Assessing dimerisation degree and cooperativity in a biomimetic small-molecule model by pulsed EPR

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Supporting Information

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1. General experimental information

Moisture- and air-sensitive reactions were performed under an atmosphere of nitrogen with the use of dry solvents, with glass-ware flame dried and cooled under nitrogen. Dry solvents (MeOH, DMF) were purchased from Sigma-Aldrich and were used as supplied. The solvent used for Sonogashira couplings (NEt$_3$) was freshly distilled from CaH$_2$, while all other solvents were used without further purification. Solvents used for Sonogashira coupling were degassed by freeze-pump-thaw cycles ($\times$ 3). Petrol refers to petroleum ether (boiling point 40-60 °C).

Room temperature (rt) refers to 20-25 °C. A temperature of 0 °C was achieved using an ice-water bath. Reflux conditions were achieved using the appropriate DrySyn heating block. The term *in vacuo* refers to the use of rotary evaporator.

Column chromatography was carried out either with silica gel 60 (Crawford Scientific) or with alumina, activated with 4% H$_2$O. Analytical thin layer chromatography was performed using precoated polystyrene TLC sheets or alumina TLC cards (Sigma-Aldrich). TLC visualization was carried out under ultraviolet lamp, followed by staining in 1% aq. KMnO$_4$ or 6% aq. vanillin.

Yields were calculated as amount of product [mmol] divided by the amount of the limiting reagent [mmol] and are given in %.

Melting points were recorded on an Electrothermal 9100 melting point apparatus and are uncorrected. Infrared spectra were acquired on a Shimadzu Fourier transform IR Affinity-1 Infrared spectrometer. NMR spectra were acquired on 300 MHz, Bruker Avance, 400 MHz, Bruker Avance, or 500 MHz, Bruker Ascend spectrometers in the deuterated solvent stated. Chemical shifts are quoted in parts per million (ppm) and referenced to the residual solvent peak(s). The abbreviations s, d, m denote singlet, doublet and multiplet respectively. Coupling constants ($J$) are reported in Hz.

Mass spectrometric ($m/z$) data were acquired by atmospheric pressure chemical ionisation (APCI), nanospray ionisation (NSI) and Matrix Assisted Laser Desorption/Ionisation (MALDI) at the EPSRC National Facility for Mass Spectrometry, Swansea.
2. Synthesis and characterisation of compound 1

(4'-iodo-[1,1'-biphenyl]-4-yl-1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrole-3-carboxylate)

Scheme S1: Synthesis of compound 1.

The title compound (0.58 g, 1.27 mmol, 80%) was prepared as described\(^1\) with minor modifications. 1-oxyl-2,2,5,5-tetramethyl-pyrroli-3-carboxylic acid (TPC) (0.30 g, 1.63 mmol) was dissolved in CH\(_2\)Cl\(_2\) (15 mL) and the solution was left stirring at 0 °C for 10 min. 4-hydroxy-4'-iodobiphenyl (0.53 g, 1.8 mmol), DMAP (0.02 g, 0.16 mmol) and EDCI.HCl (0.33 g, 1.72 mmol) were added and the reaction mixture was left stirring at 0 °C for 5 min and then at rt for 3 days in the dark. The solvent was removed in vacuo, the crude product was redissolved in CH\(_2\)Cl\(_2\) (20 mL), washed with sat. NaHCO\(_3\) (2 × 20 mL) and dist. H\(_2\)O (2 × 20 mL) and dried over Na\(_2\)SO\(_4\). The layers were separated and removal of the solvent in vacuo yielded the crude product, which was purified by column chromatography on alumina (2.5 × 16 cm, 4% H\(_2\)O) using petrol/EtOAc (8/2) as eluent to give the title compound (0.58 g, 1.27 mmol, 80%) as an intense yellow powder. Single crystals suitable for X-ray were obtained by recrystallisation from chloroform.

Formula: C\(_{21}\)H\(_{21}\)INO\(_3\).

\(v_{\text{max}}/\text{cm}^{-1}\) 754s, 802s, 999s, 1026s, 1168s, 1201s, 1730 (C=O), 2796s.

\(\delta^H (500 \text{ MHz; CD}_2\text{Cl}_2)\): 1.29 (1 H, s, 6C\(_\text{H}_3\)), 7.25 (2 H, s, ArH), 7.37 (2 H, d, ArH), 7.64 (2 H, s, ArH), 7.81 (2 H, d, ArH).

\(\delta^C (500 \text{ MHz; CD}_2\text{Cl}_2)\): 30.21 (6 C, (C\(_\text{H}_3\))\(_2\)), 31.14 (6 C, (C\(_\text{H}_3\))\(_2\)), 76.26 (1 C, C(CH\(_3\))\(_2\)), 78.57 (1 C, C(CH\(_3\))\(_2\)), 93.74 (1 C, IC(CH\(_3\))), 122.38 (2 C, CH\(_2\)(CH)CO), 128.56 (2 C, C(CH)(CH)), 129.44 (2 C, CH(CH)C), 134.88 (1 C, OCC\(_\text{vin}\)), 138.48 (2 C, IC(CH)), 138.82 (1 C, CC(CH\(_3\))\(_2\)), 140.03 (1 C, (CH\(_2\))\(_2\)CC), 144.13 (1 C, CCH\(_\text{vin}\)), 150.19 (1 C, CHCO), 160.21 (1 C, OCO).

\(m/z\) (APCI\(^+\)) 463 ([M + H\(^+\)], 100%); HRMS (APCI\(^+\)) C\(_{21}\)H\(_{22}\)INO\(_3\) ([M + H\(^+\)], found 463.0631, requires 463.0639 (− 1.7 ppm).
Found: C, 54.60; H, 4.42; N, 3.15. Calc. for C_{21}H_{21}INO_{3}: C, 54.56; H, 4.58; N, 3.03.

**Fig. S1:** IR spectrum of compound 1.

**Fig. S2:** Mass spectrum of compound 1.
Fig. S3: $^1$H NMR spectrum of compound 1.

Fig. S4: $^1$H NMR spectrum of compound 1 after reduction with phenylhydrazine.
**Fig. S5:** \(^{13}\)C NMR spectrum of the reduced (by phenylhydrazine) compound 1.

Crystal structure experimental details of compound 1:

A. Crystal Data

- **Empirical Formula**: C\(_{21}\)H\(_{21}\)INO\(_3\)
- **Formula Weight**: 462.31
- **Crystal Color, Habit**: yellow, prism
- **Crystal Dimensions**: 0.180 X 0.080 X 0.070 mm
- **Crystal System**: triclinic
- **Lattice Type**: Primitive
- **Lattice Parameters**:
  - a = 11.5839(13) Å
  - b = 15.7615(16) Å
  - c = 32.762(3) Å
  - α = 91.604(3)°
  - β = 91.020(3)°
  - γ = 101.055(3)°
  - V = 5866.7(10) Å\(^3\)
- **Space Group**: P-1 (#2)
- **Z value**: 12
B. Intensity Measurements

Diffractometer: XtaLAB P200
Radiation: MoKα (λ = 0.71075 Å)
multi-layer mirror monochromated

Voltage, Current: 45kV, 66mA
Temperature: -100.0°C
Detector Aperture: 83.8 x 70.0 mm
Data Images: 1800 exposures

ω oscillation Range (χ=45.0, φ=0.0): -100.0 - 80.0°
Exposure Rate: 5.0 sec./°
Detector Swing Angle: -10.42°

ω oscillation Range (χ=45.0, φ=90.0): -100.0 - 80.0°
Exposure Rate: 5.0 sec./°
Detector Swing Angle: -10.42°

ω oscillation Range (χ=45.0, φ=180.0): -100.0 - 80.0°
Exposure Rate: 5.0 sec./°
Detector Swing Angle: -10.42°

Exposure Rate
Detector Swing Angle
Detector Position: 45.02 mm
Pixel Size: 0.086 mm
2θmax: 50.8°

No. of Reflections Measured
Total: 114568
Unique: 21380 (Rint = 0.0397)

Corrections: Lorentz-polarization
Absorption
(trans. factors: 0.780 - 0.890)

C. Structure Solution and Refinement

Structure Solution
Direct Methods (SIR2011)

Refinement
Full-matrix least-squares on $F^2$

Function Minimized
$\Sigma w (Fo^2 - Fe^2)^2$

Least Squares Weights
\begin{equation}
    w = 1/ \left[ \sigma^2(Fo^2) + (0.0547 \cdot P)^2 \\
    + 2.4315 \cdot P \right]
\end{equation}
where $P = (\text{Max}(Fo^2,0) + 2Fe^2)/3$

$2\theta_{\text{max}}$ cutoff
50.8°

Anomalous Dispersion
All non-hydrogen atoms

No. Observations (All reflections)
21380

No. Variables
1478

Reflection/Parameter Ratio
14.47

Residuals: R1 (I>2.00$\sigma$(I))
0.0267

Residuals: R (All reflections)
0.0583

Residuals: wR2 (All reflections)
0.1042

Goodness of Fit Indicator
0.868

Max Shift/Error in Final Cycle
0.002

Maximum peak in Final Diff. Map
0.89 e$^{-}\text{Å}^3$

Minimum peak in Final Diff. Map
-0.84 e$^{-}\text{Å}^3$
3. Synthesis and characterisation of compound 2

(4′-[4-[2-(trimethylsilyl)-1-ethynyl]phenyl]-2,2′:6′,2″-terpyridine)

Scheme S2: Synthesis of compound 2.

The synthesis of 4-[(trimethylsilyl)ethynyl]benzaldehyde was performed as described\(^2\) with minor modifications. Trimethylsilylacetylene (TMS-acetylene) (5.7 mL, 41.0 mmol) and PPh\(_3\) (0.22 g, 0.83 mmol) were dissolved in NEt\(_3\) (15 mL) and degassed by freeze-pump-thaw cycles (× 3). 4-bromobenzaldehyde (5.0 g, 27.0 mmol), PdCl\(_2\)(PPh\(_3\))\(_2\) (0.24 g, 0.34 mmol) and CuI (0.09 g, 0.49 mmol) were dissolved in NEt\(_3\) (70 mL) and were degassed by freeze-pump-thaw cycles (× 4). The solution of TMS-acetylene was added drop-wise to the solution of the aldehyde over the course of 30 min and the reaction was left stirring at 88 °C for 2 h. After cooling to rt, the solvent was removed *in vacuo* and the black crude product was redissolved in CH\(_2\)Cl\(_2\) (150 mL), washed with dist. H\(_2\)O (2 × 150 mL) and brine (150 mL). The layers were separated, the organic phase was dried over Na\(_2\)SO\(_4\), the solvent was removed *in vacuo* and the crude product was purified by column chromatography on silica (4 × 8 cm, eluent petrol/EtOAc (1/1)) and further sublimated at 45 °C to yield the title compound (3.84 g, 19 mmol, 70%) as white crystalline solid.

Formula: C\(_{12}\)H\(_{14}\)OSi.

δ\(_H\) (300 MHz; CDCl\(_3\)) 0.27 (9 H, s, SiCH\(_3\)), 7.62 (2 H, d, J 8, ArH), 7.82 (2 H, d, J 8, ArH), 10.00 (1 H, s, CHO). The proton chemical shifts are in agreement with published data.\(^2\)

4-[(trimethylsilyl)ethynyl]benzaldehyde (3.83 g, 19 mmol) was treated with 0.1 eq. of K\(_2\)CO\(_3\) (0.26 g, 1.9 mmol) in anhydrous MeOH (30 mL) under an atmosphere of N\(_2\) as described\(^2\) with minor modifications. The solution was left stirring overnight at rt, the solvent was removed *in vacuo*, the
orange crude product was redissolved in CH$_2$Cl$_2$ (70 mL) and washed with sat. NaHCO$_3$ (3 × 70 mL),
dist. H$_2$O (2 × 70 mL) and brine (70 mL). The layers were separated, the organic phase was dried over
MgSO$_4$, the solvent was removed in vacuo and the crude product obtained was purified by
sublimation at 50 °C yielding 4-ethynylbenzaldehyde (1.96 g, 15.0 mmol, 78%) as white powder.

Formula: C$_9$H$_6$O.

δ$_H$(300 MHz; CDCl$_3$) 3.30 (1 H, s, CCH), 7.64 (2 H, d, $J_{8, \text{Ar}H}$), 7.85 (2 H, d, $J_{8, \text{Ar}H}$), 10.02 (1 H,
s, CHO). The proton chemical shifts are in agreement with published data.$^2$

4'- (4-ethynylphenyl)-2,2';6',2''-terpyridine was prepared according to the procedure described
for similar terpyridine based compounds$^3$ with minor modifications. NaOH pellets (0.91 g, 22.7 mmol)
were suspended in PEG-300 (30 mL) and after cooling of the solution to 0 °C, 2-acetylpyridine (4.2
mL, 32.0 mmol) was added. The solution was left stirring at 0 °C for 10 min and the previously
synthesised 4-ethynylbenzaldehyde (1.96 g, 15.0 mmol) was added. The mixture was left stirring at
0 °C for 1 h and at rt for further 2 h. During this time the colour of the solution turned from yellow to
brown. 35% conc. NH$_3$ (39 mL, 979 mmol) was added and a red solid formed immediately. The
solution was left stirring overnight at rt. The pink precipitate formed was filtered and washed with
chilled EtOH (3 × 30 mL) yielding the product (1.55 g, 4.7 mmol, 31%) as a beige powder.

Formula: C$_{23}$H$_{15}$N$_3$.

ν$_{max}$/cm$^{-1}$ 792s, 831s, 1002s, 1165br, 1288s, 1600s (-C≡CH), 1735s. The IR data are in agreement with
published data.$^4$

δ$_H$(500 MHz; CDCl$_3$) 3.19 (1 H, s, CCH), 7.32 (2 H, ddd, $J_{2.5, J_2, 8, \text{Ar}H}$), 7.59 – 7.67 (2 H, d, $J_{2, ArH}$), 7.84 – 7.92 (4 H, m, $J_{2, J_2, 8, \text{Ar}H}$), 8.67 (2 H, d, $J_{8, \text{Ar}H}$), 8.70 – 8.76 (4 H, m, $ArH$). The
proton chemical shifts are in agreement with published data.$^4$

m/z (NSI$^+$) 334 ([M + H]$^+$, 100%); HRMS (NSI$^+$) C$_{23}$H$_{16}$N$_3$ ([M + H]$^+$), found 334.1333, requires
334.1339 (+ 1.0 ppm).
Fig. S6: IR spectrum of compound 2.

Fig. S7: Mass spectrum of compound 2.
4. Synthesis and characterisation of compound 3

\( (4\text{'}-(4\text{'}-(2,2\text{'}:6\text{'}:2\text{''}-\text{terpyridin}-4\text{'}-yl)phenyl)ethynyl)-1\text{'}-biphenyl]-4-yl-1-oxyl-2,2,5,5-
\text{tetramethyl}-2,5-\text{dihydro}-1\text{H}-\text{pyrrole-3-carboxylate} \)

A solution of 4'-iodo-[1,1'-biphenyl]-4-yl-1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1\text{H}-pyrrole-3-carboxylate 1 (0.23 g, 0.5 mmol) and PdCl\(_2\)(PPh\(_3\))\(_2\) (0.06 g, 0.08 mmol) in NEt\(_3\) (80 mL) and anhydrous DMF (20 mL) were degassed with freeze-pump-thaw cycles (× 4). 4'-[4-[2-(trimethylsilyl)-1-ethynyl]phenyl]-2,2':6',2''-terpyridine 2 (0.21 g, 0.63 mmol) and PPh\(_3\) (0.06 g, 0.23 mmol) in NEt\(_3\) (20 mL) and anhydrous DMF (5 mL) were degassed with freeze-pump-thaw cycles (× 4). CuI (0.06 g, 0.31 mmol) was added to the first solution followed by drop-wise addition of the first solution to the second over the course of 20 min. The resulting orange solution was left stirring overnight at rt and then heated to 60 °C for 2 h. After cooling of the solution to rt the solvents were removed in vacuo, the solid was redissolved in CH\(_2\)Cl\(_2\) (100 mL) and washed with H\(_2\)O (4 × 100 mL) and brine (4 × 100 mL). The phases were separated, the organic phase was dried over Na\(_2\)SO\(_4\) and the solvent was removed in vacuo. The crude product was purified by column chromatography on alumina (2 × 9 cm, 4% H\(_2\)O) using CH\(_2\)Cl\(_2\)/MeOH (9.7/0.3)) as eluent. The oily product was taken up in CH\(_2\)Cl\(_2\) and addition of hexane led to precipitation of the title compound, which was isolated as a beige powder. The synthesis was repeated multiple times with varying yields (18-89%). Single crystals suitable for X-ray were obtained by layering a chloroform solution of 3 with hexane.

Formula: C\(_{44}\)H\(_{35}\)N\(_4\)O\(_3\).

m.p. 188 °C.

\( \nu_{\text{max}}/\text{cm}^{-1} \) 734s, 790s, 837s, 1002s, 1166s, 1286s, 1346s, 1386s, 1465s, 1508s, 1583s, 1735s (C=O).

\( \delta_{\text{H}} \) (400 MHz; CD\(_2\)Cl\(_2\)) 7.37 (1 H, ddd, \( J = 1, 2.7, 12.3 \), ArH), 7.62 – 7.79 (6 H, m, \( J = 8.7, 32 \), ArH), 7.85 – 7.96 (3 H, m, \( J = 8, 2, 2 \), ArH), 8.64 – 8.74 (3 H, m, \( J = 4, 8 \), ArH), 8.78 (1 H, s, ArH).

Reduction of the nitroxyl radical by phenylhydrazine led to decomposition of the compound.

\( \text{m/z} \) (NSI\(^+\)) 668 ([M + H]\(^+\), 100%); HRMS (NSI\(^+\)) C\(_{44}\)H\(_{36}\)N\(_4\)O\(_3\) ([M + H]\(^+\)), found 668.2773, requires 668.2782 (− 1.3 ppm).

Found: C, 78.84; H, 5.35; N, 8.28. Calc. for C\(_{44}\)H\(_{35}\)N\(_4\)O\(_3\): C, 79.14; H, 5.28; N, 8.39.
**Fig. S8:** IR spectrum of compound 3.

**Fig. S9:** Mass spectrum of compound 3.
Fig. S10: $^1$H NMR spectrum of compound 3.

Crystal structure experimental details of compound 3:

A. Crystal Data

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<th>Value</th>
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</tr>
<tr>
<td>Formula Weight</td>
<td>710.25</td>
</tr>
<tr>
<td>Crystal Color, Habit</td>
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<tr>
<td>Crystal Dimensions</td>
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<td>Lattice Type</td>
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<tr>
<td>Lattice Parameters</td>
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<tr>
<td></td>
<td>c = 5.6924(19) Å</td>
</tr>
<tr>
<td></td>
<td>V = 7678(4) Å$^3$</td>
</tr>
<tr>
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<tr>
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<tr>
<td>D$_{\text{calc}}$</td>
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<tr>
<td>F000</td>
<td>2976.00</td>
</tr>
<tr>
<td>$\mu$(CuK$\alpha$)</td>
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</tr>
</tbody>
</table>

B. Intensity Measurements
Diffractometer: XtaLAB P200
Radiation: CuKα (λ = 1.54187 Å)
   multi-layer mirror monochromated
Voltage, Current: 40kV, 30mA
Temperature: -148.0°C
Detector Aperture: 83.8 x 70.0 mm
Data Images: 3112 exposures
Pixel Size: 0.172 mm
θ_max: 136.4°
No. of Reflections Measured:
   Total: 59425
   Unique: 6909 (R_int = 0.1388)
Corrections:
   Lorentz-polarization
   Absorption
   (trans. factors: 0.440 - 0.976)

C. Structure Solution and Refinement
Structure Solution: Direct Methods (SIR2011)
Refinement: Full-matrix least-squares on F^2
Function Minimized:
   Σ w (Fo^2 - Fc^2)^2
Least Squares Weights:
   w = 1/ [ σ^2(Fo^2) + (0.2000 · P)^2 
   + 0.0000 · P ]
   where P = (Max(Fo^2,0) + 2Fc^2)/3
2θ_max cutoff: 136.5°
Anomalous Dispersion: All non-hydrogen atoms
No. Observations (All reflections): 6909
No. Variables: 527
Reflection/Parameter Ratio: 13.11
Residuals: R1 (I>2.00σ(I)):
   0.1365
Residuals: R (All reflections):
   0.1648
Residuals: wR2 (All reflections):
   0.4042
Goodness of Fit Indicator:
   1.612
Max Shift/Error in Final Cycle:
   0.084
Maximum peak in Final Diff. Map:
   1.65 e^-Å^3
Minimum peak in Final Diff. Map:
   -0.67 e^-Å^3
5. Complexation of Zn(II) and 3 – Formation of the bis-complex
(Zn(tpyNO)$_2$)$^{2+}$)

Scheme S3: Synthesis of complex Zn(tpyNO)$_2$(PF$_6$)$_2$.

Zinc(II) chloride (0.003 g, 0.02 mmol) was dissolved in EtOH (3 mL) and ligand 3 (0.027 g, 0.04 mmol) was dissolved in CH$_2$Cl$_2$ (3 mL). After stirring of both solutions at rt for 15 min, the Zn(II) chloride solution was added to the ligand solution and the colour of the latter turned pale yellow. The reaction mixture was left stirring at rt for 1 h, then at 60 °C overnight. After cooling to rt excess NH$_4$PF$_6$ (0.04 g, 0.25 mmol) was added. A precipitate started forming immediately and the reaction mixture was left stirring at rt for overnight. The product (0.025 g, 0.015 mmol, 74%) was isolated with filtration as pale yellow powder. An alternative preparation without overnight refluxing (“mix-and-measure”) used for the titration experiment is detailed in the next chapter. For EPR sample preparation, a ten-fold excess of NaBPh$_4$ was added for increased solubility.

Formula: C$_{88}$H$_{70}$N$_8$O$_6$ZnF$_{12}$P$_2$.

m.p. >300 °C.

$\nu_{\text{max}}$/cm$^{-1}$ 790s, 830s, 1002s, 1166s, 1288s, 1427s, 1473s, 1600s, 1732s (C=O).

$m/z$ (MALDI - DCTB) 1400 ([M – 2PF$_6$]$^+$, 100%).
Fig. S11: IR spectrum of Zn(tpyNO)$_2$(PF$_6$)$_2$.

Fig. S12: Mass spectrum of Zn(tpyNO)$_2$$^{2+}$ revealing the formation of the bis-complex.
6. Optimisation of complex and sample preparation for EPR measurements

Fig. S13: Raw (left) and background-corrected (right) time traces comparing preparation methods for the Zn(II)/tpyNO complex used for titration.

Two preparation methods were tested: the reaction batch was either refluxed overnight and precipitated with hexafluorophosphate or just mixed for 1 minute by pipetting up and down before drying under vacuum. After background-correction both preparation methods gave virtually the same modulation depth. Thus, the titration samples were prepared according to the “mix-and-measure” method.

A total of 11 samples was prepared for the EPR titration experiment. The concentration of the ligand was kept constant at 100 µM final concentration, while the Zn(II)/ligand molar ratio was varied from 0.0 to 1.0 in 0.1 steps. The final volume of each reaction batch was kept constant at 100 µl (50 µl EtOH/50 µl DCM). Upon mixing the constituents by pipetting up and down for 1 minute, the solution was dried under vacuum. NaBPh₄ was added to the dried sample in ~10-fold excess before resolubilisation in 80 µl DMSO-d₆. 20 µl of a 1:1 D₂O:ethylene glycol mixture was added slowly to avoid any precipitation. The solution was briefly mixed by pipetting up and down, filled into a 4 mm EPR quartz tube (Wilmad) and immediately frozen in liquid nitrogen. A second titration series consisting of an independent set of 11 samples was prepared as described above.
7. EPR instrumentation and collection of PELDOR data

PELDOR data were recorded on an ELEXSYS E580 pulsed X-band EPR spectrometer (~9 GHz) including the second frequency option (E580-400U) from Bruker. Pulses were amplified by a 1 kW travelling wave tube (TWT) amplifier (Applied Systems Engineering). An MD5 dielectric ring resonator with standard flex line probe head was used. The established 4-pulse DEER pulse sequence $\frac{\pi}{2}(\nu_A)-\tau_1-\pi(\nu_A)-\tau_1+\tau-\pi(\nu_B)-(\tau_2-\tau)-\pi(\nu_A)-\tau_2$-echo was employed for all PELDOR experiments.\(^5\) With the following exceptions of timings and pump pulse length chosen settings and optimisation procedures were as previously described.\(^6\) For all samples, the pump pulse was set to 18 ns, $\tau_1$ to 380 ns, $\tau_2$ to 3.55 $\mu$s to measure more than one full modulation (6 $\mu$s in addition for samples with ratios 0.1, 0.5, and 1) and the shot repetition time to 3 ms, averaging the data for $\sim$35 min per sample (5 scans on average; longer averaging time for the 6 $\mu$s time window). Samples of the first titration series were measured three times, independently, to estimate the measurement error from the standard deviation, while the second titration series served as an indication of the deviations between sample preparations.
8. EPR titration series – additional PELDOR data

Given are the raw PELDOR data (Fig. S14) and corresponding distance distributions (Fig. S15) from the titration experiment (first measurement series). In addition to the main peak some artefacts potentially resulting from a worse signal-to-noise ratio could be observed in low-ratio samples (0.1 - 0.3) only. Even when using a longer time window (Fig. S16; 6 µs instead of 3.55 µs) the PELDOR experiment at ratio 0.1 showed some artefacts.

Fig. S14: Waterfall plot of the 11 raw PELDOR traces (no background correction) from the titration experiment.

Fig. S15: Derived distance distributions from the titration series as obtained by Tikhonov regularisation.
Fig. S16: Raw (top) and background-corrected (middle; using the monomer density value as described in the main text) time traces with 6 µs time window. Bottom Derived distance distributions. Note the artefact at ~2.9 nm for ratio 0.1 only.
9. Models for Zn(II)/tpy bis-complex formation

Cooperativity models: none; negative; positive
Experimental data: EPR (this study); calculated from

Fig. S17: Experimental modulation depths $\Delta$ (EPR – this study; re-calculated from NMR data) and respective cooperativity models, all based on our experimentally obtained maximum modulation depth of 0.33 at a ratio of 0.5 (first measurement series), and the corresponding error of fit (root mean square deviation).
10. **Effect of background correction and sample preparation**

![Graph showing effect of different background correction strategies](image)

**Fig. S18:** Effect of different background correction strategies using three independent measurements per sample. Results obtained with the ‘true’ background correction based on the decay constant of the monomer are shown in black. The (trained) eye-based background correction (blue) shows slightly larger differences compared to the monomer-informed correction. The background correction based on the DeerAnalysis fit option (green) shows the largest deviations, consistently leading to a larger modulation depth.
Fig. S19: Effect of different sample preparations on the observed titration curve. A second titration series gave a modulation depth of 0.30 at ratio 0.5, instead of the previously observed mean modulation depth of 0.32 for the first series (independent set of 3 measurements per sample). The shape of the titration curve is well reproducible though. We conclude that in the present case the error introduced by individual sample preparation is larger than the PELDOR measurement error within the same series (compare to Fig. S18).

Table S1: Comparison of experimentally obtained standard deviation (SD; set of 3 independent measurements per sample in series 1), error of fit (RMSD; first measurement round in series 1), and the difference in Δ between titration series 1 and 2.

<table>
<thead>
<tr>
<th>Ratio</th>
<th>SD</th>
<th>RMSD</th>
<th>Δ1 − Δ2</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>0.003</td>
<td>0.004</td>
<td>0.011</td>
</tr>
<tr>
<td>0.1</td>
<td>0.003</td>
<td>0.006</td>
<td>0.021</td>
</tr>
<tr>
<td>0.2</td>
<td>0.004</td>
<td>0.007</td>
<td>0.054</td>
</tr>
<tr>
<td>0.3</td>
<td>0.003</td>
<td>0.007</td>
<td>0.012</td>
</tr>
<tr>
<td>0.4</td>
<td>0.009</td>
<td>0.011</td>
<td>-0.006</td>
</tr>
<tr>
<td>0.5</td>
<td>0.008</td>
<td>0.014</td>
<td>0.016</td>
</tr>
<tr>
<td>0.6</td>
<td>0.010</td>
<td>0.016</td>
<td>0.056</td>
</tr>
<tr>
<td>0.7</td>
<td>0.002</td>
<td>0.013</td>
<td>0.048</td>
</tr>
<tr>
<td>0.8</td>
<td>0.003</td>
<td>0.011</td>
<td>0.064</td>
</tr>
<tr>
<td>0.9</td>
<td>0.004</td>
<td>0.010</td>
<td>0.055</td>
</tr>
<tr>
<td>1.0</td>
<td>0.001</td>
<td>0.008</td>
<td>0.066</td>
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</table>
11. Determination of dissociation constant and error propagation

While in this study we are investigating a biomimetic model system, recent work from our lab demonstrates that also biological systems can show modulation depths in good agreement with binding constants from other methods. For a non-templated dimerisation system such as a protein dimer, the dissociation constant $K_D$ can in principle be derived from the PELDOR modulation depth and the sample concentration provided a decent signal-to-noise ratio of the PELDOR trace and a reliable background correction.

The fraction of spin labels in dimers $x_D$ can be given as:

$$x_D = \frac{\Delta}{\lambda}$$

and the fraction of spin labels in monomers $x_M$ as:

$$x_M = 1 - \frac{\Delta}{\lambda}.$$

The dissociation constant $K_D$ can be expressed using the concentrations of monomer and dimer as follows:

$$K_D = \frac{[M]^2}{[D]}.$$

Thus, $K_D$ can be expressed as a function of the sample concentration $c_S$, the experimental modulation depth $\Delta$, and the pump efficiency $\lambda$:

$$K_D = \frac{2c_S \lambda (\frac{\Delta}{\lambda} - 1)^2}{\Delta}.$$

For an estimation of the error in the determination of $K_D$ from our titration curve, we assumed an error in the sample concentration of 5%, and a $\lambda$ of 0.33 ± 0.01. For each titration step between ratio 0.0 and ratio 0.4 the experimentally obtained modulation depth and its corresponding error of fit (root mean square deviation) was used (Fig. S20, black line, Table S2). In addition, a second, theoretical set of parameters was analysed, assuming the same sample concentration, but a $\lambda$ of 0.40 ± 0.02, which may come closer to values obtained for a biological system. The modulation depth was varied from 0.05 to 0.35 in 0.05 steps, with an assumed error in $\Delta$ of 0.01 (Fig. S20, red line, Table S2). As expected the $K_D$ will be most reliable when the sample concentration is in the same order of magnitude.
Fig. S20: Reliability of $K_D$ determination. Details are given within the text.

Table S2: $K_D$ determination

<table>
<thead>
<tr>
<th>$\Delta/\lambda$</th>
<th>$K_D$</th>
<th>Error</th>
<th>$\Delta/\lambda$</th>
<th>$K_D$</th>
<th>Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.083</td>
<td>$2.00 \times 10^{-3}$</td>
<td>$3.8 \times 10^{-4}$</td>
<td>0.125</td>
<td>$1.20 \times 10^{-3}$</td>
<td>$3.3 \times 10^{-4}$</td>
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<tr>
<td>0.255</td>
<td>$4.34 \times 10^{-4}$</td>
<td>$6.1 \times 10^{-5}$</td>
<td>0.250</td>
<td>$4.50 \times 10^{-4}$</td>
<td>$8.7 \times 10^{-5}$</td>
</tr>
<tr>
<td>0.406</td>
<td>$1.74 \times 10^{-4}$</td>
<td>$2.7 \times 10^{-5}$</td>
<td>0.375</td>
<td>$2.08 \times 10^{-4}$</td>
<td>$4.0 \times 10^{-5}$</td>
</tr>
<tr>
<td>0.520</td>
<td>$8.9 \times 10^{-5}$</td>
<td>$1.5 \times 10^{-5}$</td>
<td>0.500</td>
<td>$1.00 \times 10^{-4}$</td>
<td>$2.2 \times 10^{-5}$</td>
</tr>
<tr>
<td>0.652</td>
<td>$3.7 \times 10^{-5}$</td>
<td>$1.1 \times 10^{-5}$</td>
<td>0.625</td>
<td>$4.5 \times 10^{-5}$</td>
<td>$1.3 \times 10^{-5}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.750</td>
<td>$1.67 \times 10^{-5}$</td>
<td>$7.1 \times 10^{-6}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.875</td>
<td>$3.6 \times 10^{-6}$</td>
<td>$3.1 \times 10^{-6}$</td>
</tr>
</tbody>
</table>

In the above error propagation, the total error of the determination in the dissociation constant was estimated from the individual uncertainties in sample concentration, experimental $\Delta$, and the pump efficiency $\lambda$. Assuming that the uncertainty in $\Delta$ ($\sigma_{\Delta}$) dominates, the uncertainty in the determination of $K_D$ ($\sigma_K$) can be simplified to:

$$\sigma_K = \frac{2 \, c_s \, (\Delta^2 - \lambda^2)}{\Delta^2 \, \lambda} \, \sigma_{\Delta} \, .$$
12. References


