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Luminescent Biological Probes Derived from Ruthenium(II) Estradiol Polypyridine Complexes

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Four luminescent ruthenium(II) polypyridine estradiol complexes $[Ru(N^N)_2(bpy\text{-}estradiol)](PF_6)_2$ (N^N = 2,2′-bipyridine (bpy), 4,7-diphenyl-1,10-phenanthroline (Ph_2-phen); bpy-estradiol = 5-(4-(17α-ethynylestradiolyl)phenyl)-2,2′-bipyridine (bpy-ph-est), 4-(*N*-(6-(4-(17α-ethynylestradiolyl)benzoylamino)hexyl)aminomethyl)-4′-methyl-2,2′-bipyridine (mbpy-C6-est)) have been designed as new luminescent biological probes. The lipophilicity and photophysical and electrochemical properties of these complexes have been investigated. Upon photoexcitation, all the complexes exhibited intense and long-lived triplet metal-to-ligand charge-transfer (^{3}MLCT) ($d\pi(Ru) \rightarrow \pi^*$(diimine)) emission in fluid solutions at 298 K and in low-temperature glass. The binding of the complexes to estrogen receptor-α (ERα) has been studied by emission titrations. The Ph_2-phen complexes showed emission enhancement and increased lifetimes upon binding to the protein. Additionally, the cytotoxicity of the complexes toward the HeLa cell line has been examined by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay and the IC_{50} values ranged from 83.1 to 166.6 μM (cisplatin showed an IC_{50} value of 34.3 μM under the same experimental conditions). Furthermore, the cellular uptake of the complexes has been investigated by flow cytometry and laser-scanning confocal microscopy.

Introduction

Estrogens are responsible for the development of female secondary sexual characteristics, stimulation of endometrial and uterine growth, and regulation of the menstrual cycle.[1] They are also involved in bone resorption and building,[2] coagulation of blood,[3] and control of the levels of lipoproteins, triglyceride, and fat deposit.[4] Most of the biological effects of estrogens are mediated through an interaction with estrogen receptors (ERs).[5] ERs (ERα and ERβ) belong to the superfamily of nuclear receptor proteins, which are membrane or intracellular proteins[6,7] existing in a variety of tissues.[8] They are not only vital in regulating the differentiation and maintenance of neural, skeletal, cardiovascular, and reproductive tissues[9] but also participate in the development of estrogen-dependent cancer such as breast, ovarian, colon, prostate, and endometrial cancers.[10] Many clinical studies have concluded that the receptor content gives the most

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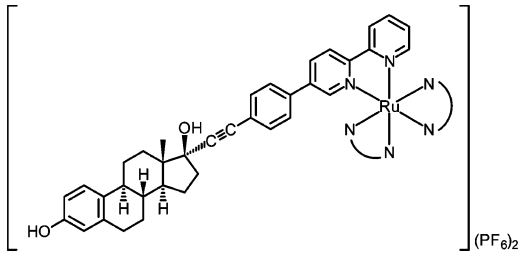
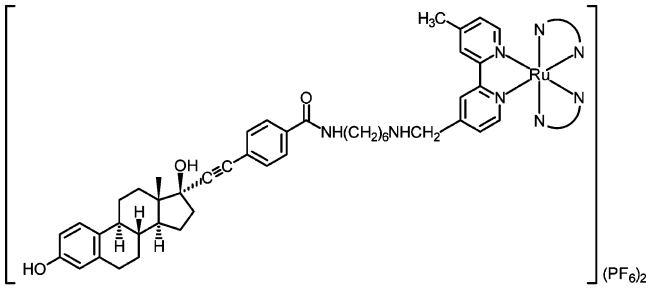
accurate index of the cancer.[11] Since the binding affinity of estradiol to ERs is the highest among all estrogens,[12] various therapeutic and diagnostic units modified with estradiol have been used to study estrogen binding and develop site-specific drugs for ER-related diseases.[12−19] These units include radioactive labels,[12,14] radiopharmaceuticals,[13] IR-active organometallic complexes,[15] and organic fluorophores.[16] In addition, biotinylated estradiol has been used to develop an enzyme immunoassay for estradiol in human plasma.[17] We have recently reported luminescent tricarbonylrhenium(I)[18] and cyclometalated iridium(III)[19] polypyridine estradiol conjugates that can recognize ERs. In view of the high photostability, low-energy absorption, and relatively long-lived luminescence of ruthenium(II) polypyridine complexes,[20] we anticipate that they are promising candidates as luminescent biological probes.

Here we report the synthesis, characterization, and photophysical and electrochemical properties of four luminescent ruthenium(II) polypyridine estradiol complexes $[Ru(N^N)_2(bpy\text{-}estradiol)](PF_6)_2$ (N^N = 2,2′-bipyridine (bpy), 4,7-diphenyl-1,10-phenanthroline (Ph_2-phen); bpy-estradiol = 5-(4-(17α-ethynylestradiolyl)phenyl)-2,2′-bipyridine (bpy-ph-est), 4-(*N*-(6-(4-(17α-ethynylestradiolyl)benzoylamino)hexyl)-aminomethyl)-4′-methyl-2,2′-bipyridine (mbpy-C6-est)) (Chart 1). The lipophilicity of these complexes has been determined by reversed-phase HPLC. The binding of the complexes to estrogen receptor-α (ERα) has been studied by emission titrations. The results have been compared to control experiments involving the estradiol-free analogues $[Ru(bpy)_3](PF_6)_2$ and $[Ru(Ph_2\text{-}phen)_2(bpy)](PF_6)_2$. Additionally, the cytotoxicity of the ruthenium(II) estradiol complexes toward the HeLa cell line has been examined by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cellular uptake of the complexes has been investigated by flow cytometry and laser-scanning confocal microscopy.

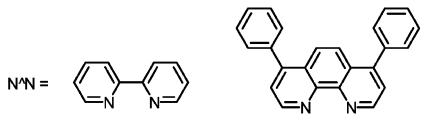
Chart 1. Structures of the Ruthenium Estradiol Complexes



$[Ru(N^N)_2(bpy\text{-}ph\text{-}est)](PF_6)_2$



$[Ru(N^N)_2(mbpy\text{-}C6\text{-}est)](PF_6)_2$



Experimental Section

Materials and Synthesis. All solvents were of analytical grade. All buffer components were of biological grade and used as received. Diethylamine (Sigma) and DMF (Lab-Scan) were freshly

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distilled over KOH and $MgSO_4$, respectively, under nitrogen before use. Ruthenium(III) chloride hydrate (Arcos), bpy (Acros), Ph_2-phen (Aldrich), 17α-ethynylestradiol (Aldrich), triphenylphosphine (Aldrich), palladium(II) chloride (Acros), copper(I) iodide (Acros), 5-(4-bromophenyl)-2,2′-bipyridine (Wako), 4-iodobenzoic acid (Acros), *N*-hydroxysuccinimide (Acros), *N*,*N*′-dicyclohexylcarbodiimide (Acros), 1,6-hexanediamine (Acros), cisplatin (Acros), MTT (Sigma), $NaBH_4$ (Acros), and KPF_6 (Acros) were used without purification. 4′-Methyl-2,2′-bipyridyl-4-carboxaldehyde,[21] *cis*-[Ru(N^N)$_2$Cl$_2$]·2H$_2$O,[22] and bpy-ph-est[19] were prepared by reported methods.

Lamb uteri cytosol was used as a source of ERα, which was purified and quantitated according to reported procedures.[15a,23] Lamb uteri tissues obtained from the HKSAR slaughterhouse in Sheung Shui, New Territories, Hong Kong, were immediately frozen after isolation and stored at −70 °C prior to purification. Before purification, they were thawed and minced. The resulting tissues were ground using mortar and pestle in 50 mM Tris-Cl with 0.25 M sucrose and 0.1% 2-mercaptoethanol, pH 7.4, at 25 °C. The homogenate was centrifuged at 14 000 rpm for 1 h at 4 °C to remove the solid residue. Uterine cytosol was made 30% saturated with ammonium sulfate and centrifuged at 14 000 rpm for 30 min at 4 °C. After removal of the supernatant, the pellets were stored at −70 °C. Before use, the receptor pellets were thawed on ice and then dissolved in ice-cold 50 mM potassium phosphate buffer. The concentration of ERα was determined by the Bradford method.[23]

Human cervix epithelioid carcinoma (HeLa) cells were obtained from the American Type Culture Collection. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin-EDTA, and penicillin/streptomysin were purchased from Invitrogen. The growth medium for cell culture contained DMEM with 10% FBS and 1% penicillin/streptomysin.

4-(*N*-(6-(4-Iodobenzoylamino)hexyl)aminomethyl)-4′-methyl-2,2′-bipyridine. A mixture of 4-iodobenzoic acid (1.00 g, 4.03 mmol), *N*-hydroxysuccinimide (0.56 g, 4.87 mmol), and *N*,*N*′-dicyclohexylcarbodiimide (1.00 g, 4.87 mmol) in 100 mL of anhydrous THF was stirred under nitrogen at room temperature for 12 h. The white solid precipitated was removed by filtration. The filtrate was evaporated to dryness to give a white solid. The solid and 1,6-hexanediamine (4.67 g, 40.26 mmol) were then dissolved in 150 mL of CH_2Cl_2, and the solution was stirred at room temperature for 12 h. The white solid precipitated was removed by filtration. The filtrate was washed with H_2O (300 mL × 3). The organic layer was dried over $MgSO_4$ and evaporated to dryness to give a white solid. The solid and 4′-methyl-2,2′-bipyridyl-4-carboxaldehyde (0.80 g, 4.03 mmol) were dissolved in 30 mL of MeOH, and the mixture was stirred at room temperature for 12 h. The white solid precipitated and was collected by filtration. The residue was washed with 30 mL of cold MeOH and was then suspended in 150 mL of MeOH. Addition of $NaBH_4$ solid (0.76 g, 20.11 mmol) led to a colorless solution, which was stirred at room temperature for 5 h. The solution was evaporated to dryness to give a white solid. The crude product was washed with water and then dried in a vacuum desiccator. Yield = 0.63 g (30%). Positive-ion ESI-MS ion cluster: m/z = 529, $\{M + H^+\}^+$.

Mbpy-C6-est. The procedure was similar to that of bpy-ph-est,[19] except that 4-(*N*-(6-(4-iodobenzoylamino)hexyl)aminomethyl)-4′-methyl-2,2′-bipyridine (396 mg, 0.75 mmol) was used instead of 5-(4-bromophenyl)-2,2′-bipyridine. The crude product was washed with cold DMF and diethyl ether. The ligand mbpy-C6-est was isolated as a pale brown solid. Yield = 236 mg (45%). ^{1}H NMR (500 MHz, DMSO-d_6, 298 K, TMS): δ = 9.03 (s, 1H, 3-OH of estradiol), 8.61−8.39 (m, 3H, bpy-4-$CH_2NHC_6H_{12}NH$ and H6 and H6′ of pyridyl rings), 8.34 (s, 1H, H3 of pyridyl ring), 8.20 (s, 1H, H3′ of pyridyl ring), 7.80 (d, 2H, J = 6.7 Hz, H2 and H6 of phenyl ring), 7.45 (d, 2H, J = 6.5 Hz, H3 and H5 of phenyl ring), 7.35 (d, 1H, J = 4.5 Hz, H5 of pyridyl ring), 7.23 (d, 1H, J = 4.7 Hz, H5′ of pyridyl ring), 7.02 (d, 1H, J = 7.6 Hz, H1 of estradiol), 6.55−6.39 (m, 2H, H2 and H4 of estradiol), 5.54 (s, 1H, 17-OH of estradiol), 3.75 (s, 2H, bpy-4-CH_2NH), 3.28−3.11 (m, 3H, bpy-4-$CH_2NHC_5H_{10}CH_2$ and bpy-4-CH_2NH), 2.78−2.49 (m, 2H, H6 of estradiol), 2.49−2.34 (m, 5H, bpy-4-CH_2NHCH_2, and CH_3 on C4′ of pyridyl ring), 2.31−2.02 (m, 4H, H9$_\alpha$, H11$_\alpha$, H12$_\beta$, and H16$_\alpha$ of estradiol), 2.01−1.59 (m, 5H, H7$_\beta$, H8$_\beta$, H11$_\beta$, H15$_\alpha$, and H16$_\beta$ of estradiol), 1.58−1.11 (m, 12H, H7$_\alpha$, H12$_\alpha$, H14$_\beta$, and H15$_\beta$ of estradiol, and bpy-4-$CH_2NHCH_2C_4H_8$), 0.78 (s, 3H, CH_3 of estradiol). IR (KBr) (ν/cm^{-1}): 3429 (br, O−H and N−H), 2196 (w, C≡C), 1669 (s, C═O). Positive-ion ESI-MS ion cluster: m/z = 697, $\{M + H^+\}^+$.

[Ru(bpy)$_2$(bpy-ph-est)](PF$_6$)$_2$. A mixture of *cis*-[Ru(bpy)$_2$Cl$_2$]·2H$_2$O (52 mg, 0.10 mmol) and bpy-ph-est (63 mg, 0.12 mmol) in 20 mL of 50% aqueous ethanol was heated at reflux for 12 h. The color of the solution turned from purple to deep red. The volume of the mixture was reduced to ca. 10 mL, and the solution was then filtered. Excess KPF_6 was added to the solution to precipitate a deep red solid. The solid was collected by filtration, washed with water, a small amount of cold MeOH, and diethyl ether. Recrystallization of the product from acetone/diethyl ether afforded the target complex as red crystals. Yield = 71 mg (58%). ^{1}H NMR (300 MHz, acetone-d_6, 298 K, TMS): δ = 8.94−8.77 (m, 6H, H3 and H3′ of pyridyl rings of bpy and bpy-ph-est), 8.49 (d, 1H, J = 8.2 Hz, H4 of pyridyl ring of bpy-ph-est), 8.32−8.01 (m, 14H, 3-OH of estradiol, H4, H4′, H6, and H6′ of bpy, H3 and H5 of phenyl ring of bpy-ph-est, and H4′, H6, and H6′ of pyridyl rings of bpy-ph-est), 7.65−7.51 (m, 7H, H5 and H5′ of bpy, H2 and H6 of phenyl ring of bpy-ph-est, and H5′ of pyridyl ring of bpy-ph-est), 7.12 (d, 1H, J = 8.5 Hz, H1 of estradiol), 6.71 (dd, 1H, J = 8.5 and 5.6 Hz, H2 of estradiol), 6.53 (s, 1H, H4 of estradiol), 4.56 (s, 1H, 17-OH of estradiol), 2.83−2.72 (m, 2H, H6 of estradiol), 2.41−2.23 (m, 4H, H9$_\alpha$, H11$_\alpha$, H12$_\beta$, and H16$_\alpha$ of estradiol), 1.95−1.72 (m, 5H, H7$_\beta$, H8$_\beta$, H11$_\beta$, H15$_\alpha$, and H16$_\beta$ of estradiol), 1.54−1.21 (m, 4H, H7$_\alpha$, H12$_\alpha$, H14$_\beta$, and H15$_\beta$ of estradiol), 0.95 (s, 3H, CH_3 of estradiol). IR (KBr) (ν/cm^{-1}): 3418 (m, O−H), 2207 (w, C≡C), 838 (s, P−F). Positive-ion ESI-MS ion cluster: m/z 1085, $\{M - PF_6^-\}^+$, 470, $\{M - 2PF_6^-\}^{2+}$. Anal. Calcd for $C_{56}H_{50}N_6O_2F_{12}P_2Ru$·2H$_2$O: C, 53.13; H, 4.30; N 6.64. Found: C, 53.38; H, 4.29; N, 6.77.

[Ru(bpy)$_2$(mbpy-C6-est)](PF$_6$)$_2$. The procedure was similar to that described for the preparation of complex [Ru(bpy)$_2$(bpy-ph-est)](PF$_6$)$_2$, except that mbpy-C6-est (84 mg, 0.12 mmol) was used instead of bpy-ph-est. Recrystallization of the crude product from acetone/diethyl ether afforded the target complex as red crystals. Yield = 57 mg (41%). ^{1}H NMR (300 MHz, acetone-d_6, 298 K, TMS): δ = 8.84−8.74 (m, 5H, H3 of pyridyl ring of mbpy-C6-est and H3 and H3′ of bpy), 8.68 (s, 1H, H3′ of pyridyl ring of mbpy-C6-est), 8.26−8.13 (m, 5H, 3-OH of estradiol and H4 and H4′ of bpy), 8.10−8.01 (m, 4H, H6 and H6′ of bpy), 7.96−7.80 (m, 4H, H6 and H6′ of pyridyl rings of mbpy-C6-est and H2 and H6 of phenyl ring of mbpy-C6-est), 7.64−7.36 (m, 9H, bpy-4-CH_2NH, H5 and H5′ of pyridyl rings of bpy and mbpy-C6-est, and H3 and H5 of phenyl ring of mbpy-C6-est), 7.12 (d, 1H, J =

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8.6 Hz, H1 of estradiol), 6.66−6.51 (m, 2H, H2 and H4 of estradiol), 4.53 (s, 1H, 17-OH of estradiol), 4.02 (s, 2H, bpy-4-CH_2NH), 3.49−3.31 (m, 2H, bpy-4-$CH_2NHC_5H_{10}CH_2$), 2.95−2.85 (m, 2H, H6 of estradiol), 2.78 (br, 1H, bpy-4-CH_2NH), 2.65−2.51 (m, 5H, bpy-4-CH_2NHCH_2 and CH_3 on C4′ of pyridyl ring of mbpy-C6-est), 2.41−2.21 (m, 4H, H9$_\alpha$, H11$_\alpha$, H12$_\beta$, and H16$_\alpha$ of estradiol), 1.94−1.74 (m, 5H, H7$_\beta$, H8$_\beta$, H11$_\beta$, H15$_\alpha$, and H16$_\beta$ of estradiol), 1.68−1.16 (m, 12H, H7$_\alpha$, H12$_\alpha$, H14$_\beta$, and H15$_\beta$ of estradiol and bpy-4-$CH_2NHCH_2C_4H_8$), 0.96 (s, 3H, CH_3 of estradiol). IR (KBr) (ν/cm^{-1}): 3428 (br, O−H and N−H), 2208 (w, C≡C), 1673 (s, C═O), 836 (s, P−F). Positive-ion ESI-MS ion cluster: *m*/*z* 1255, {M − PF_6^-}$^+$, 555, {M − 2PF_6^-}$^{2+}$. Anal. Calcd for $C_{65}H_{68}N_8O_3F_{12}P_2Ru\cdot H_2O$: C, 55.05; H, 4.97; N, 7.90. Found: C, 55.14; H, 5.12; N, 7.70.

[Ru(Ph$_2$-phen)$_2$(bpy-ph-est)](PF$_6$)$_2$. The procedure was similar to that described for the preparation of complex [Ru(bpy)$_2$(bpy-ph-est)](PF$_6$)$_2$, except that *cis*-[Ru(Ph$_2$-phen)$_2$Cl$_2$]·2H$_2$O (87 mg, 0.10 mmol) was used instead of *cis*-[Ru(bpy)$_2$Cl$_2$]·2H$_2$O. Recrystallization of the crude product from acetone/diethyl ether afforded the target complex as red crystals. Yield = 52 mg (33%). ^{1}H NMR (300 MHz, acetone-d_6, 298 K, TMS): δ = 9.03−8.90 (m, 2H, H3 and H3′ of pyridyl rings of bpy-ph-est), 8.84 (d, 1H, J = 5.1 Hz, H2 of Ph$_2$-phen), 8.71 (d, 1H, J = 5.6 Hz, H2 of Ph$_2$-phen), 8.63 (d, 1H, J = 5.6 Hz, H9 of Ph$_2$-phen), 8.59−8.47 (m, 2H, H9 of Ph$_2$-phen and H4 of pyridyl ring of bpy-ph-est), 8.38−8.12 (m, 9H, H5 and H6 of Ph$_2$-phen, H3 and H5 of phenyl ring of bpy-ph-est, and H4′, H6, and H6′ of pyridyl rings of bpy-ph-est), 8.03 (s, 1H, 3-OH of estradiol), 7.97 (d, 1H, J = 5.6 Hz, H3 of Ph$_2$-phen), 7.94 (d, 1H, J = 5.1 Hz, H3 of Ph$_2$-phen), 7.79 (d, 1H, J = 5.9 Hz, H8 of Ph$_2$-phen), 7.76 (d, 1H, J = 5.1 Hz, H8 of Ph$_2$-phen), 7.71−7.46 (m, 23H, phenyl rings of Ph$_2$-phen, H2 and H6 of phenyl ring of bpy-ph-est, and H5′ of pyridyl ring of bpy-ph-est), 7.11 (d, 1H, J = 8.5 Hz, H1 of estradiol), 6.62 (dd, 1H, J = 8.5 and 5.5 Hz, H2 of estradiol), 6.56 (s, 1H, H4 of estradiol), 4.59 (s, 1H, 17-OH of estradiol), 2.82−2.73 (m, 2H, H6 of estradiol), 2.41−2.21 (m, 4H, H9$_\alpha$, H11$_\alpha$, H12$_\beta$, and H16$_\alpha$ of estradiol), 2.03−1.68 (m, 5H, H7$_\beta$, H8$_\beta$, H11$_\beta$, H15$_\alpha$, and H16$_\beta$ of estradiol), 1.58−1.18 (m, 4H, H7$_\alpha$, H12$_\alpha$, H14$_\beta$, and H15$_\beta$ of estradiol), 0.93 (s, 3H, CH_3 of estradiol). IR (KBr) (ν/cm^{-1}): 3421 (m, O−H), 2213 (w, C≡C), 831 (s, P−F). Positive-ion ESI-MS ion cluster: *m*/*z* 1437, {M − PF_6^-}$^+$, 646, {M − 2PF_6^-}$^{2+}$. Anal. Calcd for $C_{84}H_{66}N_6O_2F_{12}P_2Ru$: C, 63.76; H, 4.20; N, 5.31. Found: C, 63.64; H, 4.02; N, 5.60.

[Ru(Ph$_2$-phen)$_2$(mbpy-C6-est)](PF$_6$)$_2$. The procedure was similar to that described for the preparation of complex [Ru(bpy)$_2$(mbpy-C6-est)](PF$_6$)$_2$, except that *cis*-[Ru(Ph$_2$-phen)$_2$Cl$_2$]·2H$_2$O (87 mg, 0.10 mmol) was used instead of *cis*-[Ru(bpy)$_2$Cl$_2$]·2H$_2$O. Recrystallization of the crude product from acetone/diethyl ether afforded the complex as red crystals. Yield = 53 mg (30%). ^{1}H NMR (300 MHz, acetone-d_6, 298 K, TMS): δ = 9.32 (s, 1H, H3 of pyridyl ring of mbpy-C6-est), 8.91 (s, 1H, H3′ of pyridyl ring of mbpy-C6-est), 8.77−8.58 (m, 2H, H2 of Ph$_2$-phen), 8.54−8.41 (m, 2H, H9 of Ph$_2$-phen), 8.31 (s, 4H, H5 and H6 of Ph$_2$-phen), 8.16 (s, 1H, 3-OH of estradiol), 8.06−7.82 (m, 4H, H2 and H6 of phenyl ring and H6 and H6′ of pyridyl rings of mbpy-C6-est), 7.81−7.19 (m, 29H, phenyl rings of Ph$_2$-phen, H3 and H5 of phenyl ring of mbpy-C6-est, H5 and H5′ of pyridyl rings of mbpy-C6-est, and H3 and H8 of Ph$_2$-phen), 7.11 (d, 1H, J = 8.3 Hz, H1 of estradiol), 6.61−6.48 (m, 2H, H2 and H4 of estradiol), 4.55 (s, 1H, 17-OH of estradiol), 3.51−3.35 (m, 4H, bpy-4-CH_2NH and bpy-4-CH_2-$NHC_5H_{10}CH_2$), 3.01−2.93 (m, 2H, H6 of estradiol), 2.86 (br, 1H, bpy-4-CH_2NH), 2.65−2.39 (m, 5H, bpy-4-CH_2NHCH_2 and CH_3 on C4′ of pyridyl ring of mbpy-C6-est), 2.38−2.21 (m, 4H, H9$_\alpha$, H11$_\alpha$, H12$_\beta$, and H16$_\alpha$ of estradiol), 1.96−1.62 (m, 5H, H7$_\beta$, H8$_\beta$, H11$_\beta$, H15$_\alpha$, and H16$_\beta$ of estradiol), 1.61−1.02 (m, 12H, H7$_\alpha$, H12$_\alpha$, H14$_\beta$, and H15$_\beta$ of estradiol and bpy-4-$CH_2NHCH_2C_4H_8$), 0.92 (s, 3H, CH_3 of estradiol). IR (KBr) (ν/cm^{-1}): 3436 (br, O−H and N−H), 2210 (w, C≡C), 1677 (s, C═O), 838 (s, P−F). Positive-ion ESI-MS ion cluster: *m*/*z* 1607, {M − PF_6^-}$^+$, 731, {M − 2PF_6^-}$^{2+}$. Anal. Calcd for $C_{93}H_{84}N_8O_3F_{12}P_2Ru$: C, 63.73; H, 4.83; N, 6.39. Found: C, 63.62; H, 4.54; N, 6.21.

Instrumentation and Methods. The instruments used for characterization and photophysical and electrochemical studies have been described previously.[20g] Luminescence quantum yields were measured using the optically dilute method[24] with an aerated aqueous solution of [Ru(bpy)$_3$]Cl$_2$ (Φ_{em} = 0.028, λ_{ex} = 455 nm)[25] as the standard solution.

Determination of Lipophilicity. The lipophilicity of the complexes, which is referred to as log $P_{o/w}$ ($P_{o/w}$ = *n*-octan-1-ol/water partition coefficient), was determined from the log k'_w values (k'_w = chromatographic capacity factor at 100% aqueous solution). Detailed procedures for the determination of lipophilicity have been described previously.[18]

Emission Titrations. Aliquots (25 μL) of an ERα solution (6.4 μM) in 50 mM potassium phosphate buffer at pH 7.4 at 298 K were added to the ruthenium(II) estradiol complex or estradiol-free complex [Ru(N^N)$_2$(bpy)](PF$_6$)$_2$ (N^N = bpy, Ph$_2$-phen) (0.92 μM) in 2 mL of 50 mM potassium phosphate buffer at pH 7.4/DMSO (8:2, v/v) at 1-min intervals. The emission spectrum of the solution was measured after each addition.

The Hill equation was used to determine the binding parameters (K_a) of the complexes to ERα:[26]

$$\log\left(\frac{Y}{1-Y}\right) = n_H \log [ER\alpha] + n_H \log K_a$$

Here $Y = (I_{obs} - I_{min})/(I_{max} - I_{min})$, I_{obs}, I_{min}, and I_{max} are the emission intensities of the apparent, free, and bound forms of the ruthenium(II) complex, respectively, and n_H and K_a are the Hill coefficient and binding constant, respectively.

Cytotoxicity Assays.[27] HeLa cells were seeded in a 96-well flat-bottomed microplate (10 000 cells/well) in growth medium (100 μL) and incubated at 37 °C under a 5% CO_2 atmosphere for 24 h. The ruthenium(II) estradiol complexes and cisplatin (positive control) were then added to the wells with concentrations ranging from 10^{-6} to 10^{-4} M in a mixture of growth medium/DMSO (99:1). Wells containing growth medium without cells were used as blank controls. The microplate was incubated at 37 °C under a 5% CO_2 atmosphere for 48 h. Then, 10 μL of MTT in PBS (5 mg mL^{-1}) was added to each well. The microplate was incubated at 37 °C under a 5% CO_2 atmosphere for another 3 h. Solubilization solution (100 μL) containing 10% SDS in 2-propanol/0.04 M hydrochloric acid (1:1, v/v) was then added to each well, and the microplate was further incubated for 24 h. The absorbance of the solutions at 570 nm was measured with a SPECTRAmax 340 microplate reader (Molecular Devices Corp., Sunnyvale, CA). The IC_{50} values of the complexes were determined from dose dependence of surviving cells after exposure to the complexes for 48 h.

Flow Cytometry. HeLa cells in growth medium (100 000 cells mL^{-1}) were seeded in a 35-mm tissue culture dish and incubated at 37 °C under a 5% CO_2 atmosphere for 48 h. The culture medium was removed and replaced with medium/DMSO (99:1, v/v)

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Table 1. Electronic Absorption Spectral Data of the Ruthenium Estradiol Complexes at 298 K

complex	solvent	λ_{abs}/nm (ϵ/dm^3 mol^{-1} cm^{-1})
$[Ru(bpy)_2(bpy\text{-}ph\text{-}est)](PF_6)_2$	CH_2Cl_2	256 (33 520), 288 (80 435), 325 (41 665), 422 sh (10 815), 456 (14 710)
	CH_3CN	254 (34 675), 288 (81 095), 325 (49 315), 425 sh (11 985), 454 (15 010)
$[Ru(bpy)_2(mbpy\text{-}C6\text{-}est)](PF_6)_2$	CH_2Cl_2	257 (36 560), 287 (74 320), 327 sh (9220), 358 sh (5250), 398 sh (5120), 423 sh (9470), 456 (12 115)
	CH_3CN	256 (37 780), 286 (69 680), 326 sh (10 010), 360 sh (5960), 397 sh (5925), 424 sh (10 225), 454 (12 565)
$[Ru(Ph_2\text{-}phen)_2(bpy\text{-}ph\text{-}est)](PF_6)_2$	CH_2Cl_2	279 (97 005), 317 sh (46 560), 344 sh (32 600), 433 sh (20 110), 463 sh (21 820)
	CH_3CN	278 (96 365), 318 sh (46 720), 344 sh (30 325), 436 sh (20 700), 462 sh (21 895)
$[Ru(Ph_2\text{-}phen)_2(mbpy\text{-}C6\text{-}est)](PF_6)_2$	CH_2Cl_2	280 (117 725), 327 sh (21 075), 434 sh (23 575), 465 (24 320)
	CH_3CN	279 (112 320), 326 sh (19 500), 439 sh (23 390), 467 (22 905)

containing the ruthenium(II) estradiol complexes at a concentration of 5 μM. After incubation for 24 h, the medium was removed and the cell layer was washed gently with PBS (1 mL × 3). The cell layer was then trypsinized and added up to a final volume of 3 mL with PBS. The samples were analyzed by a FACSCalibur flow cytometer (Becton, Dickinson and Co., Franklin Lakes, NJ). The cell samples were excited with an argon laser at 488 nm, and the emission was monitored at 585 ± 21 nm. The number of cells analyzed for each sample was between ca. 9000 and 10 000.

Live-Cell Confocal Imaging. HeLa cells were grown on sterile glass coverslips in a 35-mm tissue culture dish. The sample preparation procedure was similar to that of the flow cytometry. After washing with PBS, the coverslips were mounted onto slides for measurements. Imaging was performed using a confocal microscope (Carl Zeiss, LSM510) with an excitation wavelength at 488 nm. The emission was measured using a long-pass filter at 505 nm.

Results and Discussion

Synthesis. 17α-Ethynylestradiol was chosen as the starting material because the rigid ethynyl group at position 17α of estradiol directs the substituent away from the 17β-hydroxyl group without conformational flexibility and thus allows the probe to have strong binding affinity to ERs.[13b,c] The diimine ligands bpy-ph-est and mbpy-C6-est were prepared from Sonogashira coupling of 17α-ethynylestradiol with the aryl halides 5-(4-bromophenyl)-2,2′-bipyridine and 4-(*N*-(6-(4-iodobenzoylamino)hexyl)aminomethyl)-4′-methyl-2,2′-bipyridine, respectively, in diethylamine in the presence of a palladium(II) catalyst and a copper(I) cocatalyst. The ruthenium(II) polypyridine estradiol complexes were obtained from the reactions of *cis*-$[Ru(N\text{^}N)_2Cl_2]\cdot 2H_2O$[22] with the corresponding bpy-estradiol ligands in refluxing aqueous ethanol, followed by anion exchange with KPF_6 and recrystallization from a mixture of acetone and diethyl ether. All the complexes were characterized by ^{1}H NMR spectroscopy, positive-ion ESI-MS, and IR spectroscopy and gave satisfactory microanalysis.

Electronic Absorption and Emission Properties. The electronic absorption spectral data of all the complexes are listed in Table 1. The electronic absorption spectra of the complexes in CH_3CN at 298 K are shown in Figure 1. All the spectra featured intense absorption bands at ca. 254−288 nm (ϵ on the order of 10^4 dm^3 mol^{-1} cm^{-1}), which have been assigned to spin-allowed intraligand (^{1}IL) ($\pi \rightarrow \pi^*$) (diimine) transitions.[20a,c,e,28−33] The bpy-ph-est complexes revealed an intense absorption band at ca. 317−325 nm (Table 1 and Figure 1), which is associated with ^{1}IL ($\pi \rightarrow \pi^*$) (bpy-ph-est) transitions because a similar absorption band has been observed in ruthenium(II) complexes containing 5-phenyl-substituted bpy ligands.[34] The moderately intense bands of all the complexes in the visible region (ca. 422−467 nm) have been attributed to spin-allowed metal-to-ligand

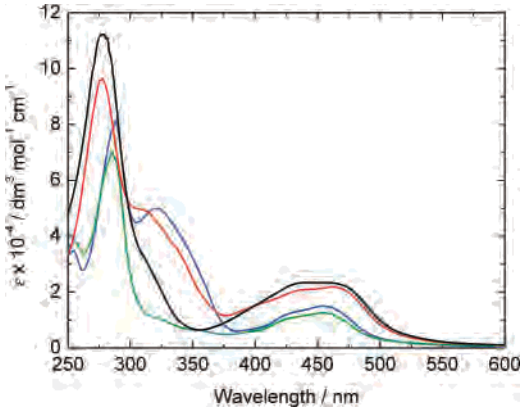

Figure 1. Electronic absorption spectra of $[Ru(bpy)_2(bpy\text{-}ph\text{-}est)](PF_6)_2$ (blue), $[Ru(bpy)_2(mbpy\text{-}C6\text{-}est)](PF_6)_2$ (green), $[Ru(Ph_2\text{-}phen)_2(bpy\text{-}ph\text{-}est)](PF_6)_2$ (red), and $[Ru(Ph_2\text{-}phen)_2(mbpy\text{-}C6\text{-}est)](PF_6)_2$ (black) in CH_3CN at 298 K.

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Table 2. Photophysical Data of the Ruthenium Estradiol Complexes

complex	medium (T/K)	λ_{em}/nm[a]	$\tau_o/\mu s$[b]	Φ[a]
$[Ru(bpy)_2(bpy\text{-}ph\text{-}est)](PF_6)_2$	CH_2Cl_2 (298)	605	1.05	0.081
	CH_3CN (298)	619	1.30	0.067
	buffer[c] (298)	622	0.81	0.051
	glass[d] (77)	590 (max), 637	5.23	
$[Ru(bpy)_2(mbpy\text{-}C6\text{-}est)](PF_6)_2$	CH_2Cl_2 (298)	603	0.96	0.070
	CH_3CN (298)	614	0.97	0.084
	buffer[c] (298)	615	0.71	0.046
	glass[d] (77)	583 (max), 627	5.01	
$[Ru(Ph_2\text{-}phen)_2(bpy\text{-}ph\text{-}est)](PF_6)_2$	CH_2Cl_2 (298)	609	2.14	0.17
	CH_3CN (298)	615	2.35	0.13
	buffer[c] (298)	620	2.11	0.074
	glass[d] (77)	590 (max), 639	7.01	
$[Ru(Ph_2\text{-}phen)_2(mbpy\text{-}C6\text{-}est)](PF_6)_2$	CH_2Cl_2 (298)	605	3.79	0.23
	CH_3CN (298)	616	5.12	0.18
	buffer[c] (298)	619	2.88	0.10
	glass[d] (77)	594 (max), 641	8.82	

[a] Excitation wavelength = 455 nm. [b] Excitation wavelength = 355 nm. [c] 30% DMSO in 50 mM potassium phosphate buffer pH 7.4. [d] EtOH/MeOH (4:1 v/v).

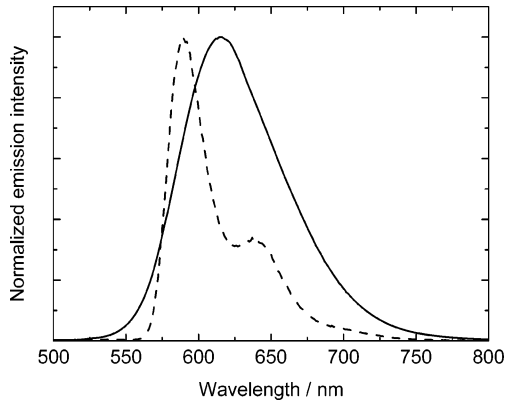

Figure 2. Emission spectra of $[Ru(bpy)_2(bpy\text{-}ph\text{-}est)](PF_6)_2$ in CH_3CN at 298 K (—) and in EtOH/MeOH (4:1, v/v) at 77 K (- - -).

charge-transfer ^{1}MLCT ($d\pi(Ru) \rightarrow \pi^*$(diimine)) transitions.[20a,c,e,28−33] The ^{1}MLCT bands of the bpy complexes occurred at higher energy than those of Ph_2-phen complexes owing to the lower lying π^* orbitals of the Ph_2-phen ligand.

Upon irradiation, all the complexes displayed intense and long-lived orange-red luminescence in fluid solutions under ambient conditions and in low-temperature alcohol glass. The photophysical data are summarized in Table 2. The emission spectra of $[Ru(bpy)_2(bpy\text{-}ph\text{-}est)](PF_6)_2$ in CH_3CN at 298 K and in alcohol glass at 77 K are shown in Figure 2. The emission maxima of all the complexes occurred at ca. 603−609 nm in CH_2Cl_2, ca. 614−619 nm in CH_3CN, and ca. 615−622 nm in aqueous buffer solution at 298 K. Upon cooling of the samples to 77 K, the emission maxima of all the complexes were blue-shifted to ca. 583−594 nm. With reference to related photophysical studies of ruthenium(II) polypyridine systems,[20a,c−e,28,30−33,35,36] the emission has been assigned to a ^{3}MLCT ($d\pi(Ru) \rightarrow \pi^*$(diimine)) excited state.

The emission energy of $[Ru(bpy)_2(bpy\text{-}ph\text{-}est)](PF_6)_2$ is slightly lower than its mbpy-C6-est counterpart $[Ru(bpy)_2(mbpy\text{-}C6\text{-}est)](PF_6)_2$ (Table 2) and $[Ru(bpy)_3]^{2+}$,[20a,c−e,28,30,35] suggesting that its emissive state possesses predominant

Table 3. Electrochemical Data of the Ruthenium Estradiol Complexes in CH_3CN (0.1 M TBAP) at 298 K (Glassy Carbon Working Electrode, Sweep Rate = 100 mV s^{-1}, All Potentials vs SCE)

complex	oxidn, $E_{1/2}$/V	redn, $E_{1/2}$ or E_c/V
$[Ru(bpy)_2(bpy\text{-}ph\text{-}est)](PF_6)_2$	+1.25[a]	−1.26, −1.49,[a] −1.66,[b] −1.79[b]
$[Ru(bpy)_2(mbpy\text{-}C6\text{-}est)](PF_6)_2$	+1.26[a]	−1.35, −1.56,[b] −1.66,[b] −1.88[b]
$[Ru(Ph_2\text{-}phen)_2(bpy\text{-}ph\text{-}est)](PF_6)_2$	+1.22[a]	−1.26, −1.43,[b] −1.64,[b] −1.79[b]
$[Ru(Ph_2\text{-}phen)_2(mbpy\text{-}C6\text{-}est)](PF_6)_2$	+1.22[a]	−1.30, −1.51,[b] −1.68,[b] −1.87[b]

[a] Quasi-reversible couples. [b] Irreversible waves.

^{3}MLCT ($d\pi(Ru) \rightarrow \pi^*$(bpy-ph-est)) character. The reason is that the π^* orbitals of bpy-ph-est are lower lying in energy than those of bpy owing to the electron-withdrawing ethynylphenyl substituent. The assignment is supported by the fact that the emission lifetime of $[Ru(bpy)_2(bpy\text{-}ph\text{-}est)](PF_6)_2$ is slightly longer than those of $[Ru(bpy)_2(mbpy\text{-}C6\text{-}est)](PF_6)_2$ (Table 2) and $[Ru(bpy)_3]^{2+}$,[30b,e−h] which is consistent with the previous finding that aryl substitutions of polypyridine ligands generally increase the emission lifetimes of ruthenium(II) polypyridine complexes.[33b,c] For the same reason, the emission lifetimes and quantum yields of the Ph_2-phen complexes are longer and higher than those of the bpy complexes (Table 2), suggesting that the Ph_2-phen ligand is significantly involved in the ^{3}MLCT emissive state of these complexes.[31,33,36] Interestingly, $[Ru(Ph_2\text{-}phen)_2(mbpy\text{-}C6\text{-}est)](PF_6)_2$ showed more intense and longer lived emission than $[Ru(Ph_2\text{-}phen)_2(bpy\text{-}ph\text{-}est)](PF_6)_2$. It is likely that the emissive state of the latter complex has mixed contributions from the Ph_2-phen and bpy-ph-est ligands because of the similar energies of their π^* orbitals. In contrast, the electron-donating methyl and aminomethyl substituents of the mbpy-C6-est ligand destabilize its π^* orbitals, resulting in a lower degree of involvement of this ligand in the emissive state of $[Ru(Ph_2\text{-}phen)_2(mbpy\text{-}C6\text{-}est)](PF_6)_2$. This is in agreement with the fact that this complex shares similar emission properties with its homoleptic counterpart $[Ru(Ph_2\text{-}phen)_3]^{2+}$.[31,33,36]

Electrochemical Properties. The electrochemical properties of the ruthenium(II) polypyridine estradiol complexes have been studied by cyclic voltammetry, and the electrochemical data are listed in Table 3. All the complexes displayed a quasi-reversible ruthenium(III/II) couple at ca. +1.22 to +1.26 V vs SCE ($i_{anodic}/i_{cathodic}$ = ca. 3.5−5.0). Since common ruthenium(III/II) couples are reversible in nature,[20e,29,30a,c−e,g,35a,d,g,37] the reduced reversibility of these

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Table 4. Lipophilicity (log $P_{o/w}$ Values) of the Ruthenium Estradiol Complexes, Estradiol, and 17α-Ethynylestradiol

compd	log $P_{o/w}$
$[Ru(bpy)_2(bpy\text{-}ph\text{-}est)](PF_6)_2$	1.33
$[Ru(bpy)_2(mbpy\text{-}C6\text{-}est)](PF_6)_2$	2.18
$[Ru(Ph_2\text{-}phen)_2(bpy\text{-}ph\text{-}est)](PF_6)_2$	7.71
$[Ru(Ph_2\text{-}phen)_2(mbpy\text{-}C6\text{-}est)](PF_6)_2$	8.58
estradiol	3.26
17α-ethynylestradiol	3.42

couples could be a consequence of the involvement of oxidation of the estradiol moiety of all the complexes and the amine group of the mbpy-C6-est complexes.[38] Also, similar irreversible waves have been observed at comparable potentials for the free ligands (ca. +1.21 V for bpy-ph-est and ca. +0.94 and +1.56 V for mbpy-C6-est). Both bpy-ph-est complexes exhibited the first reversible reduction couple at −1.26 V, which has been ascribed to the reduction of the bpy-ph-est ligand. This assignment is supported by the fact that this couple occurred at a slightly less negative potential than that of the first bpy-based reduction of $[Ru(bpy)_3]^{2+}$ (ca. −1.33 V)[30c,f,g,35a] due to the electron-withdrawing ethynylphenyl group of the bpy-ph-est ligand. The first reversible reduction couple of $[Ru(bpy)_2(mbpy\text{-}C6\text{-}est)](PF_6)_2$ at −1.35 V has been assigned to the reduction of the ancillary bpy ligand because it is more difficult to reduce the mbpy-C6-est ligand due to its electron-donating methyl and aminomethyl substituents. Owing to the lower lying π^* orbitals of Ph_2-phen compared to those of mbpy-C6-est, the first reduction couple of $[Ru(Ph_2\text{-}phen)_2(mbpy\text{-}C6\text{-}est)](PF_6)_2$ at ca. −1.30 V has been assigned to the reduction of the ancillary Ph_2-phen ligand.[30a,37]

Lipophilicity. The cellular[13d,g,39] and in vivo tissue[13a,b,d,15a,14d,40] uptake selectivity characteristics of probes and therapeutic reagents can be estimated by their lipophilicity. This is commonly referred to as the *n*-octan-1-ol/water partition coefficients (expressed in log $P_{o/w}$) of the compounds, which can be determined by reversed-phase HPLC. The log $P_{o/w}$ values of the ruthenium(II) estradiol complexes, estradiol, and 17α-ethynylestradiol are listed in Table 4. Owing to their 2+ cationic charge, the bpy complexes are less lipophilic than the natural hormone estradiol (ca. 3.26) and 17α-ethynylestradiol (ca. 3.42) (Table 4).[14d] However, the lipophilicity of the complexes can be substantially increased by incorporating more hydrophobic ligands such as Ph_2-phen, as revealed by the much larger log $P_{o/w}$ values of the Ph_2-phen complexes than those of their bpy counterparts and the neutral estrogens (Table 4). The presence of a spacer arm in the mbpy-C6-est complexes increased the log $P_{o/w}$ values by ca. 0.9. The high lipophilicity of the Ph_2-phen complexes is anticipated to facilitate the tissue and cellular uptake of these complexes.

(38) Cyclic voltammetric analysis of an equimolar solution of $[Ru(Ph_2\text{-}phen)_2(bpy\text{-}estradiol)](PF_6)_2$ and ferrocene revealed that the anodic current of the complexes at ca. +1.22 V vs SCE was ca. 1.6−1.7 times that of the ferrocene oxidation. This indicates that the wave is associated with the transfer of more than one electron, reflecting the possible involvement of the oxidation of the estradiol and amine moieties in addition to the ruthenium(III/II) oxidation.

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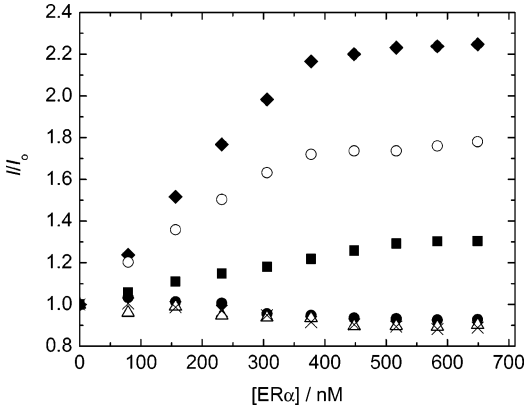

Figure 3. Emission titration curves for the titrations of $[Ru(bpy)_2(bpy\text{-}ph\text{-}est)](PF_6)_2$ (solid circles), $[Ru(bpy)_2(mbpy\text{-}C6\text{-}est)](PF_6)_2$ (triangles), $[Ru(Ph_2\text{-}phen)_2(bpy\text{-}ph\text{-}est)](PF_6)_2$ (diamonds), $[Ru(Ph_2\text{-}phen)_2(mbpy\text{-}C6\text{-}est)](PF_6)_2$ (open circles), $[Ru(bpy)_3](PF_6)_2$ (crosses), and $[Ru(Ph_2\text{-}phen)_2(bpy)](PF_6)_2$ (squares) with ERα in 50 mM potassium phosphate buffer pH 7.4 at 298 K. I_o and I are the emission intensities of the complexes in the absence and presence of ERα, respectively.

Table 5. Relative Emission Intensities and Emission Lifetimes of the Ruthenium Estradiol Complexes, $[Ru(bpy)_3](PF_6)_2$, and $[Ru(Ph_2\text{-}phen)_2(bpy)](PF_6)_2$ in the Absence and Presence of ERα in Aerated 50 mM Potassium Phosphate Buffer pH 7.4 at 298 K

complex	I/I_o[a]	τ_o[b]/μs	τ[b]/μs
$[Ru(bpy)_2(bpy\text{-}ph\text{-}est)](PF_6)_2$	0.93	0.44	0.45
$[Ru(bpy)_2(mbpy\text{-}C6\text{-}est)](PF_6)_2$	0.90	0.40	0.37
$[Ru(Ph_2\text{-}phen)_2(bpy\text{-}ph\text{-}est)](PF_6)_2$	2.25	0.74	1.96
$[Ru(Ph_2\text{-}phen)_2(mbpy\text{-}C6\text{-}est)](PF_6)_2$	1.78	0.72	1.10
$[Ru(bpy)_3](PF_6)_2$	0.89	0.37	0.36
$[Ru(Ph_2\text{-}phen)_2(bpy)](PF_6)_2$	1.30	0.71	0.92

[a] I_o and I are the emission intensities of the complexes in the absence and presence of ERα, respectively. [b] τ_o and τ are the emission lifetimes of the complexes in the absence and presence of ERα, respectively.

Emission Titrations. The ERα-binding properties of the ruthenium(II) polypyridine estradiol complexes have been investigated by emission titrations. The emission titration curves are shown in Figure 3. While the bpy complexes did not show significant changes, the emission intensities of the Ph_2-phen complexes were increased by ca. 2.3- and 1.8-fold in the presence of ERα, and their lifetimes were also elongated (Table 5). The emission spectra of $[Ru(Ph_2\text{-}phen)_2(bpy\text{-}ph\text{-}est)](PF_6)_2$ in the absence and presence of ERα are displayed in Figure 4. Interestingly, the control complex $[Ru(Ph_2\text{-}phen)_2(bpy)](PF_6)_2$ also revealed emission enhancement and lifetime elongation in the presence of ERα but the enhancement factors are relatively small (ca. 1.3). The higher amplification factors of the Ph_2-phen complexes have been attributed to the specific binding of the estradiol moieties of these complexes to ERα because similar changes were not observed when unmodified estradiol was present from the outset. The ERα-induced emission enhancement is a consequence of the increase in hydrophobicity and rigidity of the local environment of the metal complexes upon the binding event.[18,19] Unfortunately, the bpy complexes, similar to the estradiol-free complex $[Ru(bpy)_3](PF_6)_2$, did not show noticeable emission changes in the presence of ERα. This is probably due to the fact that the photophysical properties of ruthenium(II) bipyridine complexes are much less sensitive to different media compared to their substituted phenanthro-

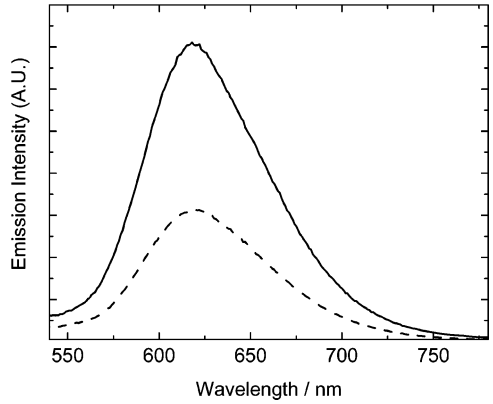

Figure 4. Emission spectra of $[Ru(Ph_2\text{-}phen)_2(bpy\text{-}ph\text{-}est)](PF_6)_2$ in the absence (- - -) and presence (—) of ERα in aerated 50 mM potassium phosphate buffer pH 7.4 at 298 K.

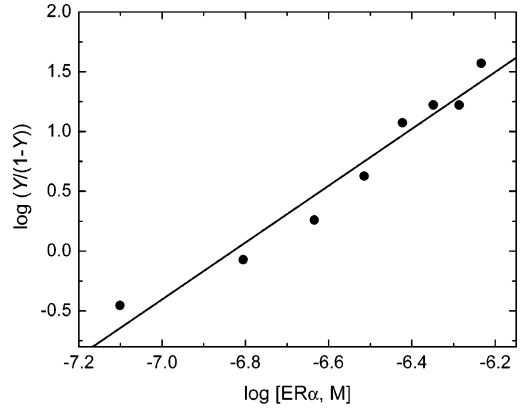

Figure 5. Hill plot for the binding of $[Ru(Ph_2\text{-}phen)_2(mbpy\text{-}C6\text{-}est)](PF_6)_2$ to ERα.

line counterparts.[20g−i,35e,41] From the titration data, the binding constants (K_a) of $[Ru(Ph_2\text{-}phen)_2(bpy\text{-}ph\text{-}est)](PF_6)_2$ and $[Ru(Ph_2\text{-}phen)_2(mbpy\text{-}C6\text{-}est)](PF_6)_2$ to ERα have been determined to be ca. 6.3×10^6 and 6.8×10^6 M^{-1}, respectively.[26] The Hill plot for $[Ru(Ph_2\text{-}phen)_2(mbpy\text{-}C6\text{-}est)](PF_6)_2$ is shown in Figure 5. The binding constants of the complexes are smaller than that of unmodified estradiol ($K_a = 5 \times 10^9$ M^{-1})[16a] but are similar to common organometallic estradiol complexes such as 17α-[(L)Re(CO)$_3$]-estradiol (L = 4′,4′-bis(ethanethio)-4′-carboxybutyn-1′-yl, 6′,6′-bis(ethanethio)-6′-carboxyhexyn-1′-yl; $K_a = 1.3 \times 10^7$ and 1.1×10^7 M^{-1}, respectively),[14f] 17α-[(C≡CCH$_2$N(CH$_3$)C$_2$H$_4$N(CH$_3$)$_2$)Pt(X)]-estradiol (X = diiodide, malonato; $K_a = 1.0 \times 10^7$ and 2.5×10^6 M^{-1}, respectively),[42] $[Re(N^N)(CO)_3(L)](CF_3SO_3)$ (N^N = diimines; L = pyridine-estradiol; $K_a = 1.5$ to 2.0×10^7 M^{-1}),[18] and $[Ir(N^C)_2(N^N)](PF_6)$ (N^C$^-$ = cyclometalating ligands; N^N = diimine-estradiol; $K_a = 1.0$ to 2.1×10^7 M^{-1}).[19] The Hill coefficients (n_H) of both $[Ru(Ph_2\text{-}phen)_2(bpy\text{-}ph\text{-}est)](PF_6)_2$ and $[Ru(Ph_2\text{-}phen)_2(mbpy\text{-}C6\text{-}est)](PF_6)_2$ (3.2 and 2.4, respectively) are >1, suggestive of cooperative binding.[16d,e,43]

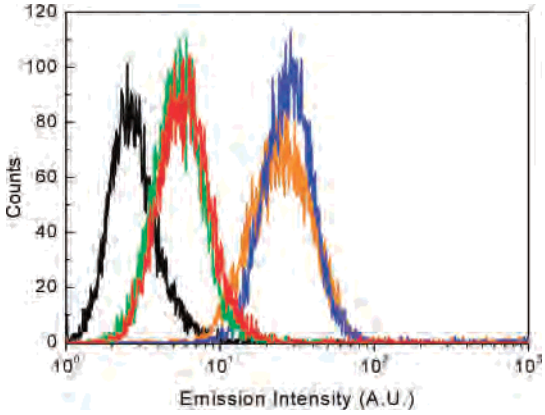

Figure 6. Results of flow cytometry of HeLa cells incubated with blank medium (black), $[Ru(bpy)_2(bpy\text{-}ph\text{-}est)](PF_6)_2$ (red), $[Ru(bpy)_2(mbpy\text{-}C6\text{-}est)](PF_6)_2$ (green), $[Ru(Ph_2\text{-}phen)_2(bpy\text{-}ph\text{-}est)](PF_6)_2$ (blue), and $[Ru(Ph_2\text{-}phen)_2(mbpy\text{-}C6\text{-}est)](PF_6)_2$ (orange) (5 μM) for 24 h.

Table 6. Cytotoxicity (IC_{50}, 48 h) of the Ruthenium Estradiol Complexes and Cisplatin toward the HeLa Cell Line

complex	$IC_{50}/\mu M$
$[Ru(bpy)_2(bpy\text{-}ph\text{-}est)](PF_6)_2$	133.4 ± 2.1
$[Ru(bpy)_2(mbpy\text{-}C6\text{-}est)](PF_6)_2$	166.6 ± 4.5
$[Ru(Ph_2\text{-}phen)_2(bpy\text{-}ph\text{-}est)](PF_6)_2$	141.6 ± 4.0
$[Ru(Ph_2\text{-}phen)_2(mbpy\text{-}C6\text{-}est)](PF_6)_2$	83.1 ± 2.2
cisplatin	34.3 ± 2.9

Cytotoxicity and Cellular Uptake Studies. The cytotoxicity of the ruthenium(II) estradiol complexes has been studied by the MTT assay using HeLa cells as the model cell line.[27] The IC_{50} values have been determined from the dose dependence of surviving cells after exposure to the complexes for 48 h. The IC_{50} values of the ruthenium(II) complexes ranged from 83.1 to 166.6 μM (Table 6), which are substantially larger than that of cisplatin (34.3 μM) under the same experimental conditions. The cytotoxicity of these complexes is also lower than that of $[Ru(^tBu_2\text{-}bpy)_2(2\text{-}appt)]^{2+}$ ($^tBu_2$-bpy = 4,4′-di-*tert*-butyl-2,2′-bipyridine, 2-appt = 2-amino-4-(phenylamino)-6-(2-pyridyl)-1,3,5-triazine) (59.7 μM), which has been identified to be a double-stranded DNA groove-binder.[44] In general, these complexes are much less cytotoxic compared to the organometallic ruthenium arene complexes $[(\eta^6\text{-arene})Ru(ethylenediamine)(X)](PF_6)_n$ (X = substituted pyridines and halides), some of which exhibit fast hydrolysis kinetics and high cytotoxicity toward the human ovarian cancer cell line A2780.[45] Since our results indicate that the ruthenium(II) estradiol complexes are relatively noncytotoxic, they are promising candidates as luminescent probes for live-cell imaging. The cellular uptake characteristics of the complexes have been investigated using flow cytometry and laser-scanning confocal microscopy. The results of the flow cytometric studies are shown in Figure 6. Upon excitation at 488 nm, all the cell samples incubated with the ruthenium(II) estradiol complexes displayed higher emission intensities compared to the autofluorescence of

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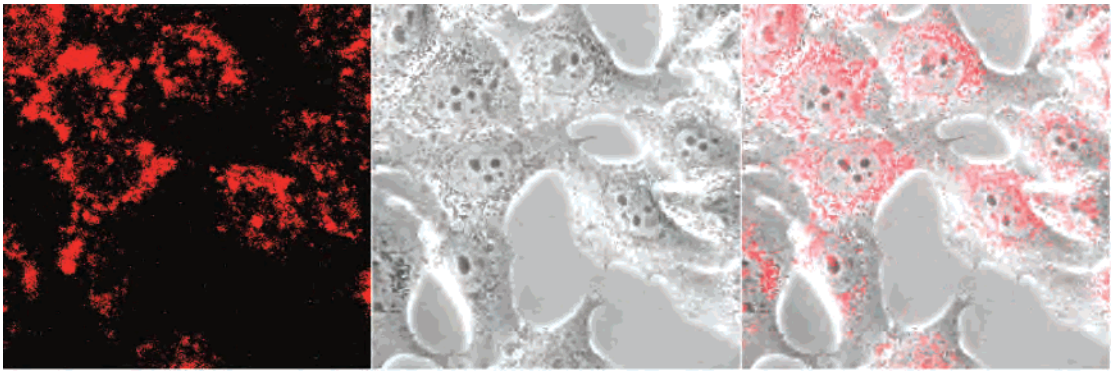

Figure 7. Fluorescence (left), brightfield (middle), and overlaid (right) images of HeLa cells incubated with $[Ru(Ph_2\text{-phen})_2(mbpy\text{-}C6\text{-}est)](PF_6)_2$ (5 μM) at 37 °C for 24 h.

untreated HeLa cells, reflecting the efficient internalization of the complexes by the cells. The emission intensities of the cells treated with the Ph_2-phen complexes are higher than those treated with the bpy complexes, which is in accordance with the relative emission quantum yields of the free complexes (Table 2). It is conceivable that the efficient internalization of the complexes by the cells is assisted by their high lipophilicity, especially in the cases of the Ph_2-phen complexes (Table 4).[39]

The possibility of the ruthenium(II) estradiol complexes as luminescent probes for live-cell imaging has been examined using $[Ru(Ph_2\text{-phen})_2(mbpy\text{-}C6\text{-}est)](PF_6)_2$ as an example. Incubation of HeLa cells with the complex at 37 °C under a 5% CO_2 atmosphere for 24 h led to efficient interiorization of the complex as observed by laser-scanning confocal microscopy with an excitation wavelength at 488 nm (Figure 7). It is interesting to note that most of the complex molecules were distributed inside the cytoplasm with a lower extent of nuclear uptake, as revealed by the much weaker luminescence intensity of the nucleus.[46] Importantly, a higher degree of localization of the complexes in the perinuclear region suggests that the complex molecules interact with hydrophobic organelles such as endoplasmic reticulum and Golgi apparatus. No interiorization was observed when the cells were incubated at 4 °C, implying that the uptake of the complex and its subsequent localization are due to energy-requiring processes such as endocytosis.[47] Investigations on the detailed internalization mechanism are underway.

Conclusions

Four ruthenium(II) polypyridine estradiol complexes have been designed as luminescent biological probes. The photophysical and electrochemical properties and lipophilicity of these complexes have been examined. The highly lipophilic Ph_2-phen estradiol complexes revealed enhanced emission intensities and extended lifetimes upon binding to ERα, rendering these complexes new homogeneous probes for the receptor. The cytotoxicity of all the complexes toward HeLa cells was relatively low compared to cisplatin. Importantly, flow cytometry and live-cell confocal imaging studies showed that these complexes were readily interiorized by HeLa cells and the emission of the complexes was maintained after the uptake. All these findings indicate that these complexes are very promising candidates as live-cell imaging reagents that could contribute to the understanding of cellular uptake of transition metal complexes.

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(46) A possible reason for the minimal nuclear uptake is that HeLa cells are ER-negative in nature. We expect that the use of cell lines such as ER-positive MCF-7 would lead to more significant interiorization of the complexes in the nucleus. Related work on other cell lines is in progress.

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