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Modification of Luminescent Iridium(III) Polypyridine Complexes with Discrete Poly(ethylene glycol) (PEG) Pendants: Synthesis, Emissive Behavior, Intracellular Uptake, and PEGylation Properties

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Abstract: We report the synthesis, characterization, and photophysical properties of a new class of luminescent cyclometalated iridium(III) polypyridine poly(ethylene glycol) (PEG) complexes $[Ir(N^C)_2(N^N)](PF_6)$ (HN^C=Hppy (2-phenylpyridine), N^N=bpy–CONH–PEG1 (bpy=2,2′-bipyridine; **1a**), bpy–CONH–PEG3 (**1b**); HN^C=Hpq (2-phenylquinoline), N^N=bpy–CONH–PEG1 (**2a**), bpy–CONH–PEG3 (**2b**); HN^C=Hpba (4-(2-pyridyl)benzaldehyde), N^N=bpy–CONH–PEG1 (**3**)) and their PEG-free counterparts (N^N=bpy–CONH–Et, HN^C=Hppy (**1c**); HN^C=Hpq (**2c**)). The cytotoxicity and cellular uptake of these complexes have been investigated by the MTT assay, ICPMS, laser-scanning confocal microscopy, and flow cytometry. The results showed that the complexes supported by the water-soluble PEG can act as biological probes and labels with considerably reduced cytotoxicity. Because the aldehyde groups of complex **3** are reactive toward primary amines, the complex has been utilized as the first luminescent PEGylation reagent. Bovine serum albumin (BSA) and poly(ethyleneimine) (PEI) have been PEGylated with this complex, and the resulting conjugates have been isolated, purified, and their photophysical properties studied. The DNA-binding and gene-delivery properties of the luminescent PEI conjugate **3**-PEI have also been investigated.

Keywords: DNA · iridium · luminescence · PEGylation probes

Introduction

PEGylation is the process of covalent modification of proteins, peptides, antibody fragments, and drug molecules with poly(ethylene glycol) (PEG). Because this derivatization procedure can significantly reduce the toxicity of the molecules without sacrificing their specific biological or therapeutic properties, PEGylation reagents with 1) a wide range of molecular weights (MWs), 2) various shapes such as linear and branched structures, and 3) different reactive functional groups for modification have been developed.[1] Many of these compounds are already commercially available. Those with fluorescence properties are particularly useful because they not only confer the aforementioned properties but also enable detection and quantization of the targets by fluorescence methods.[2] In addition to the work on the modification of cisplatin with PEG and the incorporation of related anticancer drugs into micelles composed of PEG,[3] there is an emerging interest in the use of transition-metal PEG complexes in biological studies.[4] Despite these reports, applications of luminescent transition-metal PEG complexes in biological studies are still unexplored.

The use of transition-metal complexes as cellular probes has attracted much attention recently.[5–13] In our previous bioconjugation and cellular studies of luminescent inorganic and organometallic transition-metal complexes,[14–18] we found that the solubility of the complexes in aqueous media might not be sufficiently high to fully utilize their potential in biological applications. Another observation is that most of the complexes are considerably cytotoxic to eukaryotic cell lines.[14h,i,15d,17h–m] Although this may be an advantage in the development of anticancer drugs, the high cytotoxicity may limit their use as probes in live-cell imaging studies. We anticipate that the attachment of PEG pendants to the metal complexes will circumvent these problems. To the best of our knowledge, there has been no report about the development of PEGylation reagents based on luminescent transition-metal complexes.

Herein, we report the synthesis, characterization, and photophysical and biological properties of a new class of lu-

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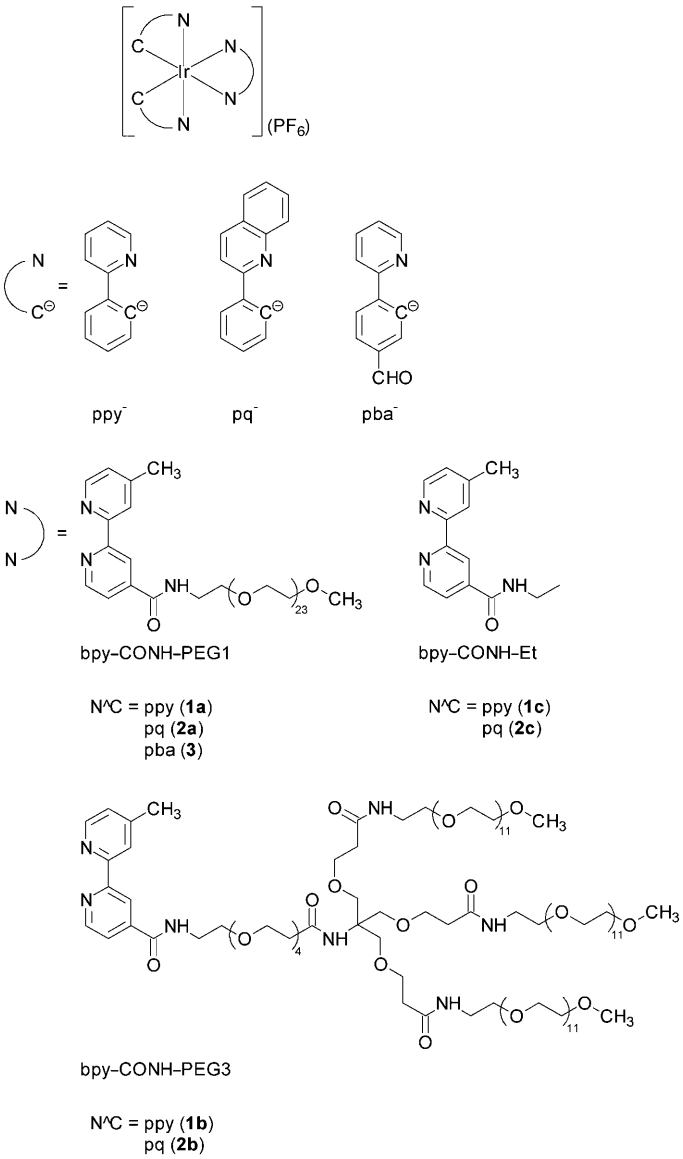
minescent cyclometalated iridium(III) polypyridine PEG complexes $[Ir(N^C)_2(N^N)](PF_6)$ (HN^C = 2-phenylpyridine (Hppy), N^N = 4-(*N*-(4,7,10,13,16,19,22,25,28,31,34,37,40,43,46,49,52,55,58,61,64,67,70,73-tetracosaoxapentaheptacontyl)aminocarbonyl)-4′-methyl-2,2′-bipyridine (bpy–CONH–PEG1; **1a**), 4-(tris(*N*-(2,5,8,11,14,17,20,23,26,29,32,35-dodecaoxaheptatriacontyl)aminocarbonyl-(2-oxabutyl))-methylamino-*N*-(3,6,9,12-tetraoxapentadecanoyl)aminocarbonyl)-4′-methyl-2,2′-bipyridine (bpy–CONH–PEG3; **1b**); HN^C = 2-phenylquinoline (Hpq), N^N = bpy–CONH–PEG1 (**2a**), bpy–CONH–PEG3 (**2b**); HN^C = 4-(2-pyridyl)benzaldehyde (Hpba), N^N = bpy–CONH–PEG1 (**3**)) and their PEG-free counterparts (N^N = 4-(*N*-ethylaminocarbonyl)-4′-methyl-2,2′-bipyridine (bpy–CONH–Et), HN^C = Hppy (**1c**); HN^C = Hpq (**2c**); Scheme 1). The cytotoxicity and cellular uptake of these iridium(III) PEG complexes have been investigated by the MTT assay, ICPMS, laser-scanning confocal microscopy, and flow cytometry. The results showed that these complexes that are supported by the water-soluble PEG can act as biological probes and labels with considerably reduced cytotoxicity. Because the aldehyde groups of complex **3** are reactive toward primary amines, this complex has been utilized as the first luminescent PEGylation reagent. Bovine serum albumin (BSA) and poly(ethyleneimine) (PEI) have been PEGylated with this complex, and the resulting conjugates have been isolated, purified, and their photophysical properties studied. The DNA-binding and gene-delivery properties of the luminescent PEI conjugate **3**-PEI have also been investigated.



Scheme 1. Structures of the iridium(III) complexes.

Results and Discussion

Synthesis: Discrete PEG units have been selected for this work because these molecules have defined chain lengths, MWs, and molecular structures, which will result in homogeneous compounds that can be readily characterized.[19] Additionally, PEGylation reagents that are functionalized with discrete PEG units are expected to offer higher consistency in bioconjugation reactions. The diimine ligands bpy–CONH–PEG1, bpy–CONH–PEG3, and bpy–CONH–Et were synthesized from the reaction of 4-(*N*-hydroxysuccinimidyl)-4′-methyl-2,2′-bipyridine (bpy–NHS) with the amines 4,7,10,13,16,19,22,25,28,31,34,37,40,43,46,49,52,55,58,61,64,67,70,73-tetracosaoxapentaheptacontane (m-$dPEG_{24}$–amine), tris((37-*N*-amido-2,5,8,11,14,17,20,23,26,29,32,35-dodecaoxaheptatriacontane)-4-oxapentanoyl)-(15-amino-4,7,10,13-tetraoxapentadecanoylamido)methane (amino–$dPEG_4$–(m-$dPEG_{12}$)$_3$), and ethylamine, respectively, in CH_2Cl_2 in the presence of triethylamine. The ^{1}H NMR spectra of the ligands revealed that the aromatic amide proton resonated at ≈7.50 ppm. All the iridium(III) complexes **1a–c**, **2a–c**, and **3** were prepared from the reaction of $[Ir_2(N^C)_4Cl_2]$ (HN^C = Hppy, Hpq, Hpba) with the diimine ligands in a mixture of CH_2Cl_2 and MeOH (1:1, v/v) under reflux conditions, followed by anion exchange with KPF_6 and purification by column chromatography. Unfortunately, the iridium(III) PEG complexes **1a**,**b**, **2a**,**b**, and **3** were hygroscopic, rendering it impossible to check the purity of the samples by elemental analysis. However, because discrete PEG molecules have been used, the PEG complexes can be readily characterized by high-resolution (HR) ESI-TOF MS and ^{1}H NMR spectroscopy. The HR mass spectra showed the relevant isotopic patterns and MWs of all the complexes, which confirmed their identities. In contrast to common cyclometalated iridium(III) polypyridine complexes, all the PEG complexes in this work are soluble in aqueous buffers. The water solubility was determined to be >0.3 M, which is sufficiently high for common biological applications.

Electronic absorption and emission properties: The electronic absorption spectral data of all the complexes are listed in Table 1. The intense absorption bands and should-

ers at 253–352 nm have been assigned to spin-allowed intraligand (^{1}IL) ($\pi\rightarrow\pi^*$) (N^N and N^C) transitions.[17,20–23,24a,25] The less intense absorption bands and shoulders at >352 nm and the weak absorption tail beyond 450 nm have been assigned to spin-allowed and spin-forbidden metal-to-ligand charge-transfer (MLCT) ($d\pi(Ir)\rightarrow\pi^*$(N^N and N^C)) transitions, respectively.[17,20–23,24a,25] Upon photoexcitation, all the complexes displayed intense and long-lived orange to greenish-yellow emission. The photophysical data are summarized in Table 2 and the emission spectra of the PEG complexes **1a**,**b**, **2a**,**b**, and **3** are shown in Figure 1. In general, the emission maxima of ppy complexes **1a–c** occurred at ≈601–625 nm in fluid solutions with a small red-shift upon increasing the polarity of the solvent. We have tentatively assigned the emission to an excited state of ^{3}MLCT ($d\pi(Ir)\rightarrow\pi^*$-(N^N)) character.[17,20a,22,24–26] The electron-withdrawing amide substituent stabilizes the π^* orbitals of the diimine ligands and renders the emission of these complexes to occur at lower energy than their unsubstituted 2,2′-bipyridine counterparts.[17d] Interestingly, the emission spectrum of complex **1a** in degassed buffer showed an unexpected high-energy (HE) band at ≈536 nm and a low-energy (LE) shoulder at ≈615 nm (Figure 1 and Table 2). For complex **1b**, this special HE emission feature appeared as a shoulder at ≈518 nm whereas the expected LE band occurred at ≈624 nm (Figure 1 and Table 2). The lifetimes of these HE features were indistinguishable from those of the LE ones. Although PEG is commonly considered as a polar molecule, its polyether nature is less polar compared with aqueous buffer. Because the MLCT emission of this type of cyclometalated iridium(III) complex occurs at higher energy in less polar solvents, it is likely that in 50 mM phosphate buffer, the long PEG pendants are in close proximity to the relatively hydrophobic complex molecules, giving rise to a comparatively nonpolar local environment and hence the HE emission features. This is in line with the appearance of a similar HE emission band for a related iridium nondiscrete PEG complex [Ir-$(ppy)_2$(bpy−CONH−PEG)](PF_6) (bpy−CONH−PEG = 4-(*N*-{2-[ω-methoxypoly(1-oxapropyl)]ethyl}aminocarbonyl)-4′-methyl-2,2′-bipyridine, MW_{PEG} = 5000 Da, PDI_{PEG} = 1.08) (λ_{em} = 539 and 615 nm (sh); τ_o = 0.30 μs; Φ_{em} = 0.0050). This argument is supported by the observation of a less intense HE band and a dominant LE emission band for complexes **1a** and **1b** in water (Figure S1 and S2 in the Supporting Information) because the degree of PEG–complex interaction should be lower in water than in more polar 50 mM phosphate buffer. Although the pq complexes **2a–c** displayed typical ^{3}MLCT ($d\pi(Ir)\rightarrow\pi^*$(N^N)) emission properties, the

Table 1. Electronic absorption spectral data of the cyclometalated iridium(III) PEG complexes at 298 K.

Complex	Solvent	λ_{abs} [nm] (ε [$dm^3mol^{-1}cm^{-1}$])
1a	CH_2Cl_2	253 (22375), 323 sh (6390), 386 sh (3140), 500 sh (470)
	CH_3CN	253 (21205), 323 sh (5360), 389 sh (2380), 472 sh (655)
1b	CH_2Cl_2	254 (24260), 321 sh (7500), 387 sh (3630), 501 sh (600)
	CH_3CN	253 (28865), 323 sh (7365), 386 sh (3220), 480 sh (475)
2a	CH_2Cl_2	260 (29505), 284 sh (25235), 350 sh (10495), 440 sh (2840), 530 sh (365)
	CH_3CN	258 (29240), 282 sh (25545), 349 sh (9865), 440 sh (2630)
2b	CH_2Cl_2	261 (38065), 285 sh (30875), 352 sh (12930), 444 sh (3950), 530 sh (500)
	CH_3CN	259 (43185), 284 sh (34780), 349 sh (14195), 440 sh (3985)
3	CH_2Cl_2	262 (35930), 302 sh (25500), 318 sh (20620), 371 sh (5670), 452 sh (2740)
	CH_3CN	264 (33330), 297 sh (23515), 317 sh (18140), 366 sh (4990), 444 sh (2475)

Table 2. Photophysical data of the cyclometalated iridium(III) complexes and the conjugates **3**-BSA and **3**-PEI.

Complex	Medium (T [K])	λ_{em} [nm]	τ_o [μs][a]	Φ_{em}
1a	CH_2Cl_2 (298)	601	0.36	0.051
	CH_3CN (298)	607	0.22	0.038
	buffer (298)[b]	536, 615 sh	0.31	0.0053
	glass (77)[c]	477, 513 sh, 549 (max)	4.87 (54%), 2.42 (46%)	–
1b	CH_2Cl_2 (298)	603	0.28	0.062
	CH_3CN (298)	611	0.20	0.038
	buffer (298)[b]	518 sh, 624	0.31	0.0023
	glass (77)[c]	489 sh, 554	4.50 (57%), 2.82 (43%)	–
1c	CH_2Cl_2 (298)	609	0.40	0.13
	CH_3CN (298)	613	0.23	0.058
	buffer (298)[d]	625	0.34	0.0023
	glass (77)[c]	541, 560 sh	4.80	–
2a	CH_2Cl_2 (298)	562, 586 sh	3.03 (20%), 0.67 (80%)	0.19
	CH_3CN (298)	562, 595 sh	5.66 (38%), 0.57 (62%)	0.10
	buffer (298)[b]	518, 545 (max), 596 sh	11.82 (61%), 3.19 (39%)	0.0031
	glass (77)[c]	505, 539 (max), 580, 631 sh	20.61 (2%), 4.56 (98%)	–
2b	CH_2Cl_2 (298)	561 sh, 583	2.79 (18%), 0.58 (82%)	0.16
	CH_3CN (298)	564, 597 sh	5.43 (22%), 0.49 (78%)	0.087
	buffer (298)[b]	517, 546 (max), 597 sh	12.70 (62%), 3.35 (38%)	0.0048
	glass (77)[c]	502, 545 (max), 583	15.84 (5%), 3.79 (95%)	–
2c	CH_2Cl_2 (298)	595	0.67	0.18
	CH_3CN (298)	587	0.54	0.14
	buffer (298)[d]	574, 596 sh	1.63 (8%), 0.17 (92%)	0.013
	glass (77)[c]	543 (max), 585	4.69	–
3	CH_2Cl_2 (298)	532, 566 sh	3.82	0.26
	CH_3CN (298)	536, 566 sh	2.84	0.20
	glass (77)[c]	521 (max), 567, 615 sh	6.99	–
3-BSA	buffer (298)[b]	490 (max), 520	2.61	0.069
3-PEI	buffer (298)[b]	504, 525 sh	2.45 (56%), 0.87 (44%)	0.074

[a] The lifetimes were measured at the emission maxima. [b] Potassium phosphate buffer (50 mM, pH 7.4). [c] EtOH/MeOH (4:1, v/v). [d] Potassium phosphate buffer (50 mM, pH 7.4) containing 10% methanol.

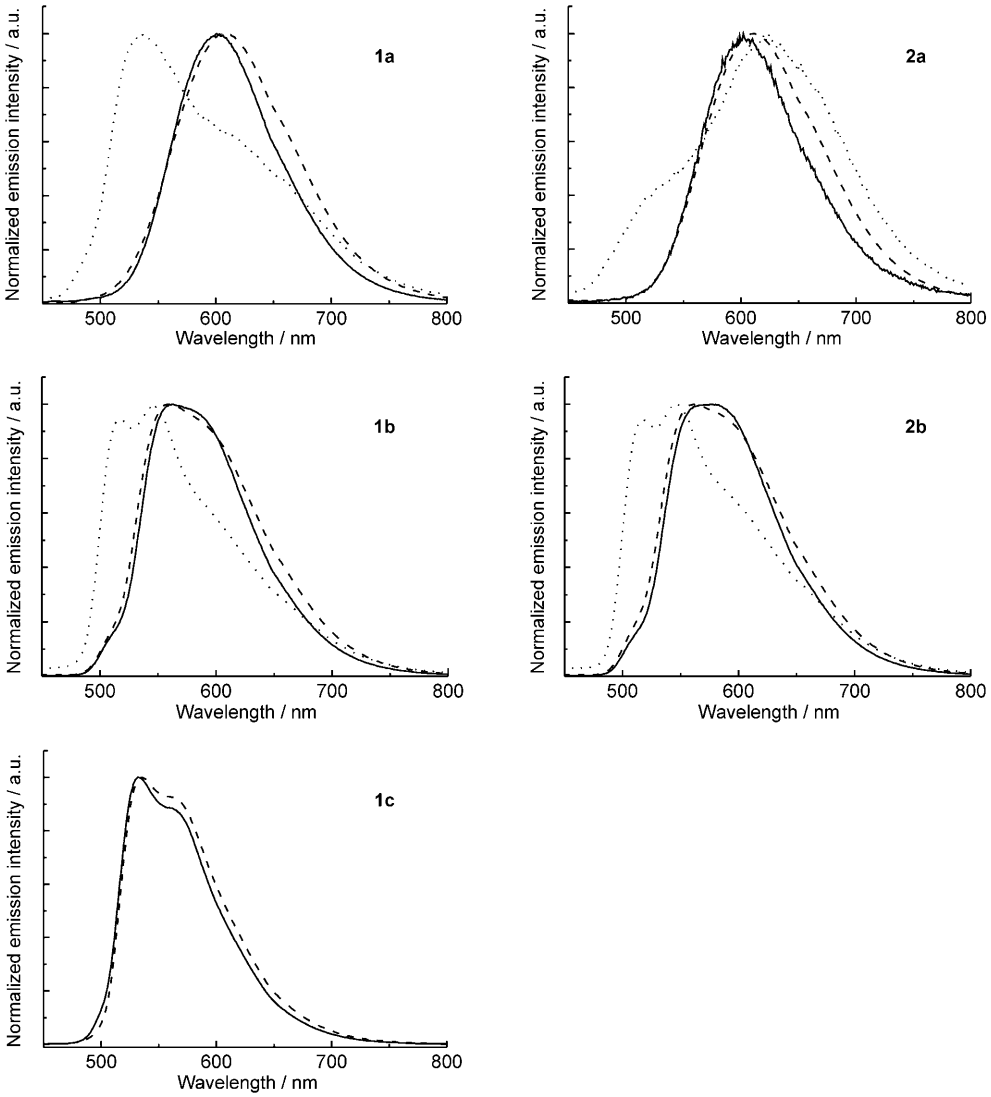


Figure 1. Emission spectra of complexes **1a**,**b**, **2a**,**b**, and **3** in degassed CH_2Cl_2 (——), CH_3CN (– – –), and potassium phosphate buffer (•••••) at 298 K.

PEG complexes **2a** and **2b** showed biphasic emission decay with lifetimes ($\tau_o \approx 3$–6 and 0.5–0.7 μs), which are noticeably longer than those of their ppy counterparts (Table 2). We have tentatively assigned the long- and short-lived components to excited states of predominant ^{3}IL ($\pi \rightarrow \pi^*$) (pq) and ^{3}MLCT ($d\pi(Ir) \rightarrow \pi^*$(N^N or pq)) character, respectively.[17c,d,f,h,i,k,m] Again, both complexes **2a** and **2b** exhibited a HE emission feature with rich vibronic structures at ≈518 nm in aqueous phosphate buffer. Lifetime measurements also revealed biphasic decay with longer lifetimes (τ_o ≈12–13 and 3–5 μs). Very similar emission spectra and lifetimes are observed for a related nondiscrete PEG complex $[Ir(pq)_2(bpy{-}CONH{-}PEG)](PF_6)$ ($\lambda_{em} = 518$ (sh), 550 and 596 nm (sh); $\tau_o = 12.36$ (37%) and 1.46 (63%) μs; $\Phi_{em} = 0.0045$). On the basis of the very long lifetime and structural band shapes, it is conceivable that the HE feature originated from a ^{3}IL state associated with the pq ligand. Unlike the ppy complexes, the emission spectra of complexes **2a** and **2b** in water (Figure S3 and S4 in the Supporting Information) are very similar to those in buffer. Probably the interactions of the PEG pendants with these complexes are similarly strong in both water and buffer, which is not unreasonable because of the higher hydrophobicity of the pq complexes compared with their ppy counterparts. Note that addition of free PEG molecules to a buffer solution of the control bpy–CONH–Et complexes **1c** and **2c** did not result in the production of such HE emission bands/shoulders. These findings resemble the interesting emission properties of related inorganic complexes incorporated with aliphatic chains.[27] All these results strongly indicate that the covalently attached PEG1 and PEG3 pendants significantly perturb the emission properties of the complexes in aqueous buffer. The pba complex **3** showed a vibronically structured emission band (Figure 1) with very long emission lifetimes in fluid solutions at 298 K and in low-temperature alcohol glass (≈3–7 μs), typical of ^{3}IL ($\pi \rightarrow \pi^*$) (pba) emission.[17b,l,18a,b]

Cytotoxicity: The cytotoxicity of all the complexes toward HeLa cells has been examined by the MTT assay and the results are listed in Table 3. The most important finding is that the IC_{50} values of the iridium(III) PEG complexes **1a**,**b**, **2a**,**b**, and **3** (ranging from ≈286.5 to 1180.0 μM) are significantly higher than those of cisplatin (10.3 μM) and the control bpy–CONH–Et complexes **1c** (14.6 μM) and **2c** (4.1 μM). We have ascribed the exceptionally low cytotoxicity of the PEG complexes to the long PEG chains which protect the complexes from 1) interacting nonspecifically with the extracellular proteins and 2) triggering immunogenicity and antigenicity inside the cells.[28,29] The ppy complexes **1a** and **1b** revealed lower cytotoxicity than their pq counterparts **2a** and **2b**, which can be accounted for by the higher lipophilicity of the latter complexes due to the more nonpolar pq ligands. A similar dependence of cytotoxicity on lipophilicity has been commonly observed for related cyclometalated iridium(III) polypyridine complexes.[17h–m]

Table 3. Cytotoxicity (IC_{50}, 48 h) of the cyclometalated iridium(III) complexes and cisplatin towards HeLa cells.

Complex	IC_{50} [μM]
1a	830.4 ± 54.5
1b	1180.0 ± 70.5
1c	14.6 ± 1.5
2a	565.9 ± 49.4
2b	286.5 ± 35.2
2c	4.1 ± 0.4
3	1050.0 ± 64.9
cisplatin	10.3 ± 0.7

Cellular uptake studies: The cellular uptake of all the complexes has been studied by ICPMS measurements and the results are listed in Table 4. The amounts of iridium taken up by the HeLa cells are of the same order of magnitude among all the complexes. Specifically, an average cell (mean volume of 3.4 pL) contained 1.38 to 4.45 fmol of iridium, which is comparable to those reported in the cellular uptake studies of other iridium[17j,k,m] and inorganic complexes.[6a,14i] Note that the intracellular iridium concentrations are in the submolar range, which is much higher than that of the free complexes in the medium before the uptake (5 μM), indicating that the complexes were concentrated within the cells. The difference of uptake efficiencies between the complexes is too small for meaningful comparison (Table 4). Thus, the similar intracellular iridium concentrations indicate that the much larger IC_{50} values of the PEG complexes **1a**,**b**, **2a**,**b**, and **3** compared with those of the control bpy–CONH–Et complexes **1c** and **2c** (Table 3) are due to the favorable effects of the PEG pendants.

Table 4. Number of moles and concentrations of iridium associated with an average HeLa cell upon incubation with the cyclometalated iridium(III) complexes (5 μM) at 37 °C for 2 h, determined by ICPMS.

Complex	Number of moles of Ir [fmol]	Concentration of Ir [μM]
1a	2.56 ± 0.44	754 ± 128
1b	3.03 ± 1.12	812 ± 330
1c	1.39 ± 0.04	408 ± 12
2a	1.38 ± 0.19	404 ± 57
2b	1.56 ± 0.06	460 ± 18
2c	4.45 ± 0.80	1308 ± 236
3	2.88 ± 0.92	848 ± 267

Laser-scanning confocal microscopy: The cellular uptake of the complexes has been investigated by laser-scanning confocal microscopy. Related cyclometalated iridium(III) polypyridine complexes that we have designed previously are quite cytotoxic and thus the complex concentration was limited to a few micromolar. However, because the current PEG complexes are almost noncytotoxic to HeLa cells, complex **2a** at higher concentrations has been used in the experiments. Incubation of the cells with the complex at 10 μM at 37 °C for 1 h resulted in efficient cellular uptake and the microscopy images revealed obvious punctate staining in the cytoplasm (Figure 2). It is likely that the complex binds to hydrophobic organelles such as the Golgi body and endoplasmic reticulum.[5c,17l,m,30] When a higher dosage (100 μM) was used, the complex was localized in the perinuclear region with negligible nuclear uptake. Most importantly, HeLa cells incubated with the complex at a relatively high concentration (200 μM) for 2 h still remained viable, and staining of the perinuclear region with very high emission intensity was observed. Interestingly, the stained HeLa cells still survived after further incubation in a complex-free growth medium. To gain more insight, we have treated four HeLa cell cultures with complex **2a** (200 μM) for 2 h at various time points in three 24 h incubation periods. The cells were washed with phosphate-buffered saline (PBS) after each incubation period before returning to the culture medium. We found that the emission intensity of the cells reduced gradually with increasing incubation time in the complex-free medium (Table 5) and the cell counts were not much different from that of the control, which was not treat-

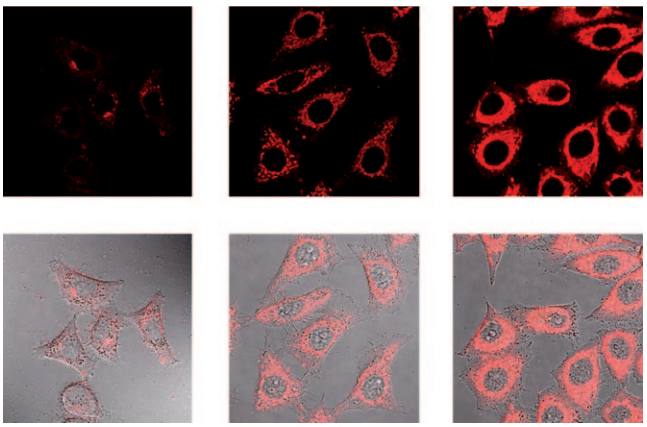


Figure 2. Confocal microscopy images of HeLa cells incubated with complex **2a** at 10 μM for 1 h (left), 100 μM for 1 h (middle), and 200 μM for 2 h (right) at 37 °C.

Table 5. Flow cytometric analysis and cell counts of HeLa cell cultures after treatment with complex **2a** (200 μM) for 2 h at various time points in three incubation 24 h periods.[a]

Entry	Incubation time before treatment with complex [h][b]	Incubation time after treatment with complex [h][b]	Emission intensity [a.u.][c]	Cell count [μL]
1	72	0	74.85±26.40	1593±297
2	48	24	17.17±6.06	1599±23
3	24	48	10.04±4.22	1347±266
4	0	72	9.93±4.16	1296±90
control[d]	NA[d]	NA[d]	3.18±1.44	1482±102

[a] Cells were washed with PBS after each 24 h incubation period. [b] Incubation in complex-free medium. [c] Data from flow cytometry. [d] Cells were not treated with the complex; total incubation time = 74 h.

ed with the complex (Table 5). These results indicate that the internalized complex did not adversely interfere with the cell-division process and was transported out of the cells.[31] These findings also highlight the low cytotoxicity and high biocompatibility of the PEG complexes, which are remarkable advantages for live-cell imaging applications.

PEGylation and transfection studies: Because the aldehyde moiety is reactive toward primary amines, complex **3** can function as a novel luminescent PEGylation reagent. We have PEGylated BSA with the complex to give the conjugate **3**-BSA. On the basis of spectroscopic data and Bradford assays,[32] the iridium-to-protein ratio was determined to be ≈2.3. The PEGylated conjugate **3**-BSA was strongly emissive in aqueous buffer under ambient conditions, and the emission spectrum was characterized by a vibronically structured band at ≈490 (max), 520 nm (Table 2). This, together with a very long lifetime (≈2.61 μs) suggests a ^{3}IL (π→π*) (N^C or N^N) emissive state.[17g,l]

Gene delivery applications that use PEI as a nonviral vector have been well documented.[33–35] The polycationic nature of PEI in aqueous media enables the formation of polyplexes with plasmid DNA (pDNA), which can be efficiently delivered into eukaryotic cells. However, there are few details about the intracellular departure of nucleic acid from the polymer owing to a lack of reporting properties. Also, because PEI is rather cytotoxic to many cell lines, it is commonly PEGylated to lower its cytotoxicity and to facilitate the transfection applications.[36–39] In this work, we have PEGylated PEI (MW = 25 kDa) with complex **3** (Scheme 2) and isolated the luminescent polymer **3**-PEI. The iridium-to-PEI ratio was determined to be ≈4.2 from the spectroscopic data. Upon photoexcitation, the PEGylated PEI polymer exhibited intense and long-lived green emission. On the basis of the photophysical data (Table 2), we have assigned the emission to a ^{3}IL (π→π*) (N^C or N^N) state,[17g,l] which is similar to that of the PEGylated BSA conjugate **3**-BSA.

The ability of luminescent PEGylated polymer **3**-PEI to form polyplexes with pDNA has been investigated. The polyplex **3**-PEI/pCMV-luc was prepared in different N/P ratios (the number of nitrogen residues of PEI per DNA phosphate; from 0.5 to 16.0) prior to analysis by agarose gel electrophoresis (Figure 3). The results showed that the

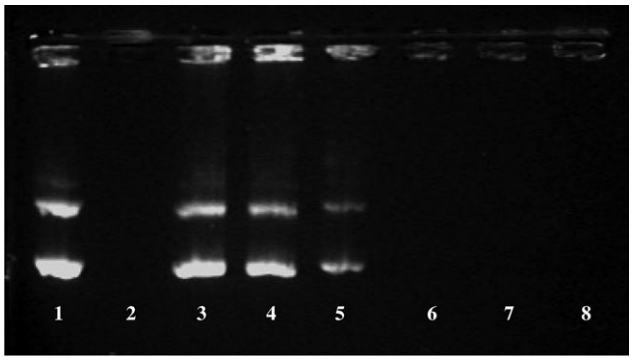
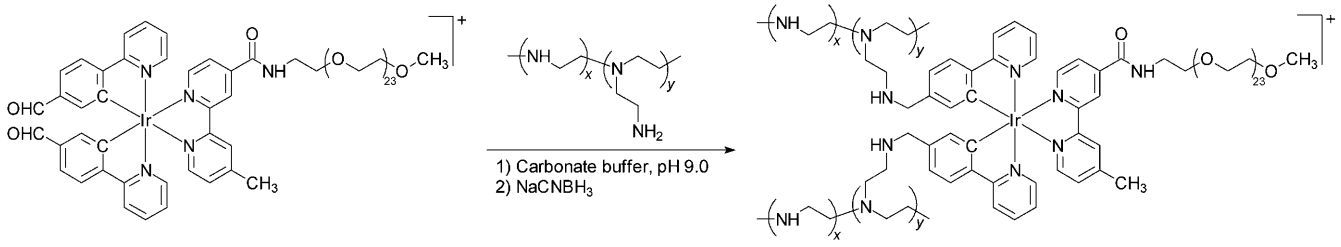


Figure 3. Gel electrophoresis of polyplexes formed from **3**-PEI and pCMV-luc. The lane numbers correspond to different N/P ratios: 1) DNA only, 2) **3**-PEI only, 3) 0.5, 4) 1.0, 5) 2.0, 6) 4.0, 7) 8.0, 8) 16.0.

pDNA band was retarded with increasing the N/P ratio, indicating that the negative charge of the plasmid was neutralized by the positively charged PEI derivative. At an N/P ratio = 4.0 (lane 6 in Figure 3), the band was completely retarded, indicating the capacity of **3**-PEI to condense DNA effectively. The zeta potentials and mean hydrodynamic diameters of the **3**-PEI/pCMV-luc polyplexes have also been studied by using dynamic light scattering (Table 6). The zeta potentials of the polyplexes changed from ≈−32 to +42 mV upon increasing the N/P ratio. At N/P ≥ 4.0, the polyplex acquired a positive zeta potential, which is in accordance with the gel electrophoresis results (Figure 3). Interestingly, the hydrodynamic diameters of the polyplexes formed were ≈490 to 540 nm with N/P being between 0.5 and 2.0 (Table 6). When N/P was ≥4.0, the polyplexes



Scheme 2. Synthetic scheme of **3**-PEI.

Table 6. Zeta potentials and mean hydrodynamic diameters of **3**-PEI/pCMV-luc in Tris-Cl buffer (50 mM, pH 7.4) at various N/P ratios.

N/P ratio	Zeta potential [mV]	Mean hydrodynamic diameter [nm]
0.5	−31.8 ± 2.87	488.5 ± 96.42
1.0	−15.1 ± 0.35	490.6 ± 35.19
2.0	−21.5 ± 2.25	540.0 ± 62.38
4.0	38.3 ± 0.36	245.5 ± 22.88
8.0	40.8 ± 0.58	218.4 ± 13.44
16.0	42.2 ± 0.46	188.0 ± 1.35

shrank to ≈190 to 250 nm in diameter, which supports the argument that the positively charged **3**-PEI condensed the negative pDNA and formed a compact polyplex at such N/P ratios.[40,41] We further assessed the transfection efficacy of PEGylated **3**-PEI by using HeLa cells and the same pDNA that expresses luciferase. The polyplex **3**-PEI/pCMV-luc was prepared in the same N/P ratios (from 0.5 to 16.0) as described above, and lipofectamine (Promega)/pCMV-luc and the naked pDNA were used as a positive and negative control, respectively. The highest transfection efficacy occurred at N/P = 4.0 and 8.0 (Figure 4), which is in agreement with the gel electrophoresis and dynamic light scattering results. The lower transfection efficacy at the highest N/P ratio we studied (16.0) is most likely a result of extensive cell death caused by the cytotoxicity of PEI.[42–44] At lower N/P ratios (0.5 to 2.0), the effect of the transfection reagent **3**-PEI is less significant. Note that the optimal N/P ratio (4.0) is lower than that of untreated PEI (≈10.0).[45] One possible explanation is that complex **3** provides additional positive charges to the PEI molecule, lowering the N/P ratio required for optimum transfection efficacy. However, the effect originating from the biocompatible PEG pendants of complex **3** should not be neglected. The cellular uptake of the polyplex **3**-PEI/pCMV-luc (4 μg pCMV-luc; N/P = 4.0) has been investigated by using confocal microscopy. The images showed extracellular luminescent granules (Figure 5), which are probably condensed polyplexes. However, some of these luminescent spots were located in the cytoplasm surrounding the nuclei with a low but nonzero intensity inside the nucleus. All these results demonstrated that complex **3** can retain the transfection properties of PEI, and the emissive behavior and intracellular uptake of the polymer can be examined by emission spectroscopy and confocal microscopy, respectively.

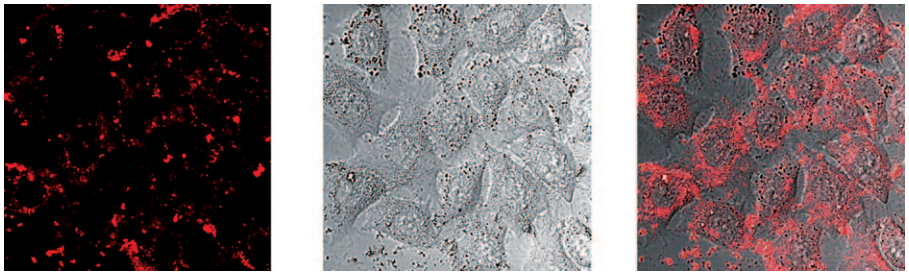


Figure 5. Fluorescence (left), brightfield (middle), and overlaid (right) microscopy images of HeLa cells incubated with **3**-PEI loaded pCMV-luc (4-μg p-CMV-luc; N/P = 4.0) at 37°C for 5 h.

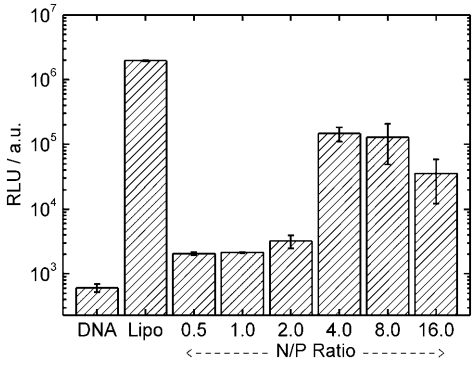


Figure 4. Luciferase activity of **3**-PEI/pCMV-luc at various N/P ratios in HeLa cells. Lipofectamine/pCMV-luc and the naked pDNA were used as a positive and negative control, respectively.

Conclusion

In summary, we have developed a new class of luminescent cyclometalated iridium(III) polypyridine PEG complexes, which showed very rich photophysical properties, high water solubility, very low cytotoxicity, excellent biocompatibility, and high cellular uptake efficiency. One of the complexes has been functionalized with amine-reactive aldehyde moieties, which can target proteins and amine-containing polymers. This renders the complex a luminescent PEGylation reagent derived from transition-metal complexes, which is the first of its kind. Related work on luminescent inorganic and organometallic transition-metal PEG complexes with a focus on biological applications is underway.

Experimental Section

Materials, synthesis, and instrumentation: All solvents were of analytical grade and purified according to standard procedures.[46] All buffer components were of biological grade and used as received. *cis*-Diamminedichloroplatinum (cisplatin), *N*,*N*′-dicyclohexylcarbodiimide, *N*-hydroxysuccinimide (NHS), KPF_6, $Na(CN)BH_4$, and triethylamine were obtained from Acros. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma. Branched PEI (MW = 25 kDa), Hpba, Hppy, Hpq, 4,4′-dimethyl-2,2′-bipyridine, and $Ir_3{\cdot}3H_2O$ were supplied by Aldrich. The PEG–amines m-$dPEG_{24}$–amine and amino–$dPEG_4$–$(m\text{-}dPEG_{12})_3$ were purchased from Quanta Biodesign. BSA was obtained from Calbiochem. PD-10 size-exclusion columns and YM-30 microcons were received from GE Healthcare and Amicon, respectively. Autoclaved Milli-Q water was used for the preparation for the aqueous

solutions. Bpy–NHS,[47] bpy–CONH–Et,[15b,48] the dichloro-bridged dimers $[Ir_2(ppy)_4Cl_2]$,[17a,c] $[Ir_2(pq)_4Cl_2]$,[17c] and $[Ir_2(pba)_4Cl_2]$,[17b] and the control complexes $[Ir(ppy)_2(bpy–CONH–Et)](PF_6)$ (**1c**) and $[Ir(pq)_2(bpy–CONH–Et)](PF_6)$ (**2c**)[17h] were synthesized according to literature procedures. HeLa cells were obtained from American Type Culture Collection. UltraPure agarose, Lipofectamine 2000 Reagent, Dulbecco's modified Eagle's medium (DMEM), reduced serum medium (Opti-MEM), fetal bovine serum (FBS), phosphate-buffered saline at pH 7.2 (PBS), trypsin-EDTA, and penicillin/streptomycin were purchased from Invitrogen. The growth medium for cell culture contained DMEM with 10% FBS and 1% penicillin/streptomycin. Tris(hydroxymethyl)methylamine (Tris) from USB was used to prepare Tris-Cl (50 mM, pH 7.4). Plasmid DNA pCMV-luc (6.8 kb) was amplified in *E. coli* and purified by HiPure Filter Plasmid Kit, and the concentration of plasmid DNA was measured spectrophotometrically. Luciferase assay kit was obtained from Promega and stored at −70°C before use. The instruments for characterization and photophysical studies have been described previously.[17j] The methods by which determination of luminescence quantum yields,[48,49] the MTT assay,[50] ICPMS,[17j] and live-cell confocal imaging[17i,j] were undertaken have also been reported previously.

Synthesis of bpy–CONH–PEG1: A mixture of bpy–NHS (57.2 mg, 183.9 μmol), m-$dPEG_{24}$–amine (100.0 mg, 91.9 μmol), and triethylamine (45.0 μL, 323.0 μmol) in CH_2Cl_2 (20 mL) was stirred under an inert atmosphere of nitrogen at room temperature for 12 h. The solution was filtered and evaporated by rotary evaporation, leaving a colorless oil. The crude product was purified by column chromatography on silica gel by using CH_2Cl_2/MeOH/NH_4OH (10:1:0.1, v/v/v) as the mobile phase. The portions containing the product were evaporated in vacuo, leaving a colorless oil. Yield: 100.2 mg (85%); ^{1}H NMR (300 MHz, [D_6]acetone, 298 K, TMS): $\delta = 8.75$ (d, $J = 4.8$ Hz, 1H; H6 pyridyl ring), 8.67 (s, 1H; H3 pyridyl ring), 8.51 (d, $J = 5.1$ Hz, 1H; H6′ pyridyl ring), 8.22 (s, 1H; H3′ pyridyl ring), 7.75 (d, $J = 4.8$ Hz, 1H; H5 pyridyl ring), 7.43 (br s, 1H; bpy–4-CONH), 7.14 (d, $J = 5.1$ Hz, 1H; H5′ pyridyl ring), 3.67–3.48 (m, 96H; OCH_2, CONH-CH_2), 3.34 (s, 3H; OCH_3), 2.41 ppm (s, 3H; CH_3 pyridyl ring); ESIMS: m/z: 1286 $[M+H]^+$.

Synthesis of bpy–CONH–PEG3: The procedure was similar to that of bpy–CONH–PEG1, except that amino–$dPEG_4$–(m-$dPEG_{12}$)$_3$ (200 mg, 90.5 μmol) was used instead of m-$dPEG_{24}$–amine. The desired product was isolated as a colorless oil. Yield: 197.2 mg (91%); ^{1}H NMR (300 MHz, [D_6]acetone, 298 K, TMS): $\delta = 8.78$ (d, $J = 5.1$ Hz, 1H; H6 pyridyl ring), 8.71 (s, 1H; H3 pyridyl ring), 8.54 (d, $J = 4.8$ Hz, 1H; H6′ pyridyl ring), 8.25 (s, 1H; H3′ pyridyl ring), 7.79 (d, $J = 5.1$ Hz, 1H; H5 pyridyl ring), 7.58 (br s, 1H; bpy–4-CONH), 7.18 (d, $J = 4.8$ Hz, 1H; H5′ pyridyl ring), 6.88 (t, $J = 5.1$ Hz, 3H; m-$dPEG_{12}$–CONH), 6.59 (s, 1H; $dPEG_4$–CONH), 3.68–3.51 (m, 166H; OCH_2), 3.43–3.42 (m, 8H; CONH-CH_2), 3.37 (s, 9H; OCH_3), 2.45 (s, 3H; CH_3 pyridyl ring), 2.40 ppm (t, $J = 5.4$ Hz, 8H; CH_2-CONH); ESIMS: m/z: 2406 $[M+H]^+$.

$[Ir(ppy)_2(bpy–CONH–PEG1)](PF_6)$ (1a): A mixture of $[Ir_2(ppy)_4Cl_2]$ (20.9 mg, 19.5 μmol) and bpy–CONH–PEG1 (50.0 mg, 38.9 μmol) in MeOH/CH_2Cl_2 (30 mL, 1:1, v/v) was heated at reflux under an inert atmosphere of nitrogen for 4 h. The reaction mixture was then cooled to room temperature and KPF_6 (20.0 mg, 0.11 mmol) was added. The mixture was stirred for 30 min and was then evaporated under vacuum. The residual orange oil was dissolved in CH_2Cl_2 and purified by column chromatography on silica gel. Subsequent recrystallization of the product from CH_2Cl_2/diethyl ether afforded complex **1a** as an orange semi-solid. Yield: 60.0 mg (80%); ^{1}H NMR (300 MHz, [D_6]acetone, 298 K, TMS): $\delta = 9.08$ (s, 1H; H3 pyridyl ring bpy–CONH–PEG1), 8.80 (s, 1H; H3′ pyridyl ring bpy–CONH–PEG1), 8.25–8.18 (m, 4H; bpy–4-CONH, H6 pyridyl ring bpy–CONH–PEG1, H3 pyridyl ring ppy), 8.01–7.80 (m, 8H; H5, H6′ pyridyl ring bpy–CONH–PEG1, H4, H6 pyridyl ring ppy, H3 phenyl ring ppy), 7.54 (d, $J = 5.4$ Hz, 1H; H5′ pyridyl ring bpy–CONH–PEG1), 7.18–7.15 (m, 2H; H5 pyridyl ring ppy), 7.04–7.00 (m, 2H; H4 phenyl ring ppy), 6.92–6.90 (m, 2H; H5 phenyl ring ppy), 6.37–6.32 (m, 2H; H6 phenyl ring ppy), 3.68–3.59 (m, 96H; OCH_2, CONH-CH_2), 3.33 (s, 3H; OCH_3), 2.60 ppm (s, 3H; CH_3 pyridyl ring bpy–CONH–PEG1); IR (KBr): $\tilde{\nu} = 3448$ (N–H), 1655 (C=O), 1109 (C–O), 842 cm^{-1} (PF_6^-); HR ESI-TOF MS: m/z calcd for $C_{83}H_{125}IrN_5O_{25}$: 1784.8293; found 1784.8266 $[M]^+$, 911.8956 $[M+K]^{2+}$.

$[Ir(ppy)_2(bpy–CONH–PEG3)](PF_6)$ (1b): The synthetic procedure was similar to that of complex **1a**, except that bpy–COHN–PEG3 (98 mg, 40.7 μmol) was used instead of bpy–CONH–PEG1. The complex was isolated as an orange semi-solid. Yield: 100.0 mg (81%); ^{1}H NMR (300 MHz, [D_6]acetone, 298 K, TMS): $\delta = 9.13$ (s, 1H; H3 pyridyl ring bpy–CONH–PEG3), 8.74 (s, 1H; H3′ pyridyl ring bpy–CONH–PEG3), 8.25–8.21 (m, 3H; H3 pyridyl ring ppy, H6 pyridyl ring bpy–CONH–PEG3), 8.07 (br s, 1H; bpy–4-CONH), 8.00–7.80 (m, 8H; H5, H6′ pyridyl ring bpy–CONH–PEG3, H4, H6 pyridyl ring ppy, H3 phenyl ring ppy), 7.56 (m, 2H; H5′ pyridyl ring bpy–CONH–PEG3, $dPEG_4$–CONH), 7.44 (br s, 3H; m-$dPEG_{12}$–CONH), 7.20–7.13 (m, 2H; H5 pyridyl ring ppy), 7.06–7.00 (m, 2H; H4 phenyl ring ppy), 6.94–6.87 (m, 2H; H5 phenyl ring ppy), 6.35–6.29 (m, 2H; H6 phenyl ring ppy), 3.68–3.51 (m, 166H; OCH_2), 3.42–3.40 (m, 8H; CONH-CH_2), 3.32 (s, 9H; OCH_3), 2.62 (s, 3H; CH_3 pyridyl ring bpy–CONH–PEG3), 2.45 ppm (br s, 8H; CH_2-CONH); IR (KBr): $\tilde{\nu} = 3432$ (N–H), 1655 (C=O), 1106 (C–O), 845 cm^{-1} (PF_6^-); HR ESI-TOF MS: m/z calcd for $C_{133}H_{221}IrN_9O_{48}$: 2906.4792; found 1472.7218 $[M+K]^{2+}$, 994.7982 $[M+2K]^{3+}$.

$[Ir(pq)_2(bpy–CONH–PEG1)](PF_6)$ (2a): The synthetic procedure was similar to that of complex **1a**, except that $[Ir_2(pq)_4Cl_2]$ (24.8 mg, 19.5 μmol) was used instead of $[Ir_2(ppy)_4Cl_2]$. The complex was isolated as an orange semi-solid. Yield: 65.0 mg (82%); ^{1}H NMR (300 MHz, [D_6]acetone, 298 K, TMS): $\delta = 8.75$ (s, 1H; H3 pyridyl ring bpy–CONH–PEG1), 8.54–8.53 (m, 4H; H3 phenyl ring pq, H3 quinoline ring pq), 8.49 (s, 1H; H3′ pyridyl ring bpy–CONH–PEG1), 8.47–8.45 (d, $J = 5.7$ Hz, 1H; H6 pyridyl ring bpy–CONH–PEG1), 8.26 (d, $J = 7.5$ Hz, 2H; H4 quinoline ring pq), 8.20–8.18 (m, 2H; bpy–4-CONH, H5 pyridyl ring bpy–CONH–PEG1), 8.04–8.02 (m, 1H; H6′ pyridyl ring bpy–CONH–PEG1), 7.96–7.92 (m, 2H; H8 quinoline ring pq), 7.57 (d, $J = 4.5$ Hz, 1H; H5′ pyridyl ring bpy–CONH–PEG1), 7.48–7.41 (m, 4H; H5, H7 quinoline ring pq), 7.20–7.12 (m, 4H; H4 phenyl ring pq, H6 quinoline ring pq), 6.86–6.80 (m, 2H; H5 phenyl ring pq), 6.58–6.52 (m, 2H; H6 phenyl ring pq), 3.69–3.58 (m, 96H; OCH_2, CONH-CH_2), 3.32 (s, 3H; OCH_3), 2.50 ppm (s, 3H; CH_3 pyridyl ring bpy–CONH–PEG1); IR (KBr): $\tilde{\nu} = 3448$ (N–H), 1655 (C=O), 1109 (C–O), 843 cm^{-1} (s, PF_6^-); HR ESI-TOF MS: m/z calcd for $C_{91}H_{129}IrN_5O_{25}$: 1884.8606; found 1884.8546 $[M]^+$, 961.9102 $[M+K]^{2+}$.

$[Ir(pq)_2(bpy–CONH–PEG3)](PF_6)$ (2b): The synthetic procedure was similar to that of complex **2a**, except that bpy–COHN–PEG3 (98 mg, 40.7 μmol) was used instead of bpy–CONH–PEG1. The complex was isolated as an orange semi-solid. Yield: 106.0 mg (80%); ^{1}H NMR (300 MHz, [D_6]acetone, 298 K, TMS): $\delta = 8.75$ (s, 1H; H3 pyridyl ring bpy–CONH–PEG3), 8.54 (m, 4H; H3 phenyl ring pq, H3 quinoline ring pq), 8.47–8.44 (m, 2H; H3′, H6 pyridyl ring bpy–CONH–PEG3), 8.37 (br s, 1H; bpy–4-CON*H*), 8.26 (d, $J = 6.6$ Hz, 2H; H4 quinoline ring pq), 8.18 (d, $J = 5.7$ Hz, 1H; H5 pyridyl ring bpy–CONH–PEG3), 8.06–8.04 (m, 1H; H6′ pyridyl ring bpy–CONH–PEG3), 7.97–7.92 (m, 2H; H8 quinoline ring pq), 7.57 (d, $J = 4.8$ Hz, 1H; H5′ pyridyl ring bpy–CONH–PEG3), 7.49–7.38 (m, 7H; m-$dPEG_{12}$–CONH, H5, H7 quinoline ring pq), 7.20–7.15 (m, 4H; H4 phenyl ring pq, H6 quinoline ring pq), 7.00 (s, 1H; $dPEG_4$–CONH), 6.84–6.80 (m, 2H; H5 phenyl ring pq), 6.57–6.52 (m, 2H; H6 phenyl ring pq), 3.69–3.59 (m, 166H; OCH_2), 3.56–3.50 (m, 8H; CONH-CH_2), 3.30 (s, 9H; OCH_3), 2.50 (s, 3H; CH_3 pyridyl ring bpy–CONH–PEG3), 2.50–2.39 ppm (m, 8H; CH_2-CONH); IR (KBr): $\tilde{\nu} = 3432$ (N–H), 1655 (C=O), 1107 (C–O), 845 cm^{-1} (PF_6^-); HR ESI-TOF MS: m/z calcd for $C_{141}H_{225}IrN_9O_{48}$: 3006.5105; found 1522.7286 $[M+K]^{2+}$, 1028.1409 $[M+2K]^{3+}$.

$[Ir(pba)_2(bpy–CONH–PEG1)](PF_6)$ (3): The synthetic procedure was similar to that of complex **1a**, except that $[Ir_2(pba)_4Cl_2]$ (23.1 mg, 19.5 μmol) was used instead of $[Ir_2(ppy)_4Cl_2]$. The complex was isolated as an orange semi-solid. Yield: 42.0 mg (55%); ^{1}H NMR (300 MHz, $CDCl_3$, 298 K, TMS): $\delta = 9.70$ (s, 2H; CHO), 8.79 (s, 1H; H3 pyridyl ring bpy–CONH–PEG1), 8.55 (s, 1H; H3′ pyridyl ring bpy–CONH–PEG1), 8.06–7.98 (m, 4H; bpy–4-CONH, H3 phenyl ring pba, H6 pyridyl ring bpy–CONH–PEG1), 7.92–7.85 (m, 5H; H3, H3′, H5, H5′, H6′ pyridyl ring bpy–CONH–PEG1), 7.72–7.65 (m, 4H; H3, H6 pyridyl ring pba),

7.63–7.53 (m, 4H; H4 phenyl ring pba, H5 pyridyl ring pba), 7.26–7.24 (m, 2H; H4 pyridyl ring pba), 6.70 (d, $J=6.3$ Hz, 2H; H6 phenyl ring pba), 3.69–3.55 (m, 96H; OCH_2, CONH-CH_2), 3.35 (s, 3H; OCH_3), 2.61 ppm (s, 3H; CH_3 pyridyl ring bpy–CONH–PEG1); IR (KBr): $\tilde{\nu}=$ 3434 (N–H), 1686 (C=O), 1107 (C–O), 844 cm^{-1} (s, PF_6^-); HR ESI-TOF MS: m/z calcd for $C_{85}H_{125}IrN_5O_{27}$: 1840.8191; found 1840.8159 $[M]^+$, 939.8861 $[M+K]^{2+}$.

Flow cytometry: HeLa cells were seeded at a density of 1000000 cells per dish in five 60 mm cell culture dishes and incubated at 37°C under a 5% CO_2 atmosphere for three 24 h periods. At a certain time point, the cells of one of the dishes were incubated with a culture medium that contained complex **2a** (200 μM) for 2 h, followed by gentle washing with PBS (1 mL×3) prior to further incubation in a complex-free medium. In addition, after each 24 h incubation period, the cells were washed with PBS and then incubated in a complex-free medium. Finally, the cell layer was trypsinized and was made up to a final volume of 2 mL with PBS. The samples were analyzed by a FACSCalibur flow cytometer (Becton, Dickinson and Co., Franklin Lakes, NJ) with excitation at 488 nm. The number of cells analyzed for each sample was ≈9000 to 10000.

Labeling of BSA with complex 3: The iridium(III) aldehyde complex **3** (3.0 mg, 1.5 μmol) in deionized water (60 μL) was added to BSA (15.0 mg, 0.227 μmol) that was dissolved in carbonate buffer (600 μL, 50 mM, pH 9.0). After the mixture was stirred slowly in the dark at room temperature for 12 h, $NaCNBH_3$ (43.4 mg, 0.69 mmol) in 1M NaOH (100 μL) was added to the solution. The yellow solution was stirred for another 24 h in the dark at room temperature. The reaction mixture was diluted to 2 mL with Tris-Cl buffer (50 mM, pH 7.4), which was then purified with a YM-30 centricon (Amicon) through successive washing with Tris-Cl buffer (50 mM, pH 7.4) and exchanged into phosphate buffer (50 mM, pH 7.4). The volume of the solution was finally reduced to 400 μL. The resulting conjugate **3**-BSA was stored at 4°C before use. The dye-to-BSA ratio was determined to be ≈2.3 by the Bradford assay.

Labeling of PEI with complex 3: The iridium(III) aldehyde complex **3** (2.6 mg, 1.3 μmol) in deionized water (60 μL) was added to branched PEI (3.4 mg, 0.136 μmol) that was dissolved in carbonate buffer (600 μL, 50 mM, pH 9.0). After the mixture was stirred slowly in the dark at room temperature for 12 h, $NaCNBH_3$ (17.0 mg, 0.27 mmol) in 1M NaOH (100 μL) was added to the solution. The yellow solution was stirred for another 24 h in the dark at room temperature. The reaction mixture was diluted to 2 mL with Tris-Cl buffer (50 mM, pH 7.4), which was then purified with a YM-30 centricon (Amicon) via successive washing with Tris-Cl buffer (50 mM, pH 7.4) and exchanged into phosphate buffer (50 mM, pH 7.4). The volume of the solution was finally reduced to 600 μL. The resulting conjugate **3**-PEI was stored at 4°C before use. The dye-to-PEI ratio was determined to be ≈4.2 on the basis of spectroscopic data.

Agarose gel electrophoresis retardation assays: The **3**-PEI/pCMV-luc polyplexes at various N/P ratios (the number of nitrogen residues of PEI per DNA phosphate, from 0.5 to 16.0) were prepared by mixing **3**-PEI and pCMV-luc at an appropriate ratio in an elution buffer. The pCMV-luc and **3**-PEI were used as a positive and negative control, respectively. After incubation for 30 min at room temperature, the polyplexes were analyzed by electrophoresis on a 0.9% (w/v) agarose gel containing ethidium bromide with Tris-acetate buffer at 100 V for 45 min. The gel was visualized by using a Bio-Rad Gel Doc imager.

Zeta potentials and mean hydrodynamic diameter measurements: A mixture of the pCMV-luc (4 μg) and **3**-PEI at various N/P ratios (from 0.5 to 16.0) in Tris-Cl buffer (80 μL, 50 mM, pH 7.4) was incubated for 30 min at room temperature. The mixture was then diluted tenfold with the same buffer before measurements. The zeta potential of the resulting polyplex was measured by using Zetasizer Nano ZS (Malvern Instruments) with the following specifications: sampling time, 10–20 s; medium viscosity, 1.0031 cP; dielectric constant, 80.4; temperature, 20°C; beam mode F(Ka)=1.50 (Smoluchowsky). Particle size was determined with the following specifications: sampling time, 180 s; medium viscosity, 1.0031 cP; refractive index (RI) medium, 1.330; RI particle, 1.450; temperature, 20°C. All the experiments were carried out in triplicate to ascertain reproducibility.

In vitro transfection (luciferase assays): HeLa cells were seeded at a density of 100000 cells per dish in a 35 mm cell culture dish and incubated for 48 h at 37°C under a 5% CO_2 atmosphere. The culture medium was replaced with DMEM (2 mL) containing 10% FBS 2 h prior to transfection. The transfection experiments were performed with 4 μg pCMV-luc. At the time of transfection, the medium was replaced with Opti-MEM (2 mL). **3**-PEI/pCMV-luc polyplexes at various N/P ratios were then incubated with the cells for 5 h. The medium was replaced with fresh growth medium (3 mL) and the cells were further incubated for 43 h. Lipofectamine/pCMV-luc polyplex and the naked pDNA were used as a positive and negative control, respectively. After the incubation, the cells were permeabilized with cell lysis buffer (200 μL) (Promega) with one freeze-thaw cycle. The luciferase activity in cell extracts was measured by using a luciferase assay kit (Promega) on a microplate reader (BMG FLUOstar OPTIMA) for an interval of 10 s. All the experiments were carried out in triplicate to ascertain the reproducibility.

Acknowledgements

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