Journal of Inorganic Biochemistry 104 (2010) 1248–1251

Contents lists available at ScienceDirect

Journal of Inorganic Biochemistry

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

A caged nicotine with nanosecond range kinetics and visible light sensitivity

Oscar Filevich, Marcelo Salierno, Roberto Etchenique *

Departamento de Química Inorgánica, Analítica y Química Física, INQUIMAE, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria Pabellón 2 Piso 3, C1428EHA, Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 1 June 2010
Received in revised form 20 July 2010
Accepted 6 August 2010
Available online 14 August 2010

Keywords:
Caged compound
Ruthenium complex
Nicotine
Leech
Neurotransmitter
nAChR

ABSTRACT

We report the synthesis, characterization and applications of a ruthenium–bipyridine based caged nicotine. The complex $[Ru(bpy)_2(nic)_2]^{2+}$ (where bpy = 2,2′ bipyridine and nic = nicotine (3-[(2S)-1-methylpyrrolidin-2-yl] pyridine)) releases nicotine with a quantum yield $\phi = 0.23$ upon irradiation with biologically harmless, blue (473 nm) or green (532 nm) light. The photolysis reaction is clean and very fast, with a time constant of 17 ns. The synthesis is simple and the obtained compound is characterized by NMR, UV-Vis spectroscopy and cyclic voltametry. We find that this compound is active in biological systems, being able to elicit action potentials in leech neurons.

1. Introduction

Nicotine is arguably one of the most addictive drugs in use today [1]. The mechanisms underlying such addiction are complex and act at many cellular and molecular levels [2] and they are still not well understood. Nicotinic acetylcholine receptors (nAChR) are pentameric ligand-gated ion channels. They exist in many combinations of several subunits (α to ε) and some drugs and toxins display different affinities for different subunit compositions, but nicotine acts on almost all of them [3]. Nicotine is an agonist of nAChR which are present in different tissues such as muscle, ganglia and brain. It also interacts with the dopaminergic system [4]. To address current, important questions about nicotine effects in living systems it is essential to be able to handle nicotine application with exquisite control over location, concentration and timing. An emerging technology for drug application is the use of caged compounds.

Caged compounds, molecular entities which release a previously bound molecule upon illumination, make possible a kind of experiments technically impossible with classical drug application methods [5]. They are preferred over traditional drug application techniques because they are non-invasive and allow a very precise spatial and temporal control, down to one micrometer and a microsecond or even better. They were used to map the location of neurotransmitter receptors with unprecedented ease [6], to measure the kinetics of the gating of cell membrane receptors [7] and to reveal the existence of multiple receptor types in a cell population [8]. It was even possible to develop an all-optical setup for neural recording and stimulation [9].

Caged compounds offer many advantages over more traditional methods of drug application, such as the picospritzer: they involve no mechanical perturbation which could otherwise loosen a patched or impalled cell. No fluid mixing is involved, so the application kinetics is only limited by the kinetics of the uncaging reaction and the intensity of incident light. As the absolute amount of uncaged drug is usually extremely small, and in a very small volume, free diffusion can "wash" the drug away of the application site in a very short time after irradiation. To shape the photorelease pattern one simply needs to shape the light cast on the biological preparation.

Our lab has recently introduced a new kind of caged compounds based in the use of Ruthenium polypiridine complexes for caging amines, including the neurotransmitters γ-aminobutyric acid (GABA) [10], glutamate [11] and serotonin [12], demonstrating that this strategy can lead to a wide family of caged compounds. Ruthenium bipyridines present a strong metal to ligand charge transfer (MLCT) band in the visible spectrum region; absorption at this band populates a triplet state that can be thermally activated to a dissociative d-d state, which in turn leads to photoproducts breaking a single metal–ligand bond with very fast kinetics [13]. Moreover, this protecting group is removed in a single step photoreaction after visible light absorption, presenting an important advantage over UV-activatable MNI-, CDMNB- or CNB-caged compounds by avoiding photodamage, and requiring only conventional microscope optics and nonexpensive light sources.

In this paper we present the synthesis and characterization of the first caged nicotine to our knowledge: the complex $[Ru(bpy)_2(Nic)_2]^{2+}$ (RuBiNic). This compound has extremely fast uncaging kinetics, superb

* Corresponding author. Tel.: +54 11 4576 3358x475; fax: +54 11 4576 3341.
E-mail address: rober@qi.fcen.uba.ar (R. Etchenique).

0162-0134/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.jinorgbio.2010.08.003

water solubility in physiological conditions, and a high quantum yield in the visible region of the spectrum. It can be photolyzed with readily available and very accessible violet (405 nm), blue (473 nm) or even green (532 nm) lasers which have better tissue penetration than UV light, not requiring expensive UV lasers or quartz optics.

2. Experimental

2.1. Syntheses

All reactants were commercially obtained and used as received without further purification. $Ru(bpy)_2Cl_2$ was synthesized according to literature [14]. UV-Vis spectra were obtained with a HP8452A (Hewlett-Packard) diode-array spectrophotometer.

$[Ru(bpy)_2(Nic)_2]Cl_2$ (RuBiNic): 100 mg of $Ru(bpy)_2Cl_2$ were dissolved in 8.5 mL of methanol. 17 mL of distilled water were added and the solution was degassed by bubbling N_2 for 15 min. The reaction was kept at 80 °C and followed with a diode-array spectrophotometer until the characteristic spectrum of $[Ru(bpy)_2(H_2O)_2]^{2+}$ was obtained [15]. All following steps were done in the dark. At this point 3 M equivalents of nicotine were added. The absorption spectrum was monitored until no changes in the position of the band were detected. The orange solution was hot-filtered, the remaining methanol evaporated, and the reaction product was precipitated at 0 °C with excess KPF_6. The precipitate was washed several times with ice-cold water and stored in a dessicator until further use. All photosensitive species were handled and kept in the dark with a yield of 79%. In order to obtain a water soluble salt of the complex, a solution of $[Ru(bpy)_2(Nic)_2](PF_6)_2$ in acetone:water 2:1 was stirred with Dowex anionic exchange resin (chloride loaded) for 12 h. The acetone was evaporated and the aqueous solution was lyophilized yielding $[Ru(bpy)_2(Nic)_2]Cl_2$. The identity of the obtained compound was confirmed by measuring proton NMR spectra in D_2O with a Bruker Avance II 500 spectrometer. 1H-RMN (500 MHz, Methanol-d_4): δ = 1.33–1.42 (m, 1H); 1.45–1.55 (m, 1H); 1.84 (s, 3H); 1.67–1.90 (m, 1H); 1.94 (s, 3H); 2.06–2.19 (m, 1H); 2.29 (q, J = 9 Hz, 1H); 3.07–3.17 (m, 1H); 7.30 (s, t, J = 7 Hz, 2H); 7.44–7.49 (m, 2H); 7.82 (d, J = 6 Hz, 2H); 7.87 (t, J = 6 Hz, 2H); 7.98 (t, J = 8 Hz, 2H); 8.06 (d, J = 6 Hz, 2H); 8.18 (t, J = 8 Hz, 2H); 8.33–8.40 (m, 4H); 8.46 (d, J = 8 Hz, 2H); 8.52 (d, J = 8 Hz, 2H); 9.14 (d, J = 5 Hz, 2H).

2.2. Electrochemistry

Redox potentials were measured by cyclic voltametry in CH_3CN/$TBAPF_6$ (0.1 M) using a three-electrode potentiostat based on a TL071 operational amplifier in current to voltage configuration [16], with acquisition software written in QB 4.5. A 1 cm platinum wire with a diameter of 500 μm was used as working electrode. An Ag/AgCl electrode was used as reference, and the measured potentials were confirmed using the Ferrocene/Ferricinium redox couple as a reference. The counter electrode was a 10 cm long Pt wire, coiled around the 2 mL cell. Scanning rate was 100 mV/s.

2.3. Flash photolysis

The fast photolysis measurements were performed using for the irradiation source the second harmonic (532 nm) of a Spectra-Physics (Indi-HG) Nd:YAG laser which generates pulses of 10 ns Full Width at Half Maximum (FWHM). A low power continuous DPDSS Nd:YAG (532 nm, 1 mW) was used as the probe laser. A 0.1 mM RuBiNic solution in water was placed in a four-sided quartz cuvette under continuous stirring to avoid photodepletion near the analyzer light path. Adequate controls were made to discard any heat effects on the measured absorbance changes. Acquisition triggering was achieved by diverting a small fraction of the photolysis pulse into a fast photodiode and measuring this signal using a 1 GHz acquisition oscilloscope. Data was acquired as the time-resolved current average of 64 photolysis pulses.

2.4. Quantum yield and UV-Vis spectra measurements

The UV-Vis spectra of RuBiNic were measured with an HP8453 diode-array spectrometer. The quantum yield measurements were performed using a Luxeon Star III Royal Blue high-power light-emitting diode (LED) centered at 450 nm, 20 nm FWHM as photolysis light. The light was collimated and sent through an optical path of 1 cm. The irradiance of the light source was determined by measuring a reference sample of $[Ru(bpy)_2(py)_2]^{2+}$ as photosubstitution standard [17] with a known quantum yield (ϕ = 0.26).

2.5. Electrophysiology

Leeches (*Hirudo medicinalis*) were obtained from a commercial supplier. An isolated leech ganglion from a segment between 7 and 14 was pinned down in a sylgard-coated, 35 mm Petri dish. Leech saline with a 7:1 Mg^+ to Ca^+ ratio was perfused, to hinder synaptic transmission [18]. Retzius and P cells were identified by their position, size, and firing behaviour. Intracellular recordings were obtained with ~20 MΩ sharp microelectrodes, a Neuroprobe 1600 (A-M Systems) amplifier and an A/D signal acquisition board at 1 kHz sampling rate running custom-made software.

3. Results and discussion

After the synthesis and ion exchange procedures, RuBiNic was obtained as an orange hygroscopic powder. Being freely soluble, aqueous solutions of this complex present absorption bands between 420 and 470 nm corresponding to MLCT transitions typical of these complexes [11]. Nicotine presents a tertiary amine group with a pKa = 8.02 for its protonated form. It is expected that the effect of a positive charge (+2) in the Ru–bpy core shifts the pKa of the coordinated ligand to lower values.

Fig. 1 shows the absorption spectra at different pH values for the complex RuBiNic in water. The position of these bands is slightly dependent on the pH. This dependence arises from the difference in donor capabilities of the ligand when it is in its neutral or protonated form. The inset was obtained using complete spectra analysis to solve the equilibrium relative concentrations. The good fit of the protonation–deprotonation curve vs. pH with a single pKa constant demonstrates the rather independent behaviour of the two amine groups on the different nicotines. As expected, the coordinated nicotine presents a pKa = 6.8

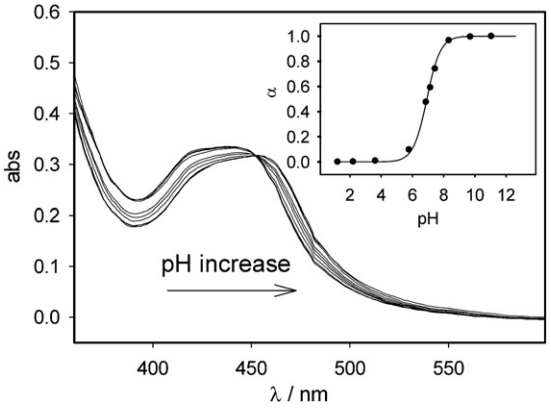


Fig. 1. Absorption spectra of RuBiNic at different pH values. The weaker donor ability of the protonated nicotine ligand shifts the MLCT band to shorter wavelengths at acidic pH. Concentration: 63 μM in water. Inset: fit of the molar fraction (α) of the deprotonated nicotine with a single equilibrium constant (pKa = 6.8).

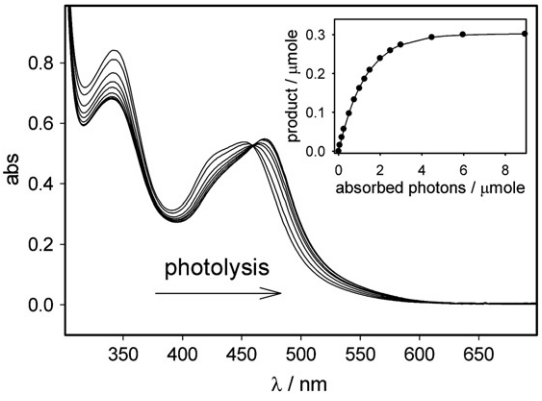


Fig. 2. Photolysis of RuBiNic in aqueous solution. Changes in RuBiNic UV-Vis spectrum in aqueous solution during irradiation with a 450 nm LED. Inset shows the number of moles of obtained products ($[Ru(bpy)_2(Nic)(H_2O)]^{2+}$ and nicotine) and the fit with a quantum yield $\phi = 0.23$ at pH = 7.

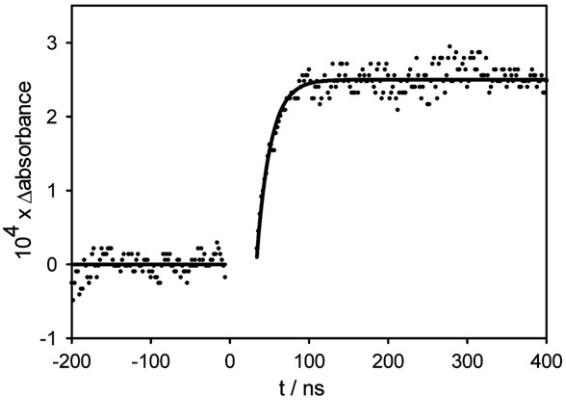


Fig. 3. Kinetics of the photolysis reaction. Dots: Flash photolysis of a 100 μM RuBiNic aqueous solution. Excitation: 532 nm Nd–YAG, 10 ns FWHM pulsed laser. Analyzer: 532 nm, 1 mW cw. laser. Solid line: fitting of the data with a monoexponential curve with a time constant of $\tau = 17.3$ ns.

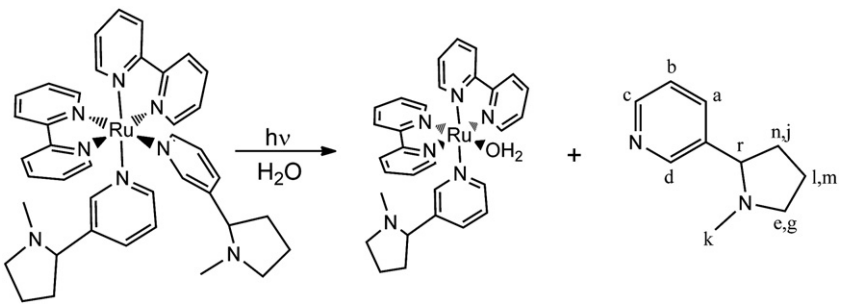
Cyclic voltametry of the compound shows a single process at 1.52 V vs. NHE in good agreement with the Ru(III)/Ru(II) parametrization given by Lever [19], for the Ru couple coordinated by 2 bipyridines and 2 alkylpyridinic ligands.

Aqueous solutions of RuBiNic undergo chemical changes upon irradiation. Fig. 2 shows the UV-Vis spectra of RuBiNic during irradiation with a 450 nm LED at pH = 7. The presence of an isosbestic point in the shown wavelength range is an indication of the existence of only two absorptive species, consistent with the expected direct photoaquation reaction depicted in Scheme 1. The only colored photoproduct is the aquo complex $[Ru(bpy)_2(Nic)(H_2O)]^{2+}$, which was synthesized to confirm its identity. After irradiation, no further changes in the UV-Vis spectra are evident after 48 h at 37 °C.

The inset in Fig. 2 depicts the progress of the photolysis reaction calculated from the complete spectra analysis. The quantum efficiency of photorelease was measured as the ratio between the number of moles of consumed RuBiNic and the number of moles of photons absorbed. The quantum yield for the photolysis reaction was calculated to be $\phi = 0.23$. The amount of product that can be photodelivered in a given procedure at low absorbance conditions is proportional to $\phi\varepsilon$, being ε the molar absorptivity of the caged compound. Given the molar absorptivity of RuBiNic of 4300 M^{-1} cm^{-1}, the product $\phi\varepsilon_{453} = 989$ is among the highest effective cross sections of any caged compound, including the ones that are only active in the UV range. As a comparison, figures for MNI-glutamate and CNB-glutamate [20] are lower, being $\phi\varepsilon_{350} = 366$ and 75 respectively, even in the UV range (350 nm). RuBiNic, on the other hand, is still active under green light. Using 532 nm irradiation, the quantum yield remains close to 0.2, but the absorptivity is much lower (ca. 500 M^{-1} cm^{-1}). However, the effective cross section of $\phi\varepsilon_{532} > 100$ is still adequate for its use in biological preparations. Preliminary experiments indicates a two-photon cross section of 0.01–0.1 GM at 800 nm for this compound. RuBiNic solutions in physiological saline behave exactly as in water. No decomposition is observed in a 0.1 mM solution of RuBiNic in saline at pH 7.6 after 2 days in the dark.

To measure the reaction kinetics, a solution of RuBiNic in water was photolysed with a 532 nm Nd–YAG pulsed laser. The results are depicted in Fig. 3. The dots indicate the absorbance during the pulse irradiation (as the average of 64 photolysis events). The continuous line is a single exponential fit with a time constant of 17.3 ns, even faster that a similar caged glutamate [11]. This fast change is consistent with the clean, one-step coordination bond rearrangement photocleavage mechanism, and with the reported excited-state lifetime of the analog complex $[Ru(bpy)_2(Py)_2]^{2+}$ [21]. This makes RuBiNic an invaluable tool not only in nicotine-related research but also to shed light into fast nicotinic acetylcholine receptor biophysics.

The photoreaction was also followed by ^{1}H-NMR spectroscopy. The non-irradiated RuBiNic spectrum is shown in Fig. 4 (top). In the aromatic region of a typical *cis*-$[Ru(bpy)_2\,L_2]^{n+}$ complex it is possible to distinguish 8 signals that correspond to the 16 bipyridine protons, each integrating for 2H due to the symmetry of the bis-substituted molecule. For this complex, however, it is sometimes possible to distinguish up to 16 different signals, due to the fact that the racemic mixture of the enantiomeric Δ and Λ forms of the Ru–bpy_2 center is converted to a roughly 50/50 mixture of diasteromers after (−)-nicotine coordination. Fortunately, the differences between the chemical shifts of the protons in the diasteromers are very small using methanol-d_4 as solvent. In the aromatic region the signals corresponding to the coordinated nicotine are apparent. No free nicotine signals are detectable. The signals of pure nicotine can be seen in the bottom trace of Fig. 4. (The signals are labeled according to Scheme 1.) After direct irradiation of the NMR tube with a 450 nm high-power LED, the middle spectrum was obtained. The doublet at 9.14 ppm appears diminished in about 80%, showing that no more



Scheme 1. Structure of RuBiNic and its photolysis products. The identity of the species was confirmed by NMR and UV-Vis spectra and by its biological effects.

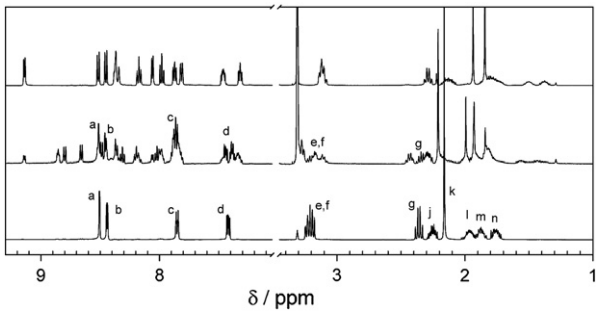


Fig. 4. NMR spectra of RuBiNic and its photolysis products. Top: 1H-NMR spectrum of RuBiNic before irradiation. Middle: The same NMR tube after 2 min of 450 nm irradiation. Bottom: Spectrum of pure nicotine. All spectra were taken in MeOD.

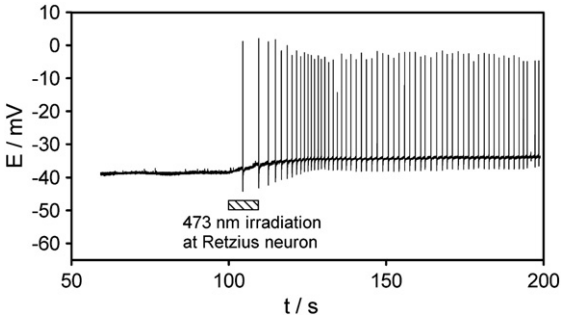


Fig. 5. Biological effects of RuBiNic photolysis on leech neurons. Irradiation with a 473 nm laser (grey bar) photoreleases nicotine in the extracellular bath, depolarizing a leech Retzius cell and inducing immediately a high frequency firing of action potentials.

than 20% of the initial compound remains. Further photolysis yields up to 100% photoproducts in a clean way. As the photoreaction proceeds, two new species are formed, being one of them free nicotine, as can be determined from comparison of the signals with those of the free nicotine ligand. As a stability test, an identical solution of RuBiNic was left in an NMR tube in the dark and measured 15 days later, obtaining the same results.

To test the biological compatibility of this caged compound, we used it to induce depolarization and action potential firing in neurons. It is known that leech Retzius neurons increase their firing rate when exposed to nicotine [22]. A leech ganglion was pinned down and a Retzius neuron impaled according to standard protocols [23]. 100 μM RuBiNic was added to the bath and kept in the dark to record a baseline. A small negative current was introduced into the cell in order to lower the spiking rate to nearly zero. After this procedure, a 6 mW, 473 nm blue laser light pulse was delivered onto a P-type medial neuron, which induced no visible changes in membrane resting potential or in action potential frequency. However, the same laser pulse delivered over a Retzius cell induced a depolarization and a sudden increase in firing frequency, as can be seen in Fig. 5. The increased firing rate lasted until the ganglion was washed with normal saline (not shown). This behaviour was robust and repeatable over many ganglia and different animals. As negative controls, the same procedure applied to a P cell (which are not nicotine sensitive) produced no changes, and the same light pulse delivered over the same neurons with no caged compound present in the bath induced no response.

4. Conclusions

We have devised a new caged compound (RuBiNic) based in the photochemistry of Ru–bpy complexes. This phototrigger releases nicotine by irradiation with visible light up to 532 nm. Its photoreaction quantum yield is $\phi = 0.23$ at pH = 7 using a 450–473 nm light source. Similar quantum yield is obtained at 532 nm, although the molar absorptivity at this wavelength is 10 times lower. Its chemistry is clean, with no other products than the free ligand nicotine and the aquo complex $[Ru(bpy)_2(Nic)(H_2O)]^{2+}$, which are photoreleased in less than 20 ns.

The compound is very stable as a solid or in aqueous solutions, showing no decomposition after months in the dark at RT. Its chloride salts are freely soluble in water and physiological solutions. RuBiNic showed no toxicity when applied to the extracellular bath of nervous tissue at 1 mM concentrations. Upon irradiation, a 0.1 mM solution of RuBiNic is capable of inducing action potentials in Retzius neurons of leech ganglia. Moreover, its molecular structure, very similar to other caged compounds of the family, strongly suggests that 2-photon activation is possible. Further research is being conducted over this issue.

Acknowledgments

We are grateful to Dr. Lidia Szczupak for providing the leeches. This work was done with funding from UBACyT, CONICET and ANPCyT. RE is a member of CONICET.

References

[1] U.S. Department of Health and Human Services, U.S. Department of Health and Human Services, Public Health Service, Centers for Disease Control, Center for Health Promotion and Education, Office on Smoking and Health, DHHS, 1988, Publication No. (CDC) 88-8406.
[2] J.A. Dani, S. Heinemann, Neuron 16 (1996) 905–908.
[3] J.-P. Changeux, Nat. Rev. Neurosci. 11 (2010) 389–401.
[4] B. Le Foll, A. Gallo, Y. Le Strat, L. Lu, P. Gorwood, Behav. Pharmacol. 20 (2009) 1–17.
[5] L. Zayat, L. Baraldo, R. Etchenique, in: R. Yuste, A. Konnerth (Eds.), Imaging in Neuroscience and Development. A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York, 2005, pp. 367–424.
[6] D.L. Pettit, S.S.H. Wang, K.R. Gee, G.J. Augustine, Neuron 19 (1997) 465–471.
[7] T. Otis, S. Zhang, L.O. Trussell, J. Neurosci. 16 (1996) 7496–7504.
[8] G. Lowe, J. Neurophysiol. 90 (2003) 1737–1746.
[9] V. Nikolenko, K. Poskanzer, R. Yuste, Nat. Meth. 4 (2007) 943–950.
[10] L. Zayat, M.G. Noval, J. Campi, C.I. Calero, D.J. Calvo, R. Etchenique, Chembiochem 8 (2007) 2035–2038.
[11] M. Salierno, E. Marceca, D.S. Peterka, R. Yuste, R. Etchenique, J. Inorg. Biochem. 104 (2010) 418–422.
[12] L. Zayat, M. Salierno, R. Etchenique, Inorg. Chem. 45 (2006) 1728–1731.
[13] S. Campagna, F. Puntoriero, F. Nastasi, G. Bergamini, V. Balzani, Top. Curr. Chem. 280 (2007) 117–214.
[14] C. Viala, C. Coudret, Inorg. Chim. Acta 359 (2005) 984.
[15] B. Durham, S.R. Wilson, D.J. Hodgson, T.J. Meyer, J. Am. Chem. Soc. 102 (1980) 600–607.
[16] A.J. Bard, L.R. Faulkner, Electrochemical Methods. Fundamentals and Applications, Wiley, New York, 1980.
[17] D.V. Pinnick, B. Durham, Inorg. Chem. 23 (1984) 1440–1445.
[18] J.G. Nicholls, D. Purves, J. Physiol. Lond. 209 (1970) 647–667.
[19] A.V.P. Lever, Inorg. Chem. 29 (1990) 1271–1285.
[20] O.D. Fedoryak, J.-Y. Sul, P.G. Haydon, G.C.R. Ellis-Davies, Chem. Commun. 29 (2005) 3664–3666.
[21] B. Durham, J.V. Caspar, J.K. Nagle, T.J. Meyer, J. Am. Chem. Soc. 104 (1982) 4803–4810.
[22] J. Edgar, M.L. Peralta, W.B. Kristan Jr., J. Exp. Biol. 201 (1998) 1895–1906.
[23] K.J. Muller, J.G. Nicholls, G.S. Stent, Neurobiology of the Leech, Cold Spring Harbor, New York, 1994.