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Synthesis and evaluation of the europium^{III} and zinc^{II} complexes as luminescent bioprobes in high content cell-imaging analysis

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ABSTRACT

Novel phenanthroline derivatives and their europium(III) and zinc(II) complexes have been prepared in up to 92%. In contrast to the stable zinc complexes, the europium compounds exhibit a strong luminescence in THF solution. However, quenching of the emission is observed in DMSO indicating complete dissociation of the complexes back to free ligands in this solvent. ^{1}H NMR studies of the Eu(III)-complexes **5** and **6** also confirmed the existence of different states depending on the solvent used. Moreover, it was found that compound **5** is stable in EtOH–PBS solutions; here a strong signal in the emission spectra corresponding to the europium ion was detected. No spectral changes were observed for the zinc(II) complexes, they were shown to be stable in the media. These metal complexes can be used as fluorescence markers for the diagnosis of oesophageal squamous carcinoma (OE21) cells at low concentrations. Cell images were acquired using the compounds **5**, **7**–**9** as luminescent agents. The first images were taken already after 20 min incubation time at a very low concentration range (0.7–1.6 μM).

1. Introduction

In past decades, molecular imaging technology has become a central tool in biology, particularly in bioassays and cell imaging, cancer research, and clinical trials in medicine [1–5]. The development of novel luminescent probes for intracellular analysis, that are efficient and kinetically stable, is one of the vital goals. Rare earth metal complexes are now versatile targets in many areas of research, such as photochemistry, biomedicine and nanotechnology [6–13]. Special attention has been given to the luminescence properties of lanthanide ions emitting at a millisecond lifetime-scale and in the visible region for europium(III) and terbium(III) complexes [14–17]. These remarkable photophysical properties are key factors to make them molecules of choice for cellular imaging and diagnostics [18–21]. A variety of the different Ln(III) complexes have been synthesised, however a major problem is their instability in aqueous media. The lanthanide ion and its neutral ligands are linked via weak coordination bonds and generally exist in an equilibrium of "free ligand" ⇆ "metal complex", which can be shifted in a medium via solvent exchange [22–23]. The metal complexes are prone to dissociation in the presence of solvent molecules. Disadvantageously, in some cases this can lead to an irreversible dissociation into the "free ligand" form in water through completion of the first coordination sphere [22–25] of a lanthanide ion with solvent molecules. This normally prevents the use of Ln(III) complexes as luminescent markers for bioassays. Nevertheless, only limited information is available on the real state of such metal complexes in different media.

Zinc compounds are known to be highly fluorescent and present an alternative to lanthanide complexes. Thus, many photochemical studies targeted at modelling the natural situation use more stable zinc complexes. The propensity of zinc to act as an acceptor atom is used in many supramolecular approaches and/or for the modulation of chromophore properties via ligand binding. Due to their biological relevance, technical importance and good stability, they have found significant use as industrial pigments, electron transfer components, for photochemical transformations and in photobiotechnology [26].

Here, we have studied the stability and photophysical efficiency of europium and zinc complexes of phenanthroline derivatives. These molecules are strongly absorbing chromophores often used as antennas for indirect excitation of metal ions, where direct excitation of a metal is inefficient. Phenanthroline itself is known to inhibit metallopeptidases and its various metal complexes have been prepared and tested as bioprobes [27–37]. Here we present results based on the synthesis, behaviour indifferent solvents monitored by NMR spectroscopy, photophysical analysis and cellular imaging of novel metal complexes at low concentrations.

2. Experimental

2.1. Materials and methods

A 1 mM stock solution was prepared in THF, MeOH, DMSO and EtOH for each compound. Dilutions were carried out directly before starting the analyses. For cell stain imaging a stock solution prepared

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in EtOH was diluted with phosphate buffered saline (PBS) to different concentrations. Buffer solutions prepared at the desired concentrations were added directly to the cell culture. Total EtOH concentration was lower than 0.1%. After adding compounds, cells were incubated at 37 °C in 5% CO_2 for 20 min to 24 h. Cells (human esophageal squamous carcinoma OE21) were routinely cultured in RPMI 1640 (Hyclone, USA) with 10% inactivated foetal bovine serum (Hyclone, USA) and 1% Penicillin/(Hyclone, USA). These cultures were grown in sterile filtered top cell culture flasks (Nunc, Denmark). Cultures were split 1:8 when 70–80% confluency was reached. Cells were split using typsin 0.25% solution, with EDTA (Hyclone, USA) and kept at 37 °C and 5% CO_2 in a humidified atmosphere. Culture medium was changed every 3 to 4 days until confluency. The cell lines were plated at a concentration of 1.0×10^5 cells/mL into sterile 96-well plates (Nunc, Denmark) and left to attach overnight.

2.2. General

^{1}H NMR spectra were recorded on a Bruker DPX 400 (400 MHz and 600 MHz for ^{1}H NMR). Chemical shifts are reported in ppm referred to TMS set at 0.00 ppm. All photophysical measurements were performed in THF, MeOH, EtOH, DMSO and PBS. All emission spectra were recorded on Fluorolog Horiba Jobin Yvon spectrometer. High resolution mass-spectra (HRMS) were measured on MaldiQ-Tof Premier Micromass and Micromass/Waters Corp. USA liquid chromatography time-of-flight spectrometer equipped with ES source. UV–visible (UV–vis) measurements were performed on Specord 250 Analytik Jena instrument. Melting points were acquired on Stuart SMP10 melting point apparatus and are uncorrected. Thin layer chromatography (TLC) was performed on silica gel 60 F_{254} (Merck) precoated aluminium sheets. Spectroscopy grade solvents were used in all cases. All commercial chemicals and solvents were supplied by Sigma-Aldrich and used without further purification. Abbreviations "s" — singlet, "d" — doublet, "dd" — doublet of doublets, "m" — multiplet, "br" — broad, ES MS — electrospray mass-spectrometry, TOF MS — time-of-flight mass-spectrometry, THF — tetrahydrofuran, MeOH — methanol, EtOH — ethanol, DMSO — dimethylsulfoxide, PBS — phosphate buffered saline were used.

2.3. Synthesis of 1,10-phenanthroline-5,6-dione [38] 1

To a dry mixture of 1,10-phenanthroline (2.0 g, 0.01 mol) and KBr (10.0 g, 0.08 mol), H_2SO_4 (40 mL) followed by HNO_3 (20 mL) was added dropwise at 0 °C. The resulting mixture was heated at 100 °C until the bromine vapours disappeared. The solution was poured carefully onto ice and slowly neutralised to pH>7 with Na_2CO_3 (saturated solution and powder). The product was extracted with dichloromethane and dried over Na_2SO_4. The solvents were evaporated to give a yellow solid that was dried under vacuum (88–95%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.16 (dd, $J=4.6$ Hz, $J=1.7$ Hz, 2H); 8.54 (dd, $J=7.9$ Hz, $J=1.6$ Hz, 2H); 7.62 (dd, $J=7.9$ Hz, $J=4.7$ Hz, 2H).

2.4. Synthesis of imidazo[4,5-f]1,10-phenanthroline derivatives 2–4

1,10-Phenanthrolin-5,6-dione **1** (1.0 g, 4.76 mmol) and ammonium acetate (11.7 g, 0.15 mol) were dissolved in hot glacial acetic acid (50 mL) at ca. 70 °C and an appropriate aldehyde (4.76 mmol), dissolved in 20 mL of glacial acetic acid, was added. The resulting mixture was heated at 70–80 °C for 2 h (TLC-control). Then the solution was allowed to cool to room temperature and was neutralised with 1 N NaOH. The yellow compound was filtered, washed with water and dried under vacuum.

2.4.1. 2-(4-Bromophenyl)imidazo[4,5-f]-1,10-phenanthroline 2

Yellow solid (1.43 g, 80%); analytical data were in accordance with the literature [39].

2.4.2. 2-{4-[1′-(2′-Trimethylsilyl)ethynyl]phenyl}imidazo[4,5-f]-1,10-phenanthroline 3

Yellow solid (1.24 g, 66%). Mp 258 °C; δ_H (400 MHz, DMSO-d_6) 9.06 (m, 2H, H_{phenan}), 8.92 (d, $J=6.9$ Hz, 2H, H_{phenan}), 8.29 (d, $J=8.4$ Hz, 2H, H_{Ph}), 7.86 (dd, $J=8.0$ Hz, $J=4.3$ Hz, 2H, H_{phenan}), 7.70 (d, 2H, $J=8.4$ Hz, H_{Ph}), 0.27 (s, 9H, TMS); δ_C (150.2 MHz, DMSO-d_6) 149.9, 148.3, 144.0, 132.6, 130.4, 129.9, 126.6, 123.3, 105.2, 96.5 (m); *m/z* (TOF MS) 393.152 (M + H. $C_{24}H_{21}N_4Si$ requires 393.153); UV–visible (UV–vis) (CH_3OH): λ_{max} (lgε) 283 (4.3), 335 (4.4).

2.4.3. 2-(4-Pyridinyl)imidazo[4,5-f]-1,10-phenanthroline [40] 4

Brown solid (1.07 g, 76%); δ_H (400 MHz, DMSO-d_6) 9.20 (m, 2H, H_{phenan}), 8.92 (m, 4H, H_{phenan} and H_{Py}), 8.27 (m, 2H, H_{Py}), 7.97 (m, 2H, H_{phenan}). UV–visible (UV–vis) (CH_3OH): λ_{max} (lgε) 273 (4.6), 322 (4.3), 387 (3.6).

2.5. Synthesis of the imidazo[4,5-f]-1,10-phenanthroline europium (III) complexes 5–7

To an appropriate imidazo[4,5-*f*]-1,10-phenanthroline derivatives **2**–**4** (0.63 mmol) was dissolved in hot ethanol (100 mL) and an ethanolic solution of $Eu(NO_3)_3{\bullet}5H_2O$ (90 mg, 0.21 mmol) was added. The resulting mixture was heated at reflux until the starting material disappeared (TLC-control, ca. 30 h). The yellow solid formed was filtered, washed with ethanol and dried under vacuum to give the desired product **5**–**7**.

2.5.1. 2-(4-Bromophenyl)imidazo[4,5-f]-1,10-phenanthroline europium (III) complex 5

Yellow solid (0.47 g, 58%). Mp>300 °C; δ_H (400 MHz, THF-d_8, TMS) 13.19 (3H, br s, NH), 8.86 (6H, br m, H_{phenan}), 8.08 (16H, br m, H_{phenan} and H_{Ph}), 6.50 (8H, br m, H_{phenan}); *m/z* (TOF MS) 1274.971 (M. $C_{57}H_{33}Br_3EuN_{12}$ requires 1274.976).

2.5.2. 2-{4-[1′-(2′-Trimethylsilyl)ethynyl]phenyl}imidazo[4,5-f]-1,10-phenanthroline europium(III) complex 6

Yellow solid (0.77 g, 92%). Mp>300 °C; δ_H (400 MHz, THF-d_8, TMS) 13.00 (br s, 3H, NH), 8.75 (br m, 6H, H_{phenan}), 8.18 (br m, 10H, H_{phenan} and H_{Ph}), 7.63 (m, 6H, H_{Ph}), 6.26 (br m, 8H, H_{phenan}), 0.20 (s, 27H, TMS); *m/z* (TOF MS) 1302.292 (M-3Me + H_2O. $C_{69}H_{53}EuN_{12}OSi_3$ requires 1302.299.

2.5.3. 2-(4-Pyridinyl)imidazo[4,5-f]-1,10-phenanthroline europium(III) complex 7

Yellow solid (0.36 g, 55%). Mp>300 °C; δ_H (400 MHz, D_2O + DMSO-d_6, TMS) 7.82 (br m, 30H, H_{phenan} and H_{Ph}), 0.52 (br, 3H, NH); *m/z* (ES) 968.17 (M-Py + H. $C_{50}H_{30}EuN_{14}$ requires 968.21), *m/z* (TOF MS) 906.18 (M-2Py + H_2O). $C_{44}H_{27}EuN_{13}O$ requires 906.17, 1044.23 (M). $C_{54}H_{33}EuN_{15}$ requires 1044.44, 1064.30 (M + H_2O + 2H). $C_{54}H_{37}EuN_{15}O$ requires 1064.25.

2.6. Synthesis of imidazo[4,5-f]-1,10-phenanthroline zinc complexes 8–10

To an appropriate imidazo[4,5-*f*]-1,10-phenanthroline derivatives **2** or **4** (0.54 mmol) dissolved in 10–20 mL of THF–MeOH (1:1, v/v) was added a solution of $ZnCl_2$ (0.27 mmol) in MeOH. A yellow precipitate was formed immediately after addition of the metal salt. The mixture was stirred at room temperature for 24 h and filtered. The solid residue was washed with ethanol and dried in vacuum.

2.6.1. 2-(4-Pyridinyl)imidazo[4,5-f]-1,10-phenanthroline zinc(II) complex 8

Pale yellow solid (36 mg, 20%). Mp>300 °C; δ_H (600 MHz, DMSO-d_6) 10.09 (s, 2H, NH), 9.16 (br, 8H, H_{phenan}), 8.96 (d, $J=4.6$ Hz, 4H,