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Fluorescent Polylactides with Rhenium(bisimine) Cores for Tumour Diagnostics

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Dedicated to Professor Wolfgang Kläui on the occasion of his 65th birthday

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Early tumour detection, for example, by passive delivery of a polymer-based diagnostic agent, can greatly improve the life expectancy of cancer patients. Here, we present the synthesis of biodegradable 3-hydroxymethylpyridine-functionalised polylactides coordinated to $Re(CO)_3$(bisimine) cores [bisimine = bipy (**2**) phen (**3**), dppz (**4**)]. The $Re(CO)_3$(bisimine) polymers **2** and **3** show fluorescence at 560 nm and 572 nm, respectively, with large Stokes shifts, which makes them distinguishable from tissue autofluorescence and therefore suitable diagnostic probes for fluorescence imaging.

Introduction

The early detection of tumours is crucial to the effective treatment of cancer. At this point, there are several strategies to improve tumour diagnostics. One of them is the use of macromolecules that deliver the diagnostic agent directly to the tumour tissue. This is possible because macromolecules cannot pass the vascular walls the way small drugs do. Thus they are not distributed throughout the body, but can only leave the blood stream through defects in the blood vessel walls, which occur typically in tumour vasculature. The macromolecules accumulate inside the tumour tissue as a result of the so-called enhanced permeability and retention (EPR) effect.[1,2]

An important diagnostic tool is fluorescence microscopy.[3] The use of bisiminetricarbonyl rhenium compounds as fluorochromes is of particular interest for diagnostic applications because of their fluorescence in the red region of the visible light spectrum and large Stokes shifts, which makes them distinguishable from the interfering autofluorescence of biological material.[4] Targeting these dyes to tumour tissue will enhance the signal-to-noise ratio. Thus, attachment of these rhenium cores, for example, to ligand-functionalised polylactides leads to biodegradable fluorescent polymers. Polylactides are useful, since they are degraded to lactic acid in vivo, which is nontoxic and easily metabolised. Some fluorescent ligands also double as DNA-intercalating agents, which bind directly to the DNA and could thus be useful for localising the fluorescent dye in the nucleus.[5,6]

Using rhenium as the coordination centre is interesting, because rhenium itself can be applied in radiotherapy ($^{186/188}$Re), and its lower congener technetium in its metastable form (^{99m}Tc) is the most commonly used medical isotope for real-time in vivo imaging, such as SPECT (single photon emission computer tomograpy).[7–9] In this paper, we present functionalised polylactides, which carry the $Re(CO)_3$ moiety with different bisimine ligands. Their synthesis, characterisation and fluorescence properties are presented.

Results and Discussion

Polymer Synthesis

The synthesis of biodegradable polylactides (PLAs) was carried out by using ring-opening polymerisation of L-lactide. We adapted Saatchi and Häfeli's approach of opening lactides with hydroxy-functionalised tridentate ligands. Instead of a tridentate ligand, the monodentate 3-hydroxymethylpyridine was used as the initiator.[10,11] A monodentate end-group at the polylactide instead of a tridentate ligand leaves two coordination sites on the rhenium tricarbonyl core for further functionalisation with a bidentate ligand. This way, for example, fluorescent probes or targeting moieties can also be attached, bringing additional functionalities to the molecule. This concept, the so-called "[2+1] approach" has already been successfully applied for medical

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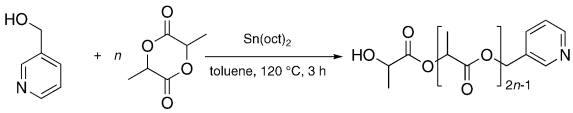
purposes.[12–14] Additionally, the distal hydroxy group of the polylactide can be used for further derivatisation, for example, for attachment to biomolecules.

The synthesis of the polylactide is simple and straightforward. 3-Hydroxymethylpyridine (3-HMP) was used to initiate the ring-opening polymerisation of L-lactide in the presence of the FDA-approved $Sn(oct)_2$[15] as catalyst (Scheme 1).



Scheme 1. Ring opening polymerisation of L-lactide initiated by 3-HMP in the presence of stannous octoate yields PLA-HMP (compound **1**).

The polymers are obtained as oils or waxes, depending on their chain length. The polymer size can be varied by adjusting the monomer/initiator ratio. Polylactides with chain lengths of about twenty or more repeating units can easily be purified by precipitation from dichloromethane with diethyl ether. The PLA-HMP polymers were characterised by ^{1}H NMR, and IR spectroscopy as well as MALDI-TOF analysis.

^{1}H NMR signals of the PLA backbone as well as signals of the pyridyl ligand appear in the spectrum as well-resolved signals. The integration ratios of the methine signal of the repeating unit to those of the pyridyl ligands can be used to estimate the number of repeating units. The calculated average polymer chain length by ^{1}H NMR spectroscopy is in good agreement with that calculated from the MALDI-TOF data (Table 1).

Table 1. Summary of monomer/initiator ratios and average number of repeating units (n) of **1** calculated by ^{1}H NMR spectroscopy and from m/z_{max} by MALDI-TOF analysis.

n_{eq} L-lactide	n_{eq} initiator (3-HMP)	n (NMR)	n (MALDI–TOF)
2	1	5[a]	4[a]
5	1	10[a]	10[a]
5	1	18[b]	18[b]
10	1	20[b]	20[b]
18	1	27[b]	26[b]
29	1	42[b]	40[b]

[a] Oil. [b] Precipitate.

It is important to note that low-molecular-weight polylactides remain in solution if the product is precipitated from dichloromethane/diethyl ether. ^{1}H NMR spectroscopic and MALDI-TOF analyses of the precipitate will therefore not reflect the actual average molecular mass of the polymer if a low monomer/initiator ratio (ca. 5) is used. IR spectra of the polylactide show the broad -OH vibrational signal at about 3550 cm^{-1}, the sharp C=O stretching vibration at 1759 cm^{-1} as well as the pyridyl C=C valence vibration at about 1600 cm^{-1}.

Coordination to [Re(CO)$_3$(bisimine)]$^+$

Rhenium tricarbonyl bromide complexes of 2,2′bipyridine, 1,10-phenanthroline as well as dipyrido[3,2-*a*:2′,3′-*c*]-phenazine (dppz) were prepared. These bisimine complexes can easily be synthesised from $Re(CO)_5Br$, but, more importantly for in vivo applications, also from $[Re(CO)_3(H_2O)_3]Br$.[16] The $Re(CO)_3$(bisimine)Br fluorochromes were bound to the polymer via the pyridyl function after halide abstraction (Figure 1).

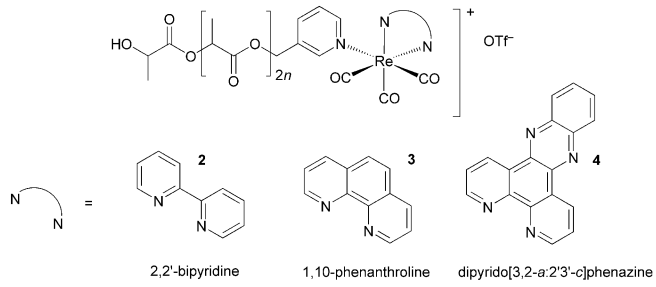


Figure 1. Structures of the prepared PLA-HMP-Re(CO)$_3$(bisimine) polymers (compounds **2**, **3** and **4**).

The precipitated polymers were characterised by ^{1}H NMR spectroscopy and MALDI-TOF analysis (Figure 2), as well as IR spectroscopy. In the case of **2** and **3**, all signals in the ^{1}H NMR spectra are baseline-separated signals. Coordinating the dppz complex to PLA-HMP (**4**), however, does not result in a complete conversion. The ^{1}H NMR spectrum shows an overlap of coordinated and uncoordinated rhenium(bisimine) and pyridyl signals in the aromatic region.

In the MALDI-TOF spectra, the peaks correspond to the masses of the polymers with different repeating units (n) added to the molecular weight of the Re(CO)$_3$(bisimine) fragment. The intensity of even-numbered peaks is higher, representing one lactide monomer for every 2n repeating units. Signals for the sodium salt adducts are also observed in the MALDI-TOF spectra. Incomplete conversion of the lactide results in two intermeshed curves of peaks, one for the unreacted lactide, which is shifted to lower masses, and the other for the coordinated polymer.

The IR spectra of the rhenium bisimine coordinated polymers show the lactide signals, as well as the prominent ν(CO) vibrational bands of the facially coordinated carbonyl groups of the rhenium complex. Two bands are observed for the ν(CO) vibrations (A and E band) at 2035 and 1929 cm^{-1}, which indicate a local C_{3v}-like symmetry at the Re(CO)$_3$ core.

Fluorescent Polymers

The absorption and fluorescence properties of the three synthesised polylactide-rhenium(I) bisimine complexes were measured in acetone solution. As expected, the polymers carrying 2,2′-bipyridine and 1,10-phenanthroline complexes show large Stokes shifts resulting from a metal-to-ligand charge transfer (MLCT) (Table 2).[17] Their Stokes shifts amount to about 11100 and 11550 cm^{-1} respectively, which makes them easily distinguishable from tissue autofluorescence. No fluorescence was detected from the polymer-bound Re(CO)$_3$(dppz) complex. It is known that dppz

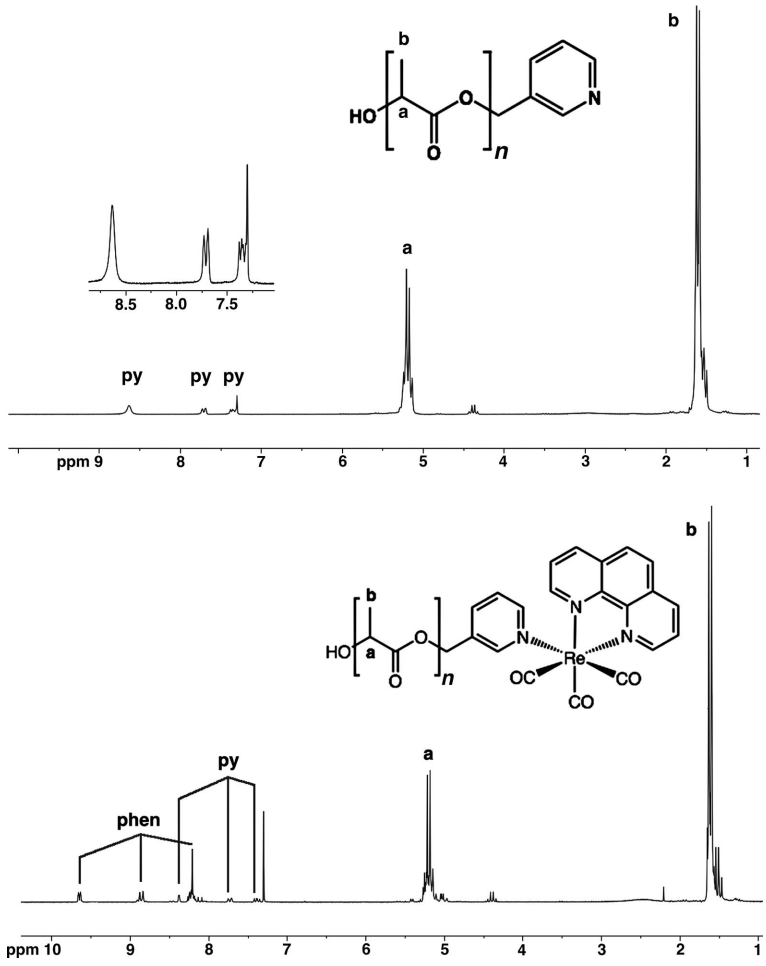


Figure 2. ^{1}H NMR spectra of the polymer (PLA-HMP) before and after coordination to the $Re(CO)_3$(phen)Br complex.

complexes of Ru^{II} serve as light switches for non-aqueous environments, because hydrogen bonding with water quenches the luminescence.[18] To investigate whether something similar applies to the Re^I complex or whether this can be attributed to an effect of the polymer, the free complex was also investigated. For this purpose, fluorescence spectra of $Re(CO)_3$(dppz)Br in acetone, dichloromethane and DMF were recorded. Fluorescence was observed only in DMF, but in very low quantum yields. Absorption and emission values found for the dppz complex are in accordance with the findings of Kurz et al.[8] Comparing the fluorescence data of the free complexes to those of the polymer-bound $Re(CO)_3$(bisimine) cores (Table 2) shows that the complexes retain their absorption and emission properties when attached to the polylactide carrier (Figure 3).

Table 2. Fluorescence and IR spectroscopic data.

	λ_{max} abs. [nm][a]	λ_{max} em. [nm]	ν(CO) [cm^{-1}][b]
[PLA-Re(CO)$_3$(bipy)]OTf	350 nm	572	2035, 1929
[PLA-Re(CO)$_3$(phen)]OTf	340	560	2035, 1926
[PLA-Re(CO)$_3$(dppz)]OTf	–[c]	–[c]	2034, 1912

[a] In acetone solution. [b] KBr pellets. [c] No fluorescence observed.

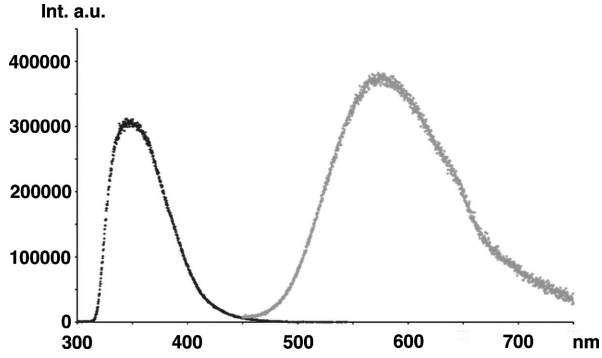


Figure 3. Absorption and emission spectra of [PLA-Re(CO)$_3$-(bipy)]OTf.

Confocal Fluorescence Microscopy

Confocal fluorescence microscopy was used to record images of high spatial resolution without interference from diffracted light. Several images were recorded of polymer **3** and its corresponding free complex, [Re(CO)$_3$(phen)(3-HMP)]OTf, after an incubation period of two hours with 10 μM solutions of the respective compound. Optical slices stacked on top of each other, so-called *z*-stacks, were com-

bined to generate a 3D image to determine the localisation of fluorescent polymer **3** (see Supporting Information).

The images in Figure 4 show the accumulation of the polymer-bound complex in the plasma membranes, which was expected with regard to the lipophilic nature of the polylactide and corresponds to the findings reported by Fernandez-Moreira et al.[19] In contrast, the free complex accumulates within the cells, as shown in Figure 5.

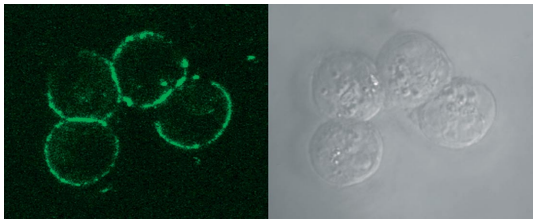


Figure 4. Confocal fluorescence and bright field images of A2780 cells incubated for two hours with compound **3**.

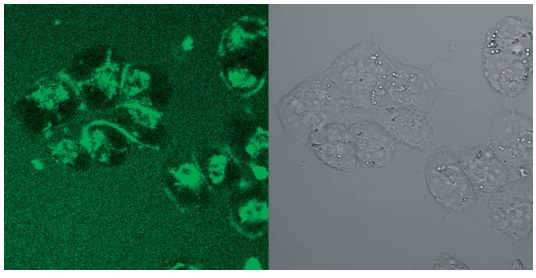


Figure 5. Confocal fluorescence and bright field images of A2780 cells incubated with $[Re(CO)_3(phen)(3\text{-}HMP)]OTf$.

In contrast, confocal images of polymer **3** after an incubation period of 72 h shows increased fluorescence inside the cell and less fluorescence in the cell membranes (Figure 6). This is probably due to hydrolytic degradation of the oligolactide. Hydrolysis of the polymer releases the free complex, which can then enter the cells.

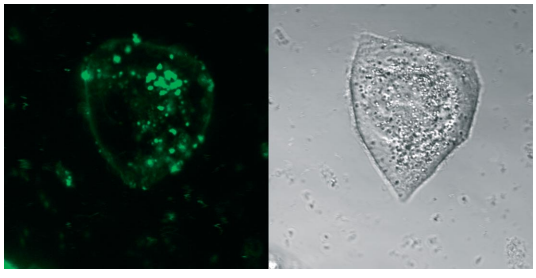


Figure 6. Confocal fluorescence and bright field images of A2780 cells incubated for 72 h with compound **3**.

The degradation kinetics of these polylactides can be calculated as published by van Nostrum et al.[20] The calculations predict a degradation half life of about 15 min in aqueous environment at 37 °C and pH 7.2 (oligolactides with a number of 20 repeating units and a free hydroxy chain end group). This means that hydrolysis would take place fairly quickly upon intravenous injection, giving the polymer little time to accumulate in the tumour. However, integrating the lactides as side chains into larger polymeric carrier systems can solve this problem. Derivatisation of the hydroxy chain end group leads to a degradation half-life of 1.56 h, which is enough for the polymer to accumulate in the tumour tissue.[21]

Cell Membrane Integrity, Viability and Cytotoxicity

Accompanying the confocal microscopy, a trypan blue test was performed in A2780 cells to determine whether the fluorescent polymer disrupts cell membrane integrity. Since this particular dye cannot pass through intact cell membranes, only damaged and dead cells are stained and can thus be distinguished from viable cells. There was no notable trypan blue uptake in the cells after two hours of incubation with compound **3**, as well as with 0.5% DMSO, proving membrane integrity.

The cytotoxicity of **3** as well as that of the free complex $[Re(CO)_3(phen)(3\text{-}HMP)]Br$ towards the human ovarian carcinoma cell line A2780 have been evaluated by MTT assay. After incubation for 72 h with increasing concentrations of either of the two compounds, the IC_{50} was found to be 6 μM for the polymer and 12 μM for the free complex (pIC_{50}: 5.2 ± 0.04 (mean ± SEM) and 4.9 ± 0.03 respectively). For comparison, the IC_{50} of cisplatin is 1.7 μM (pIC_{50}: 5.8).[22] Compound **3** and the free complex $[Re(CO)_3(phen)(3\text{-}HMP)]Br$ show some cytotoxicity in vitro (compared to cisplatin), but the cytotoxicity should be low enough to be suitable for diagnostic purposes in vivo.

Physiological Stability

An important requirement for the in vivo application of the fluorescent polymers is physiological stability for at least the duration of the drug effect. After injection into the bloodstream, these compounds have to be inert against ligand exchange. Typical competing ligands are N- and S-functional groups of proteins, for example, nitrogen-donor ligands like histidines. In order to test the inertness of the complex, two ligand-challenge NMR spectroscopic studies were carried out, in which histidine methyl ester and cysteine methyl ester were added in large excess to $[Re(CO)_3(bipy)(3\text{-}HMP)]OTf$ in $[D_6]DMSO$.[23] ^1H NMR spectra were recorded in increasing time intervals. The ratio of the area under the curve of uncoordinated ligand to the that of sum of coordinated and uncoordinated ligands was calculated to monitor the decomposition of the complex. In the case of histidine, no decomposition was observed within the first four hours. After 24 h about 15% and after a week about 24% decomposed. In the case of cysteine, no decomposition was observed in the first four hours. After 24 h about 13% and after a week about 30% decomposed. Therefore, the polymer conjugate is sufficiently stable towards ligand exchange for the purposes of this application, as the accumulation of macromolecules in the tumour tissue mainly occurs within the first hour.[21]

Conclusions

Bisiminetricarbonyl rhenium conjugates of polylactides with properties suitable for fluorescence microscopy have been prepared. Their monodentate coordination of the organometallic fluorophore to the polymer is sufficiently inert for in vivo application. The polymer itself is degradable into lactic acid in vivo, which is nontoxic and easily metabolised. The MLCT-fluorescence of the bisiminetricarbonyl rhenium complexes shows large Stokes shifts, which precludes reabsorption and makes them distinguishable from tissue autofluorescence. The possibility of using radioactive rhenium or technetium isotopes instead of the stable isotopes, $^{185/187}$Re, makes these compounds interesting for cancer therapy or real-time imaging. The prepared polymers could thus be useful for either therapeutic or diagnostic purposes.

Experimental Section

All chemicals were used as purchased from Aldrich and Fluka unless mentioned otherwise. $[Re(CO)_3(H_2O)_3]Br$,[16] $[Re(CO)_3(bisimine)]Br$[24] and $[Re(CO)_3(bipy)(3\text{-HMP})]OTf$[4,24] were prepared according to literature procedures with minor modifications. Reactions carried out under inert conditions were performed by standard Schlenk techniques. ^{1}H NMR spectra were recorded with a Bruker AM 200 and a Bruker DRX 500 spectrometer. The spectra were calibrated against the residual proton signals of the solvents as internal references. The ESI mass spectra were recorded with a Finnigan LCQ Deca Ion-Trap-API mass spectrometer, MALDI-TOF mass spectra were recorded with a Bruker Ultraflex TOF mass spectrometer. IR spectra were recorded with a Bruker IFS 66 FTIR spectrometer. Fluorescence spectra were recorded with a Fluoromax-3 instrument. Imaging of preincubated cells was performed by means of an Olympus FV1000 Confocal Laser scanning microscope (Olympus GmbH, Hamburg, Germany) equipped with a 60× water immersion objective, NA 1.2. The fluorescent polymer was excited at 405 nm by using a diode laser at an output power of 10%. The fluorescence signal was detected between 540 and 640 nm with a spectral detector.

UV/Vis spectra for the MTT assay were measured with a FLUOstar (bmg, Offenburg) microplate reader. GraphPadPrism© 4.0 software (San Diego, CA, USA) was used to calculate the sigmoidal dose-response curves by means of the four-parameter logistic equation with variable Hill slope for nonlinear regression.

PLA-HMP: 3-(Hydroxymethyl)pyridine (0.1 g, 0.9 mmol) and (3S)-cis-3,6-dimethyl-1,4-dioxane-2,5-dione (2.40 g, 16.6 mmol) were added to dry toluene (50 mL) under a nitrogen atmosphere. The solution was heated to 120 °C, and stannous octoate (50 μL) was added. The solution was heated at reflux for 3 h at 120 °C. The solvent was removed in vacuo, and the residue was dissolved in dichloromethane, which was extracted with dilute HCl, brine and H_2O. The organic layer was separated, dried with Na_2SO_4 and concentrated to approximately 3 mL. The polymer was precipitated and washed with diethyl ether. The product was obtained as a white powder, which was dried under high vacuum. (1.65 g, 66% yield). ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 8.65 (s, 2 H, pyC2-H, pyC6-H), 7.73 (d, $^3J_{HH}$ = 7.9 Hz, 1 H, pyC4-H), 7.37 (m, 1 H, pyC5-H), 5.20 (q, $^3J_{HH}$ = 7.10 Hz, 30 H, PLA-CH-), 1.62 (d, $^3J_{HH}$ = 7.10 Hz, 90 H, PLA-CH_3) ppm. MS (MALDI-TOF): *m*/*z* = 615, 686, 758, 830, 902, 974, 1046, 1118, 1190, 1262, 1334, 1406, 1478, 1551, 1623, 1695, 1767, 1839, 1911, 1983, 2055, 2127, 2199, 2271, 2343, 2415, 2487, 2559, 2631, 2703, 2775, 2847, 2919, 2991, 3064, 3136, 3279, 3423. IR (KBr, excerpt): $\tilde{\nu}$ = 2998, 2948, 1759 cm^{-1}.

General Procedure for the Preparation of [PLA-HMP-Re(CO)$_3$(bisimine)]OTf: $[Re(CO)_3(bisimine)]Br$ (1 equiv.) and silver triflate (1 equiv.) were dissolved in dry acetone under a nitrogen atmosphere and heated at reflux for 1 h. The silver bromide precipitate was filtered out with Celite, and the filtrate was added to an acetone solution of PLA-HMP (0.5 equiv.). The mixture was heated at reflux for 2 h and kept at room temperature for 8 h with stirring. The solvent was removed in vacuo, and the residue was dissolved in the minimum amount of dichloromethane. The product precipitated upon addition of diethyl ether. It was washed twice with diethyl ether and dried under high vacuum.

(bipy): ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 9.13 (d, 2 H, bipyC6-H), 8.78 (d, $^3J_{HH}$ = 8.22 Hz, 2 H, bipyC3-H), 8.34 (m, 2 H, bipyC4-H), 8.26 (d, J_{HH} = 1.72 Hz, 1 H, pyC2-H), 8.08 (m, 1 H, pyC6-H), 7.78 (m, 3 H, bipyC5-H, pyC4-H), 7.41 (m, 1 H, pyC5-H), 5.19 (q, $^3J_{HH}$ = 7.10 Hz, nH, PLA-CH), 1.60 (d, $^3J_{HH}$ = 7.10 Hz, 3nH, PLA-CH_3) ppm. MS (MALDI-TOF): *m*/*z* = 830, 968, 1112, 1256, 1400, 1544, 1688, 1833, 1977, 2121, 2265, 2409, 2553, 2697, 2840, 2986. IR (KBr, excerpt): $\tilde{\nu}$ = 2035, 1929, 1759 cm^{-1}.

(phen): ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 9.64 (m, 2 H, phenC2/9-H), 8.86 (m, 2 H, phenC4/7-H), 8.38 (m, 1 H, pyC2-H), 8.27–8.18 (m, 5 H, phenC5/6-H, pyC6-H, phenC3/8), 7.73 (m, 1 H, pyC4-H), 7.39 (m, 1 H, pyC5-H), 5.14 (q, $^3J_{HH}$ = 7.10 Hz, PLA-CH), 1.62 (d, $^3J_{HH}$ = 7.10 Hz, PLA-CH_3) ppm. MS (MALDI-TOF): *m*/*z* = 1424, 1496, 1568, 1640, 1712, 1785, 1857, 1929, 2001, 2073, 2145, 2217, 2289, 2361, 2433, 2505, 2577, 2721, 2865. IR (KBr, excerpt): $\tilde{\nu}$ = 2035, 1926, 1759 cm^{-1}.

(dppz): For the sparingly soluble $[Re(CO)_3(dppz)]Br$, methanol was used as a solvent, and the heating time was increased to three hours until complete precipitation of silver bromide was observed. The silver bromide was filtered out, and the filtrate was added to a solution of the lactide in acetone. The solution was heated at reflux for two hours and stirred for 72 h. Coordination of the lactide was incomplete, which resulted in an overlap of the NMR signals in the aromatic region. MS (MALDI-TOF): *m*/*z* = 1118, 1190, 1262, 1335, 1406, 1479, *1526*, 1550, 1623, *1670*, 1695, 1767, *1815*, 1839, 1910, *1959*, 1983, *2102*, 2127, 2270, 2414, 2558. IR (KBr, excerpt): $\tilde{\nu}$ = 2034, 1912, 1758 cm^{-1}.

Trypan Blue Test: Fifty thousand cells (A2780) per well were seeded in 24-well plates, grown overnight at 37 °C (5% CO_2) and then exposed to either compound **3** (10 μM), DMSO (0.5%) as a negative control or DMSO (30%) as a positive control. After two hours, the supernatant was removed, and the cells were incubated with a 20:80 (%) mixture of RPMI cell-culture medium and trypan blue solution (0.1% in PBS) for 20 min. The control cells incubated with DMSO (30%) showed a considerable blue staining.

Cytotoxicity Assay: Eleven thousand cells (A2780) per well were seeded into a 96-well plate, incubated at 37 °C (5% CO_2) overnight and exposed to various concentrations of either compound **3** or the corresponding free complex for 72 h. After adding a MTT solution (20 μL, 5 mg/mL in PBS) to each well, cells were incubated at 37 °C (5% CO_2) for approximately 30 min. The supernatant was then removed, and the cells were dissolved in DMSO (50 μL per well). Formazan absorption was measured at 544 nm. Background absorption was measured at 690 nm and subtracted. Sigmoidal dose/response curves were obtained by using the four-parameter logistic equation with variable Hill slope for nonlinear regression.

Supporting Information (see footnote on the first page of this article): The preparation and analysis of $[Re(CO)_3(bipy)]Br$, $[Re(CO)_3$-

(phen)]Br, [Re(CO)$_3$(dppz)]Br and [Re(CO)$_3$(bipy)(3-HMP)]OTf, fluorescence data for [PLA-HMP-Re(CO)$_3$(phen)]OTf and [PLA-HMP-Re(CO)$_3$(dppz)]Br, bright field images of the trypan blue test as well as the data from the MTT assay.

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