Nanoparticles from Block Copolymer Encapsulating Re(phen) as Bifunctional Agents for Cell Imaging and Gene Transfection

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Materials and methods

1. Materials

1,10-phenanthroline, tetrakis(triphenylphosphine)platinum(0), fluorene, pentacarbonylchlororhenium(I), carbazole, 1,1,4,7,10,10-hexamethyl triethylenetetramine (HMTETA), 2-bromoisobutyryl bromide (BiBB), 2-(dimethylamino)-ethyl methacrylate (DMAEMA) and poly(ethylene glycol)methyl ether methacrylate (PEGMA) were purchased from Sigma Chemical Co. DMAEMA and PEGMA were passed through a basic alumina column and then distilled prior to polymerization. All other reagents were of analytical grade and were used as received. Plasmid DNA pEGFP-C2 (4.7 kb) was purified from E.coli DH5α. The concentration of plasmid DNA was measured through spectrophotometric analysis using a DU-7 Spectrophotometer (Beckman). DNA was kept in TE buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH = 8.0).

2. Instruments

NMR spectra were recorded on a Bruker spectrometer at 400 (^{1}H NMR) MHz and 75 (^{13}C NMR) MHz. Chemical shifts (δ values) were reported in ppm down field from internal Me_4Si (^{1}H and ^{13}C NMR). FTIR spectra (KBr pellet) were recorded on a 5DX FT-IR spectrometer (Nicolet, USA). Positive ion FAB mass spectra were recorded on a ZAB-HS mass spectrometer (VG, U. K.). Molecular weight of the block copolymer were determined by gel permeation chromatography(GPC), which consists of a Waters 515 liquid chromatography pump (1 ml min^{-1}) and four Polymer Standards Service columns (102 Å, 103 Å, 104 Å, 105 Å) in series and a Waters 2400 differential refractometer. Elemental analyses were performed on a Vanio-EL elemental analyzer (Analysensysteme GmbH, Germany). The crystallographic data were collected with a Rigaku Saturn CCDX area detector diffractometer, which was equipped with graphite monochromatic Mo Kα radiation (λ = 0.71073 Å). UV absorption spectra were recorded on a UV-3600 UV-VIS-NIR spectrophotometer (Shimadzu, Japan). Fluorescence measurements were performed using an F-4500 fluorescence spectrophotometer (Hitachi, Japan) equipped with a plotter unit and a quartz cell (1 cm × 1 cm). Transition electron microscope (TEM) images were recorded on a Tecnai G^2 20-S-TWIN TEM

(Philips, Holland). A fluorescence microscope TE 2000-U (Nikon, Japan) equipped with Spot software was applied to observe the gene transfection result.

3. Synthesis of Re(phen) complexes and amphiphilic polymer

3.1 Synthesis of Re(phen) complex A

$[Re(CO)_5Cl]$ (181 mg, 0.5 mmol) and 3-[2-(9,9-dihexylfluorenyl)]phenanthroline (**L1**)[1] (256 mg, 0.5 mmol) were added to toluene (20 mL) under a nitrogen atmosphere. The suspension was heated under reflux for 2 h, during which the starting materials dissolved gradually to give a yellow solution, which subsequently changed to an orange-yellow solution. After removal of the solvent under reduced pressure, the residue was purified by column chromatography with dichloromethane/methanol (10:1 v/v) as eluent. Bright yellow crystals of complex **A** were obtained by slow diffusion of n-hexane into a dichloromethane solution of the complex. Yield: 303 mg, 0.37 mmol, 74%. ^{1}H NMR (400 MHz, $CDCl_3$, TMS): δ=9.64 (d, *J*=2.0 Hz, 1H), 9.42 (d, *J*=4.8 Hz, 1H), 8.70 (d, *J*=1.6 Hz, 1H), 8.56 (d, *J*=8.4 Hz, 1H), 8.07 (dd, *J*=8.8 Hz, 14.4 Hz, 2H), 7.92 (d, *J*=8.0 Hz, 1H), 7.87 (dd, *J*=12.4 Hz, 3.2 Hz, 1H), 7.81–7.80 (m, 1H), 7.76 (dd, *J*=1.6 Hz, 6.0 Hz, 1H), 7.73 (s, 1H), 7.43–7.37 (m, 3H), 2.17–2.02 (m, 4H), 1.15–1.08 (m, 12H), 0.76 (t, *J*=7.0 Hz, 6H), 0.72–0.64 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$, TMS): δ=196.9, 189.5, 153.0, 152.9, 152.6, 152.3, 151.2, 147.2, 145.4, 143.1, 140.2, 139.8, 137.9, 134.7, 133.6, 130.8, 130.5, 128.1, 127.8, 127.1, 126.9, 125.5, 123.1, 122.0, 120.9, 120.4, 55.6, 40.4, 31.5, 29.7, 23.9, 22.6, 13.9; IR (KBr): ν=2026, 1918, 1881 cm^{-1}; FAB-MS (*m*/*z*): 782.8 (M^+-Cl); Elemental analysis calcd (%) for $C_{40}H_{40}ClN_2O_3Re{\cdot}H_2O$: C, 57.44; H, 5.06 ; N, 3.35. Found: C, 57.53; H, 5.15; N, 3.28.

3.2. Synthesis of Re(phen) complex B

The complex B was synthesized and purified by a procedure similar to that used for complex **A**, except that 3-{2-[9,9-bis[6-(N-carbazolylhexyl)fluorenyl]]}phenan-throline (**L2**) (422 mg, 0.5 mmol) was used in place of **L1** in the substitution reaction. The complex **B** was obtained as bright yellow crystals. Yield: 390 mg, 0.34 mmol; 68 %. ^{1}H NMR (400 MHz, $CDCl_3$, TMS) *δ*=9.59 (d, *J*=1.6 Hz, 1H), 9.40 (d, *J*=4.4 Hz, 1H), 8.52 (t, *J*=3.4 Hz, 2H), 8.03 (dd, *J*=3.6 Hz, 7.6 Hz, 4H), 7.95 (dd, *J*=8.8 Hz, 15.2 Hz, 2H), 7.86 (d, *J*=7.6 Hz, 1H), 7.83 (t, *J*=8.0 Hz, 1H), 7.76 (d, *J*=7.2Hz, 1H), 7.68 (d, *J*=8.0 Hz, 1H), 7.64 (s, 1H), 7.38 (t, *J*=8.0 Hz, 5H), 7.32 (t, *J*=7.2 Hz, 1H), 7.28 (d, *J*=8.4 Hz, 5H), 7.16 (t, *J*=7.4 Hz, 4H), 4.15 (t, *J*=7.2 Hz,

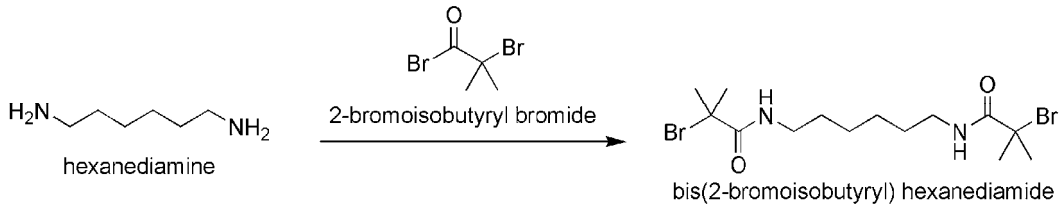
[1] X. S. Zeng, M. Tavasli, I. F. Perepichka, A. S. Batsanov, M. R. Bryce, C. J. Chiang, C. Rothe, A. P. Monkman, *Chem. Eur. J.* **2008**, *14*, 933.

4H), 2.10–1.93 (m, 4H), 1.68 (t, *J*=6.4 Hz, 4H), 1.20-1.12 (m, 8H), 0.65-0.61 (m, 4H), ^{13}C NMR (75 MHz, $CDCl_3$, TMS) δ=196.9, 189.6, 153.0, 152.2, 150.9, 147.1, 145.3, 143.0, 140.3, 140.2, 139.9, 137.9, 134.7, 133.7, 130.7, 130.5, 128.2, 127.8, 127.7, 125.5, 122.9, 122.8, 122.7, 121.9, 121.0, 120.4, 120.2, 118.6, 118.5, 108.6, 55.4, 42.9, 40.2, 29.6, 28.7, 26.7, 23.6. IR (KBr): ν=2018, 1918, 1897 cm^{-1}; FAB-MS (*m*/*z*): 1113.2 (M^+-Cl); Anal. Calcd for $C_{64}H_{54}ClN_4O_3Re$: C, 66.91; H, 4.74; N, 4.88. Found: C, 67.45; H, 4.88; N, 4.75.

3.3. Synthesis of the triblock copolymer PDMAEMA-b-poly(PEGMA)-b-PDMAEMA C

3.3.1. Synthesis of bis(2-bromoisobutyryl) hexanediamide as the bis-initiator

Under a nitrogen atmosphere, a stirred suspension of hexanediamine (1.16 g, 10 mmol) and anhydrous dichloromethane (300 mL) was cooled to 0 °C using an ice bath. The solution of BiBB (2.3 g, 10 mmol) and dichloromethane (10 mL) was then added dropwise. The mixture was subsequently stirred for 12 h at room temperature. After filtration, the solvent was removed with rotary evaporation. The obtained white powder was dissolved in 20 mL dichloromethane and extracted with saturated aqueous solution of sodium carbonate for three times. The organic layer was dried over anhydrous magnesium sulfate and filtered. The solvent was removed under reduced pressure to give the desired product bis(2-bromoisobutyryl) hexanediamide (Scheme S1). Yield: 0.82 g (31 %). ^{1}H NMR (400 MHz, $CDCl_3$, TMS): δ=6.76 (s, 2H, NH), 3.60 (m, 4H, *J*=6.0 Hz, *NH*-*CH*$_2$-), 1.95 (s, 12H, C(*CH*$_3$)$_2$Br), 1.56 (m, 4H, NH-CH$_2$-*CH*$_2$-), 1.38 (m, 4H, -CH$_2$-CH$_2$-*CH*$_2$-).

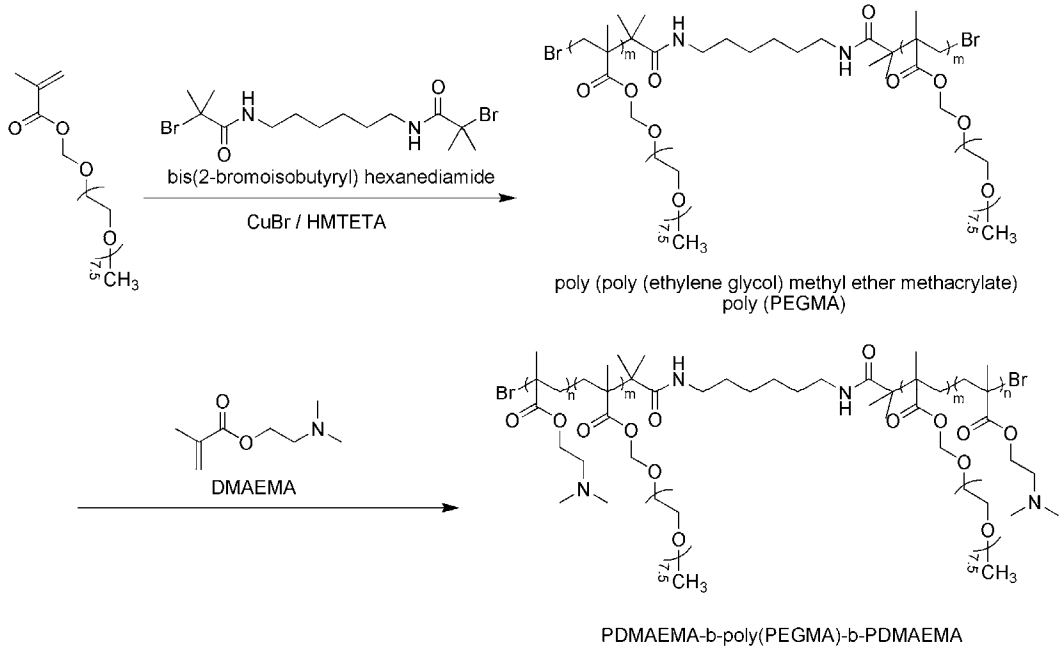


Scheme S1. Synthesis of bis(2-bromoisobutyryl) hexanediamide.

3.3.2. Synthesis of the triblock copolymer PDMAEMA-b-poly(PEGMA)-b-PDMAEMA C

Atom transfer radical polymerization (ATRP) of the copolymer **c** (Scheme S2) was carried out in solution with bis(2-bromoisobutyryl) hexanediamide as the bis-initiator. In a typical experiment, monomer PEGMA (21.6 mmol, 9.5 mL), CuBr (0.36 mmol, 51.6 mg) and THF (200 mL) were charged to a round-bottom flask. The flask was degassed with nitrogen for 3 h before being sealed with a rubber septum. Then HMTETA (0.36 mmol, 98 μL) was added by a microsyringe. When CuBr was completely dissolved and the solution became transparent, bis(2-bromoisobutyryl) hexanediamide (0.18 mmol, 73.6 mg) was added.

After 6 h at 40 °C, the second monomer DMAEMA (35.6 mmol, 6 mL) was added to the solution and polymerized for another 12 h. The mixture was diluted with THF, passed through a silica gel column using acetone as eluent, precipitated in petroleum ether and then dried under vacuum for 24 h. ^{1}H NMR (400 MHz, D_2O, TMS): δ=4.19 (-*COO-C<u>H</u>$_2$-CH$_2$-N(CH$_3$)$_2$*, -*COO-<u>CH</u>$_2$-CH$_2$-O*-), 3.72 (-*O-<u>CH</u>$_2$-<u>CH</u>$_2$-O*-), 3.36 (-*O-C<u>H</u>$_3$*), 2.76 (-*O-CH$_2$-C<u>H</u>$_2$-N(CH$_3$)$_2$*), 2.36 (-*N(C<u>H</u>$_3$)$_2$*). GPC: Mn=47,113, Mw/Mn=1.14. According to the ratio of the terminal methyl groups of DMAEMA to that of PEGMA, the composition of the triblock copolymer is DMAEMA$_{50}$-b-PEGMA$_{66}$-b-DMAEMA$_{50}$.



Scheme S2. Synthesis of PDMAEMA-b-poly(PEGMA)-b-PDMAEMA.

3.4 Preparation of the fluorescent nanoparticles.

In a typical procedure, copolymer **c** (10 mg, 0.7 μmol) and Re(phen) complexes (0.85 μmol) in 500 μL of CH_2Cl_2 were mixed thoroughly in a glass test tube. The solvent was then removed at 60 °C. PBS (10 mL) was added to the film, and then stirred for 1 h. The solution was extruded through a polycarbonate polymer membrane filter (Alltech) with a pore size of 0.45 μm and then used for microscopy and emission studies.

3.5. Electrophoretic mobility shift assays.

The plasmid pEGFP-C2 was prepared by an alkaline lysis procedure. 5 μL of purified plasmids (40 ng/μL) were incubated with the 5 μL of nanoparticles in PBS at pH 7.2 for 30 min at room temperature to allow the plasmid being absorbed sufficiently onto the nanospheres' surface. The N/P ratio is 0.06, 0.12, 0.30, 0.60, 1.20, 3 and 6, respectively. The native DNA and pure nanoparticles without DNA were used as

controls. Then all the samples were loaded into a 0.8% agarose gel containing 1 μg/mL ethidium bromide for electrophoresis assay. The data was analyzed using software UVP BioImaging Systems Lab Works 4.5TM.

3.6 Cell culture.

The HEK293T cells (a human kidney cell line) were cultured in Dulbecco's modification of Eagles media (DMEM) supplemented with 10 % heat-inactivated fetal bovine serum. Cells were seeded onto 24-well plates at a density of 8×10^4 cells per well for cell imaging and gene transfection. After 48 h, the cells were incubated with 160 μL of the as-prepared fluorescent nanoparticles for cell imaging. For gene transfection, 2.0 μg of plasmid pEGFP-C2 was mixed with 160 μL of the fluorescent nanoparticles and the mixture was incubated for 30 min at room temperature. Then the mixture was added to each well. 48 h later, the gene transfection result was observed by using a TE 2000-U fluorescence microscope (Nikon Japan) equipped with Spot software.

3.7 X-ray structural data of B.

Single crystals of **B** are obtained by layering diethyl ether on a concentrated dichloromethane solution of the complex. There are two independent formula units for **B** in one crystallographic unit (a selective drawing is depicted in Figure S1). The structure of **B** consists of a rhenium(I) atom octahedral coordinated by a chelating phenanthroline ligand **L2**, a chloride ion and three carbonyl groups in a facial conformation. The bond distances of Re-C and Re–N are 1.909-2.457 Å, 2.174-2.199 Å respectively, and with the N-Re-N bond angles of 75.7° and 75.6° in the two formula units. Data collection on a Nonius Kappa CCD, equipped with graphite-monochromated Mo_{Ka} radiation (λ = 0.71073 Å). Data for complex **B**: $C_{65.5}H_{59.5}Cl_4N_4O_{4.25}Re$, Mr = 1298.67, triclinic, P_{-1}, a = 16.419(3) Å, b = 14.416(4) Å, c = 21.944(4) Å, β = 74.41(3)°, V = 6077(2) Å^3, Z = 4, $\rho_{calcd.}$ = 1.419 mg m^{-3}, T = 113(2) K, $R1$ = 0.0716, $wR2$ = 0.1881 for 41600 reflections with $I > 2o(I)$. CCDC 733850 contain the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

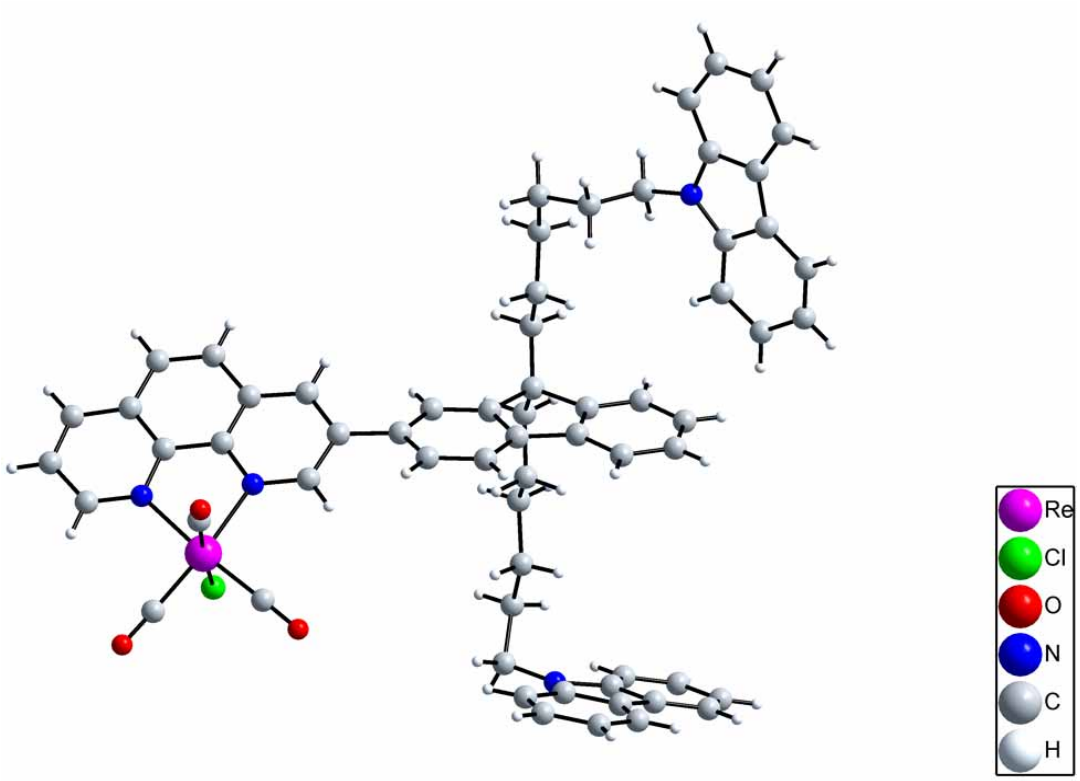


Figure S1. A selective crystal structure of complex **B**

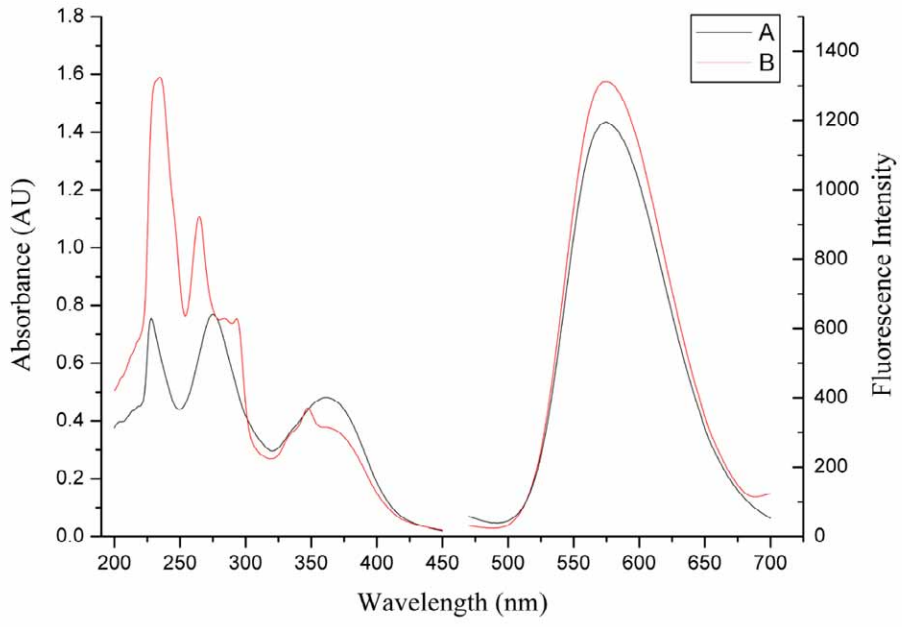


Figure S2. Electronic absorption and emission spectra of complex **A** and complex **B** in degassed CH_2Cl_2 at room temperature.

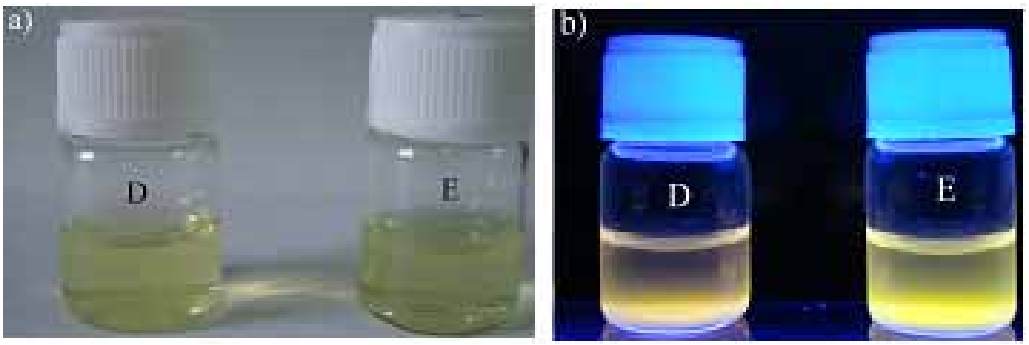


Figure S3. The photo of nanoparticles **D** and **E** under normal (a) and UV (b) excitation. in PBS.

Table S1. Photophysical Date of Complexes

complex	medium (T/K)	λ_{em}/nm	ϕ
A	CH_2Cl_2 (298)	575	0.00097
D	PBS (298)	563	0.0060
B	CH_2Cl_2 (298)	575	0.00070
E	PBS (298)	557	0.01345

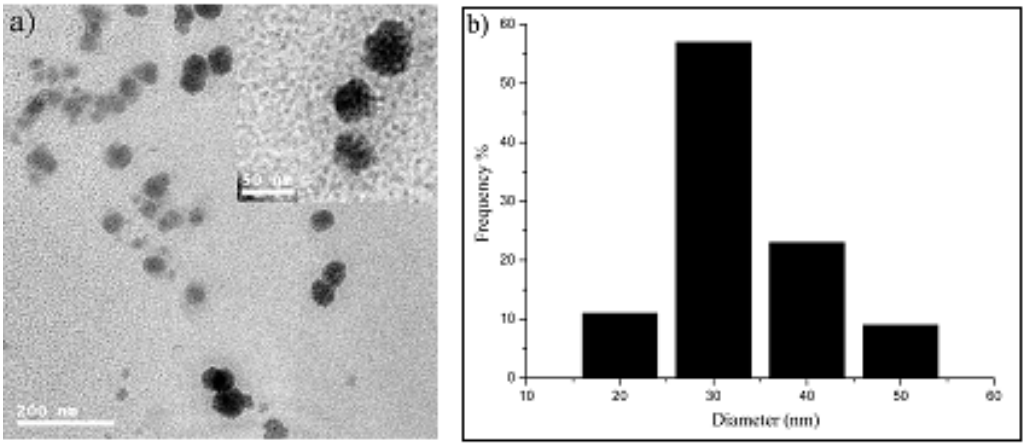


Figure S4. TEM iamge of the nanoparticles **E** (a) and the size distribution of diameter (b).

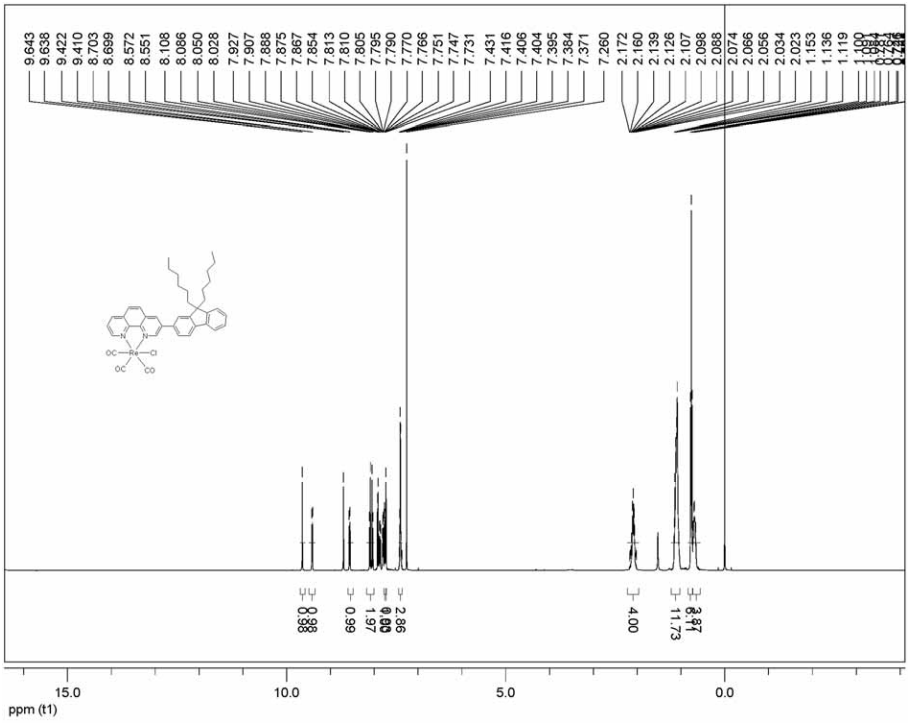


Figure S5. ^{1}H NMR of complex **A**

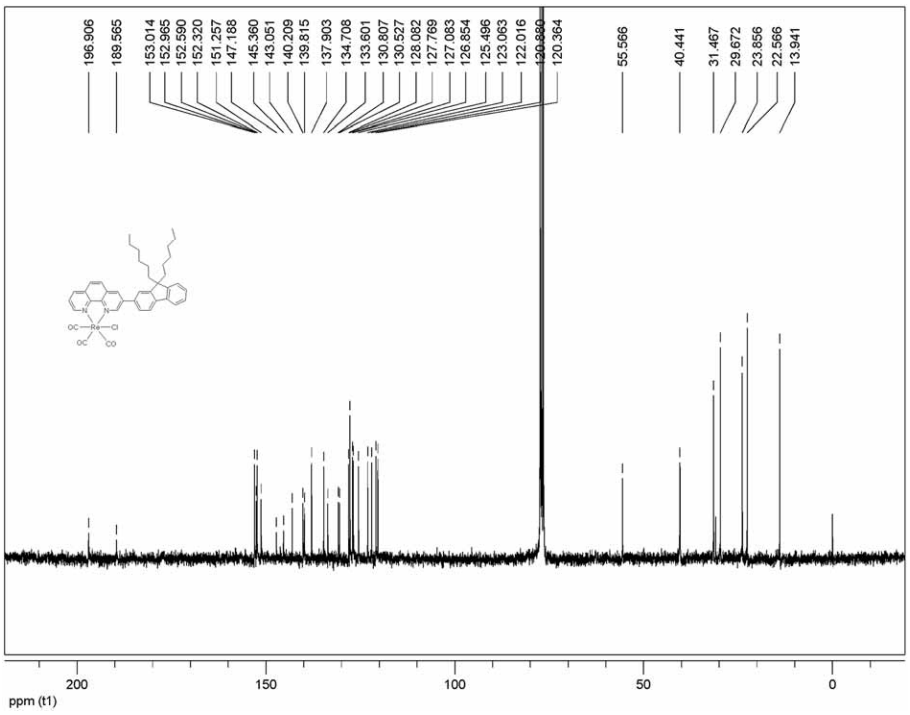


Figure S6. ^{13}C NMR of complex **A**

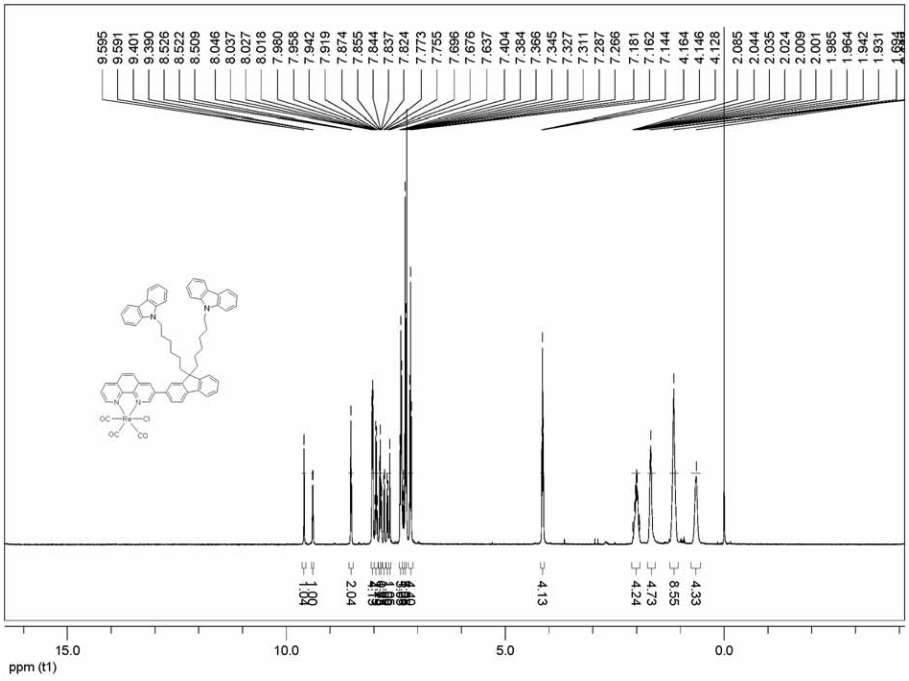


Figure S7. ^{1}H NMR of complex **B**

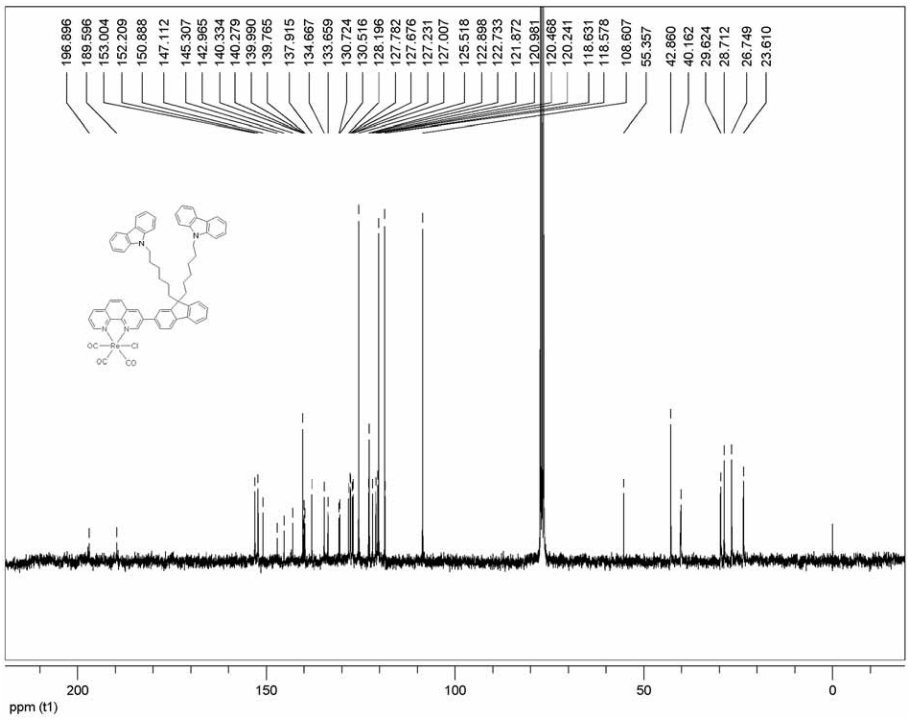


Figure S8. ^{13}C NMR of complex **B**