short-range Auger electrons, near the cellular DNA in a conjugate with the intercalator pyrene.[6] A more rational approach is to use the cellular machinery for the accumulation of metal ions in the nucleus. Nuclear localization signals (NLS) are small peptides which, upon binding to cargo proteins, translocate into the cellular nucleus.[7] The simian virus SV40 antigen NLS has been extensively studied. It has the primary sequence Pro-Lys-Lys-Lys-Arg-Lys-Val and has been used in nuclear translocation studies of a variety of cargos, e. g. proteins, DNA, RNA, peptide nucleic acid (PNA), and even gold nanoparticles.[8]

We recently reported a study on the nuclear localization of cobaltocenium NLS bioconjugates using fluorescence microscopy.[9] It might be argued that the fluorescent dye, which is necessary for detection, might influence the cellular uptake and nuclear localization of the conjugate. A more elegant approach would be direct detection of the metal complex. To this end, atomic absorption spectroscopy (AAS),[10,11] inductively coupled plasma mass spectrometry (ICP-MS),[12,13] X-ray fluorescence,[14,15] and visible-light fluorescence microscopy have been suggested for nonradioactive metal ions and their complexes.[16,17] The latter method is not generally applicable as it requires that the metal complex itself is fluorescent. X-ray fluorescence, on the other hand, requires a powerful synchroton source and sophisticated equipment to achieve the required spatial resolution.[15] Moreover, the cells need to be fixed, which may cause intracellular redistribution of compounds, as has recently been demonstrated for small, cationic cell-penetrating peptides.[18] This leaves AAS and ICP-MS as the most feasible and general methods for direct studies of metal-ion distribution inside living cells.

Herein, we describe the use of peptides and PNAs as biological vectors to enhance cellular uptake and nuclear localization of cobalt complexes. AAS is the method of choice to directly quantify uptake and cellular localization of these conjugates. Cobalt was used in this study because it is not particularly toxic,[19] its natural background is low, and an excellent comparison is available in the form of cyanocobalamine (vitamin B_{12}). The bis(picolyl)amine (bpa) ligand **1** was

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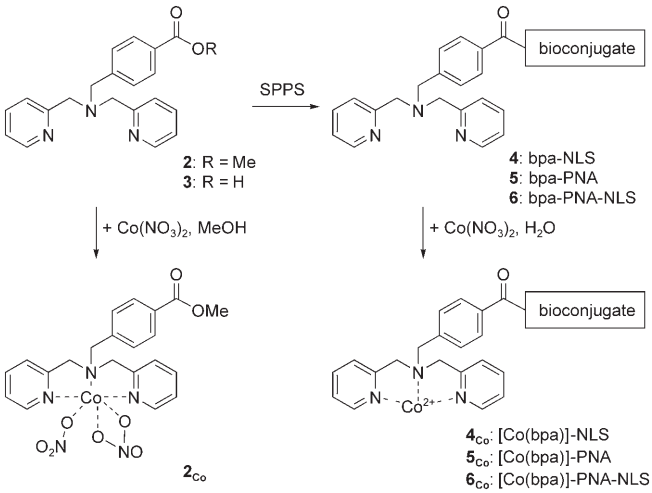
chosen because it forms stable complexes with a variety of metal ions, the properties of which have been extensively studied.[20,21] Moreover, carboxylato-functionalized bpa ligands **2** (ester) and **3** (free acid) are readily available (Scheme 1).[5,22]



Scheme 1. Synthesis of the compounds used in this study, see Table 1 for details of the bioconjugates **4**–**6**. SPPS = solid-phase peptide synthesis, Ahx = ω-aminohexanoic acid, NLS = Pro-Lys-Lys-Lys-Arg-Lys-Val-NH_2, PNA = tgttatcc. All metal complexes are denoted with the subscript "Co".

The synthesis of the conjugates studied is shown in Scheme 1. The oligomers bpa-NLS **4**, bpa-PNA **5**, and bpa-PNA-NLS **6** were prepared by standard 9-fluorenylmethoxycarbonyl (Fmoc) solid-phase peptide synthesis (SPPS) using the bpa acid **3**. All new conjugates were purified by reversed-phase HPLC and characterized by MALDI-TOF mass spectrometry.[5,9,23] The sequences of the oligomers are given in Table 1.

Table 1: Composition of the compounds used in this study.[a]

No.	Abbreviation	Sequence/Compound
1	bpa	Bis(picolyl)amine
2		bpa-CH_2-(*p*-C_6H_4)-C(O)-OMe
3		bpa-CH_2-(*p*-C_6H_4)-C(O)-OH
4	bpa-NLS	bpa-CH_2-(*p*-C_6H_4)-C(O)-Ahx-Pro-Lys-Lys-Lys-Arg-Lys-Val-NH_2
5	bpa-PNA	bpa-CH_2-(*p*-C_6H_4)-C(O)-Ahx-tgttatcc-Lys-NH_2
6	bpa-PNA-NLS	bpa-CH_2-(*p*-C_6H_4)-C(O)-Ahx-tgttatcc-Gly-Gly-Pro-Lys-Lys-Lys-Arg-Lys-Val-NH_2
7	DNA_{18}	5′-TAG**GGATAACA**GGGTAAT-3′

[a] Small letters (a, c, t, g) indicate PNA and capital letters DNA (A, C, G, T) monomer units; the three-letter code is used for amino acids. All peptide sequences are written from N to C terminus (left to right). The complemantary (to PNA **5** and **6**) part of DNA **7** is shown in bold.

The PNA-containing oligomers **5** and **6** were also characterized by UV melting experiments with a complementary DNA oligomer **7**.[24] The results show a hybridization in both **5**·**7** ($T_m = 29.7\,°C$) and **6**·**7** ($T_m = 36.1\,°C$) duplexes (see the Supporting Information, Figure S1). The difference in melting temperature $\Delta T_m = 6.3\,°C$ can be ascribed to an attractive electrostatic interaction between the positively charged NLS peptide and the negatively charged overhanging DNA at the 5′-end in duplex **6**·**7**.

The bpa ligand readily forms complexes with divalent metal ions. The [Co(bpa)] complexes studied herein were obtained in situ by mixing equimolar amounts of bpa ligand conjugates and $Co(NO_3)_2$. To gain insight into the coordination chemistry and structural parameters of these complexes, the Co^{II} complex $\mathbf{2_{Co}}$ of the bpa ester (see also Scheme 1) was isolated and characterized by X-ray crystallography. An ball-and-stick representation of $\mathbf{2_{Co}}$ is shown in Figure 1. In the solid state, $\mathbf{2_{Co}}$ is a discrete mononuclear complex with a hexacoordinated Co center. The N_3O_3 coordination is achieved by three nitrogen atoms of the bpa ligand and three oxygen atoms of two nitrate counterions. The bpa ligand coordinates in a facial fashion, and the angle between the two planes of the pyridine rings is 72.4°.

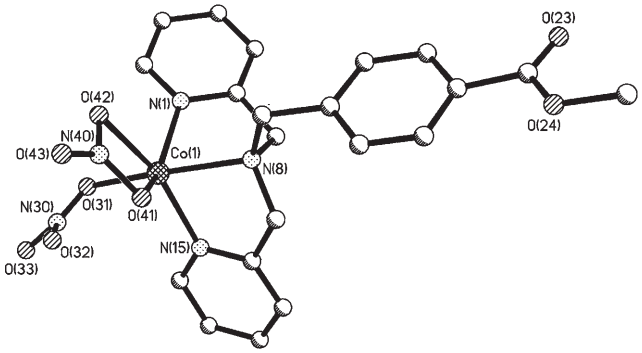


Figure 1. Crystal structure of $\mathbf{2_{Co}}$. Hydrogen atoms are omitted for clarity. Selected bond lengths [Å] and angles [°]: Co(1)–N(1) 2.074(6), Co(1)–N(8) 2.197(5), Co(1)–N(15) 2.109(5), Co(1)-O(31) 2.140(6), Co(1)–O(41) 2.120(5), Co(1)–O(42) 2.208(5), N(1)-Co(1)-N(15) 111.7(2), N(8)-Co(1)-N(1) 77.6(2), N(8)-Co(1)-N(15) 76.54(19), N(8)-Co(1)-O(41) 85.87(18), N(8)-Co(1)-O(31) 157.7(2), N(8)-Co(1)-O(42) 108.20(18), O(31)-Co(1)-O(42) 89.69(19), O(41)-Co(1)-O(42) 59.22(19).

Cellular uptake experiments on the cobalt complexes were performed in HT-29 colon carcinoma cells after exposure to 50 μM of the compounds over an incubation period of 6 or 24 h (Figure 2a). The uptake of metal compounds into cells and nuclei was then quantified by determination of the cobalt content of isolated cells or nuclear pellets by AAS. This value was normalized to the total protein content of the cells (pmol cobalt per μg cellular protein, Figure 2a). In the cell uptake studies, cyanocobalamin (**8**, vitamin B_{12}) was used as a reference. In separate experiments, the nuclei were separated by density-gradient centrifugation, and the cobalt content of the nuclei was determined by AAS after 24 h. In this case, the amount of Co was normalized to the nuclear protein content (pmol cobalt per μg nuclear protein, Figure 2b). The ratio of nuclear to cellular protein content may vary from preparation to preparation. While the cobalt-to-protein ratio is reliable and reproducible for each determination, there is no universal

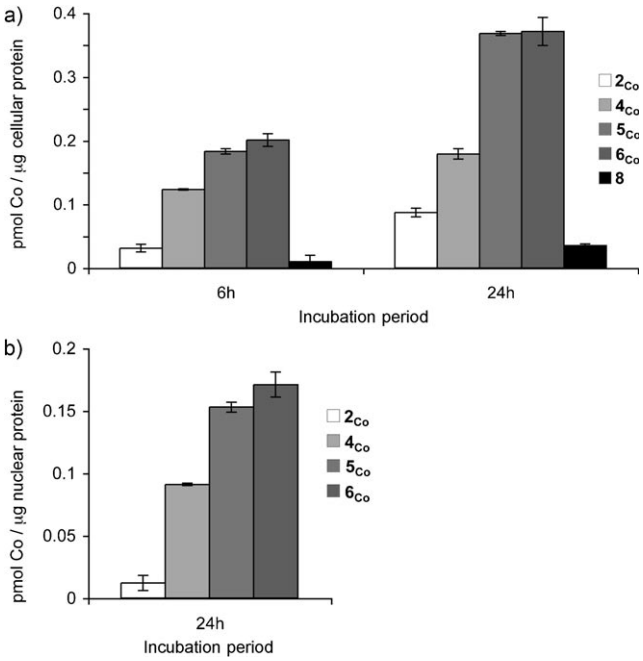


Figure 2. a) Cellular uptake into HT-29 cells after 6 and 24 h exposure to 50 µM solutions of $\mathbf{2}_{Co}$, $\mathbf{4}_{Co}$, $\mathbf{5}_{Co}$, $\mathbf{6}_{Co}$, and $\mathbf{8}_{Co}$ ($n=2$); b) uptake into the nuclei of HT-29 cells after 24 h exposure to 50 µM solutions of $\mathbf{2}_{Co}$, $\mathbf{4}_{Co}$, $\mathbf{5}_{Co}$, and $\mathbf{6}_{Co}$ ($n=2$). Please note the different *y* axis, which refers to cellular protein mass in (a) but to nuclear protein content in (b). See the text and Experimental Section for details.

conversion factor between the cobalt content of Figure 2a and Figure 2b. As a rough estimate, the nuclear protein content is on the order of 10–20% of the total cellular protein mass.

The detection limits of cobalt in our AAS experiments were 0.014 µM (0.82 µg L^{-1}) for a typical cell suspension as used for cellular uptake studies and 0.023 µM (1.35 µg L^{-1}) for suspensions of nuclei. The results in Figure 2 are presented as mean values calculated from the data of two independent experiments (different day, different passage of cells, freshly prepared solution of compounds). The higher detection limit for Co in the nuclear fraction is due to the higher systematic background in all measurements. This background results from the fairly high phosphate content of the DNA, which is, naturally, only present in nuclear preparations.

For the purpose of this study, vitamin B_{12} (**8**) may be regarded as a stable, nontoxic Co complex and is thus a more appropriate comparison for cellular uptake studies than, for example, simple metal salts would be. Indeed, $Co(NO_3)_2$ was internalized by HT-29 cells in much higher amounts than all compounds used herein, presumably by binding to transferrin or by uptake through ion channels (data not shown). Neither mechanism is available to the metal complexes, and therefore, simple metal salts do not serve as relevant comparisons. Figure 2 shows that the amount of Co inside the cells is almost doubled between 6 and 24 h, independent of the nature of the Co compound. Interestingly, all bpa-containing compounds were found in higher amounts in the cells than vitamin B_{12}. Cellular uptake of the [Co(bpa)] complexes increases in the series $\mathbf{2}_{Co} < \mathbf{4}_{Co} < \mathbf{5}_{Co} \approx \mathbf{6}_{Co}$. Absolute differences are even more pronounced for nuclear uptake. Thus, $\mathbf{5}_{Co}$ was found in approximately 14-fold higher concentration in the nuclei than $\mathbf{2}_{Co}$, but only about four times more in the whole-cell analysis.

On the basis of distinct cellular parameters such as the mean cellular protein content and mean cellular volume, the molar concentrations can be estimated from the metal concentration (pmol metal per µg protein).[25] For the compounds with the highest uptake rates ($\mathbf{5}_{Co}$ and $\mathbf{6}_{Co}$ after 24 h), intracellular concentrations greater than 70 µM were calculated, whereas only 6 µM are reached for the complex with the lowest cellular accumulation ($\mathbf{2}_{Co}$ after 6 h). All other compounds also reached cellular levels well below 50 µM, which is the extracellular concentration during the incubation period. Intracellular concentrations greater than 50 µM may indicate an active cellular uptake process for conjugates $\mathbf{5}_{Co}$ and $\mathbf{6}_{Co}$. This result is of special interest, as the intracellular levels of even very active drugs, such as cisplatin, hardly exceed the extracellular concentration.[13,26] As we reported previously, the cellular uptake of metal-based drugs can be significantly influenced by the properties of the ligand and the charge of the metal atom.[27] In this context, the somewhat low overall uptake of the target compounds presented herein may be the consequence of the hydrophilic nature of the peptide-containing ligands and the positive charge of the cobalt complexes after replacement of the nitrato ligands by water upon dissolution. Interestingly, the uptake was significantly improved by the presence of the PNA sequence in the ligands. This aspect is surprising in light of earlier reports on rather poor uptake of PNA oligomers into mammalian cells and will be further discussed below.

It is conceivable that increased uptake results from cytotoxic effects of the conjugate. Therefore, cytotoxicity experiments were performed on $\mathbf{2}_{Co}$ and $\mathbf{4}_{Co}$. No toxic effects were noted up to the highest concentration of 100 µM (data not shown). This finding indicates that metal uptake into the cells and nuclei was not facilitated by apoptotic or necrotic processes, which might have caused higher permeability of the nuclear membranes.

Several conclusions can be drawn from the above data. First of all, AAS as a detection method is selective for the metal and has an appropriate sensitivity. Typical experimental values are on the order of 0.2–0.6 µM Co, and thus at least ten times higher than the detection limit of the method. Significantly different metal concentrations are observed across the bioconjugates. Therefore, we conclude that the peptides remain coordinated to the Co ions throughout the study. Significantly improved uptake is observed for the NLS conjugates over the simple [Co(bpa)] complex. This result may seem somewhat surprising, as the NLS peptide alone is not a good vector for cellular uptake in bioconjugates.[28] On the other hand, we have shown in previous work that attachment of cationic organometallic complexes to the N terminus of NLS drastically improves the uptake efficiency of the conjugates.[9] Moreover, we show that the nuclear localization properties of the NLS peptide are not affected by the attached metal complex.

It may further seem surprising that PNA conjugates are internalized even better than the [Co(bpa)-NLS] conjugate.

The combination of NLS peptide and PNA oligomer, on the other hand, does not yield any improvement in uptake efficiency over PNA alone. The properties of the conjugate are thus dominated by the PNA part of the molecule, not the NLS peptide, at least with respect to cellular uptake. Limited cellular uptake has seriously hampered the use of PNA oligomers as antisense agents, and numerous solutions to this problem were proposed.[28,29] Krämer and co-workers reported enhanced cellular uptake of PNA oligomers with N-terminally attached Zn^{2+} terpyridine complexes.[17] These results were, however, obtained by flow cytometry and fluorescence microscopy of organic dyes and therefore constitute indirect, nonquantitative evidence. In a comprehensive study with numerous peptides, Nielsen and co-workers recently evaluated the uptake efficiency of PNA–peptide conjugates by a splice-correction assay.[28] In terms of antisense PNA delivery into cells, this work is certainly most relevant, because it uses a biological effect as the read-out. On the other hand, antisense efficiency in this system will depend on additional factors, such as RNA binding and the response of the cellular machinery. Thus, even this system is again only an indirect one to determine the numbers of molecules that were internalized into the cell.

There is a significant difference between the various conjugates studied herein with respect to nuclear localization. Our data show that enhanced transport of metals into nuclei is possible by choice of appropriate peptide vectors. While the ratios $\mathbf{4_{Co}}/\mathbf{6_{Co}}$ and $\mathbf{5_{Co}}/\mathbf{6_{Co}}$ are similar in the nuclei and whole cells, the situation is very different for the nonpeptide complex [Co(bpa)], for which the ratio $\mathbf{2_{Co}}/\mathbf{6_{Co}}$ is 23% in the whole cells but only 7% in the nuclei. Clearly, bioconjugation with either PNA or NLS (or both) favors nuclear localization. This result is in good agreement with recent studies reporting the nuclear accumulation of NLS conjugates with cobaltocenium, nucleotide, and platinum moieties.[9,30] Again, there is no difference between the PNA conjugates $\mathbf{5_{Co}}$ and $\mathbf{6_{Co}}$, but both show a higher nuclear localization that the NLS conjugate $\mathbf{4_{Co}}$. Finally, only a relatively small fraction (less than 10%) of the conjugates reaches the nucleus. This result, however, is in line with findings for other metal complexes like cisplatin, of which also only about 5% is found in the nucleus of treated cells.

In summary, this work investigates the use of peptides and peptide nucleic acid oligomers (PNA) as vectors for the cellular uptake and nuclear delivery of metal complexes. AAS is used as the metal-specific detection method. Compared to previous studies, which use indirect methods such as fluorescence spectroscopy or splice-correction assays, metal-specific AAS is a method which allows accurate determination of intracellular concentrations of the conjugates. It is thus most suited to establish uptake efficiencies for any metal-containing bioconjugate and is applied herein to study the cell uptake of PNA oligomers and peptides for the first time. For the best systems in this study, an accumulation 150% higher than in the cell culture medium was achieved.

We have recently investigated the use of metal–bpa complexes for DNA cleavage.[20] The results herein show that it is possible to direct metal complexes for which a biological activity such as DNA cleavage was demonstrated ex vivo into the nucleus of living cells, where they could exert their activity on the cellular DNA in vivo.

Experimental Section

Full experimental details are provided in the Supporting Information.

4: $C_{66}H_{107}N_{19}O_9$, $M_r = 1310.68\ g\,mol^{-1}$, MS (ESI-pos.): $m/z = 655.7$ $[M+2H]^{2+}$, 437.26 $[M+3H]^{3+}$; **5**: $C_{118}H_{151}N_{47}O_{30}$, $M = 2707.76\ g\,mol^{-1}$, MS (MALDI): $m/z = 2707.1$ $[M+H]^+$; **6**: $C_{156}H_{221}N_{61}O_{38}$, $M = 3558.81\ g\,mol^{-1}$, MS (MALDI) = 3558.2 $[M+H]^+$.

X-ray crystallography: Crystal data for $\mathbf{2_{Co}}$: Pink crystals of $\mathbf{2_{Co}}$, $C_{21}H_{21}CoN_5O_8$, $M_r = 530.36\ g\,mol^{-1}$, $0.12 \times 0.12 \times 0.06\ mm^3$, monoclinic, space group $P2_1/c$, $Z = 4$, $a = 9.8665(6)$, $b = 14.2680(7)$, $c = 16.7896(8)$ Å, $\beta = 106.13(1)°$, $V = 2270.5(2)$ Å^3, $Mo_{K\alpha}$ radiation ($\lambda = 0.71073$ Å), $\mu(Mo_{K\alpha}) = 6.45\ mm^{-1}$, $\rho = 1.551\ g\,cm^{-3}$, $T = 100(2)$ K, 11041 reflections measured, 3386 independent reflections ($R_{int} = 0.043$), 3212 observed reflections ($F_o > 4\sigma(F_o)$), $2\theta_{max} = 62.85°$, $R = 0.0897$ ($F_o > 4\sigma(F_o)$), $R = 0.0946$ (all data), $wR = 0.1661$ ($F_o > 4\sigma(F_o)$), 311 parameters, refinement against F^2, ShelXTL 6.14 Bruker AXS program suite. CCDC-648421 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

Uptake into HT-29 cells and nuclei: HT-29 human colon carcinoma cells were maintained in Eagle's MEM cell culture medium (Sigma, Germany) supplemented with $NaHCO_3$ ($2.2\ g\,L^{-1}$), sodium pyruvate ($110\ mg\,L^{-1}$), gentamycin ($50\ mg\,L^{-1}$), and 10 vol% fetal calf serum according to standard procedures. For uptake studies the cells were grown in 75-cm^2 culture flasks at 37°C in an atmosphere of 5% CO_2 and 95% air until at least 70% confluency. The medium was replaced with 10 mL medium containing the freshly prepared complexes in a concentration of 50 μM. Solutions of the metal complexes were prepared by mixing equimolar amounts of 50 mM solutions of compounds **4**–**6** and $Co(NO_3)_2$ in water. 10 μL of this mixture was added to 10 mL MEM to give the desired concentration with MEM. After further incubation for 6 or 24 h hours the cells were detached by trypsinization, resuspended in 10 mL phosphate-buffered saline pH 7.4 (PBS), pelleted by centrifugation (2000 rpm, 5 min), and washed twice with PBS. For cell uptake studies the isolated pellets were resuspended in 1.0 mL twice-distilled water and lysed using a sonotrode (Bandelin Sonoplus GM 70). The cobalt content and protein content of the lysates was determined according to a reported procedure.[10] The molar cellular concentrations were calculated according to the literature.[25] For quantification of the uptake into the nuclei, the isolated pellets were treated as previously described.[11]

Cytotoxicity experiments: Cell growth inhibitory effects were evaluated as previously described.[25]

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