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Self-assembled nanoparticles as multifunctional drugs for anti-microbial therapies

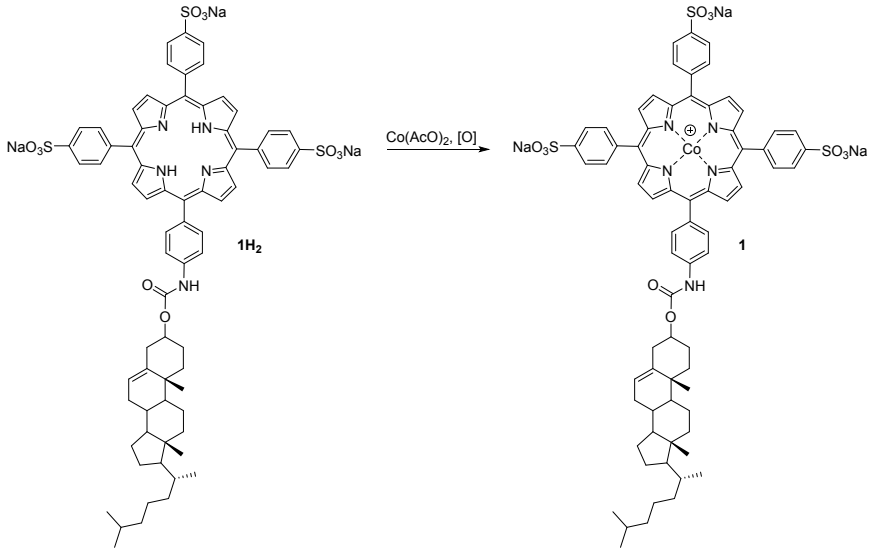
Manpreet Bhatti, Timothy D. McHugh, Lilia Milanesi, and Salvador Tomas

Supplementary Information

General experimental methods. Chemicals and solvents were obtained from commercial sources and used without further purification. Chromatographic purification was performed on a self-packed column using Silica 60A, particle size 40-63 microns as a stationery phase. UV-Vis absorbance spectra were recorded with a Thermoelectron Corporation UV1 Thermospectronic UV-Vis spectrometer. ^{1}H NMR spectroscopy was performed with a Bruker AMX 500 instrument. Stock solution of amphiphile **1** (5.00 mM) and pyrazinoic acid ester **2** (50.0 mM) were generated by dissolving the appropriate amount of the compounds in HPLC-grade methanol. These stock solutions were kept at -20 ^{0}C and used for the preparation of all the samples within a month of the stock preparation. In these conditions the compounds do not experience methanolysis to any detectable degree. In all cases, the working buffer was a solution of NaCl 100 mM in water with the pH adjusted to 5.6 using HCl 1M.

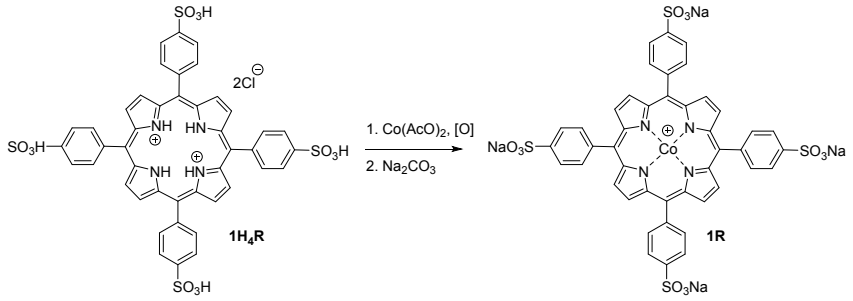
Synthesis. Porphyrin **1H$_4$R** (Supplementary Fig. 2) and pyrazionic acid were obtained from commercial sources. Porphyrin **1H$_2$** (Supplementary Fig. 1) was synthesized using a method described elsewhere (see ref. 15 in the main text).

Synthesis of amphiphile 1. (Supplementary Fig. 1) Amphiphile **1** was synthesized by dissolving 15.0 mg (0.016mmol) of porphyrin **1H$_2$** in 2 mL of MeOH containing 15.0 mg (0.085mmol) of Co(AcO)$_2$. The mixture was stirred at room temperature under nitrogen atmosphere for 18 hours. After this time, the methanol was removed under pressure and the solid was re-disolved into 2.5 mL of distilled water. The solution was applied to PD-10 desalting column and eluted with distilled water to remove excess Co salts and acetic acid. The solvent was removed under reduced pressure, yielding 12.7 mg of amphiphile **1** as a deep red solid (85 % yield). ^{1}H NMR (500 MHz, CD_3OD) δ = 8.93 (d, J = 4.6 Hz, 2H), 8.83 (s, 4H) 8.84 (d, J = 4.6 Hz, 2H), 8.29 (d, J = 8.0 Hz, 6H), 8.24 (d, J = 8.0 Hz, 6H), 8.16 (d, J = 8.6 Hz, 2H), 7.88 (d, J = 8.6 Hz, 2H), 5.04 (broad, 1H, 6-CH-cholesterol), 4.46 (m, 1H, 3-CH-cholesterol), 0.6-2.5 (m, 43H, cholesterol protons) ppm. HRMS (Cl^+) m/z: found 1423.3480; calcd. 1423.3437 $(M+H_2O)^+$



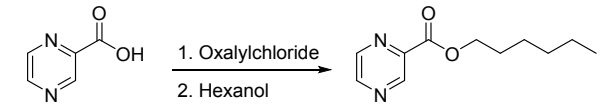
Supplementary Figure 1. Synthesis of amphiphilic porphyrin **1**

Synthesis of 1R. (Supplementary Fig. 2) 100 mg of **$1H_4R$** (0.10 mmol) were dissolved in 5 mL of methanol to which 177 mg of $Co(AcO)_2$ (1 mmol) were added. The solution was stirred under N_2 atmosphere for 18 hours, after which time the solvent was evaporated under reduced pressure. The solid was re-dissolved in 5 mL of a solution of Na_2CO_3 0.5 M in water. A red precipitated of Co salts is formed and was removed by centrifugation. The solvent was removed under reduced pressure and re-suspended in 5 mL of methanol. Excess Na_2CO_3 was removed by filtration and the solvent removed under reduced pressure. The crude was further purified by means of preparative RP-HPLC, using water as eluent. The fractions containing the porphyrin were evaporated, yielding a deep red solid (64.8 mg, 60% yield of tretrasodium salt). ^{1}H NMR (500 MHz, D_2O) δ = 7.35 (t, *J* = 0.8 Hz, 2H), 7.00 (t, *J* = 1.3 Hz, 2H), 6.84 (t, *J* = 1.1 Hz, 2H), 4.31 (s, 4H) ppm. HRMS (CI^+) m/z: found 1097.9359; calcd. 1097.9330 $(M+HDO)^+$



Supplementary Figure 2. Synthesis of porphyrin **1R**

Synthesis of ester 2 . (Supplementary Fig. 3). Pyrazionic acid (2.20g, 18mmol) was suspended into 130 mL of anhydrous CH_2Cl_2, to which 2 drops of dimethylformamide and 4.57 g (36mmol) of oxalyl chloride were added. The mixture was stirred at room temperature for 2 hours and the solvent was removed under pressure. The resulting solid was added to 1.02 g (10 mmol) of hexanol and triethylamine (1.01g, 10 mmol). The solution was then stirred for 18 hours at room temperature under a nitrogen atmosphere. After this time, 130 mL of CH_2Cl_2 was added and the solution which was stirred for a further hour. The solution was applied to a short silica gel column and the product eluted with CH_2Cl_2. The solvent was removed under pressure which afforded **2** as a waxy solid (1.47 g, 67.2% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ = 9.32 (d, *J* = 1.3 Hz, 1H), 8.77 (d, *J* = 2.4 Hz, 1H), 8.74 (dd, *J* = 1.3 and 2.4 Hz, 1H), 4.45 (t, *J* = 7.0 Hz, 2H) 1.82 (m, *J* = 7.0 Hz, 2H) 1.34 (br, 6H) 0.90 (t, *J* = 7.0 Hz, 3H)ppm. HRMS (CI^+) m/z: found 208.1203; calcd. 208.1212 $(M+H)^+$



Pyrazinoic acid **2**

Supplementary Figure 3. Synthesis ester 2

Binding model. The data from the binding constant determination experiments (see below) is consistent with up to two molecules of **2** binding to each molecule of **1** by formation of a coordination bond between the ligand and metal centre of the porphyrin. We use therefore a binding model that takes into account the formation of two complexes with stoichiometry 1 to 1 and 1 to 2 (i.e., complexes **1•2** and **1•2**$_2$ for **1** and **1R•2** and **1R•2**$_2$ for **1R**). For the binding to the nanoparticles, the binding constants are referred to the concentration of **1** rather to the concentration of nanoparticle, and are defined as follows:

1 + **2** $\rightleftharpoons$ **1•2** (Supplementary Equation 1)

$$K_1 = \frac{[1 \cdot 2]}{[1][2]}$$ (Supplementary Equation 2)

1•2 + **2** $\rightleftharpoons$ **1•2$_2$** (Supplementary Equation 3)

$$K_2 = \frac{[1 \cdot 2_2]}{[1 \cdot 2][2]}$$ (Supplementary Equation 4)

The binding constants K_1 and K_2 can then be determined from the changes in observed absorbance (A_o) by solving the system of equations defined by the mass balances and binding constants, and the relationship of the concentration of coloured species and A_0:

$$K_1 = \frac{[1 \cdot 2]}{[1][2]} \qquad \text{(Supplementary Equation 2)}$$

$$K_2 = \frac{[1 \cdot 2_2]}{[1 \cdot 2][2]} \qquad \text{(Supplementary Equation 4)}$$

$$[1]_0 = [1] + [1 \cdot 2] + [1 \cdot 2_2] \qquad \text{(Supplementary Equation 6)}$$

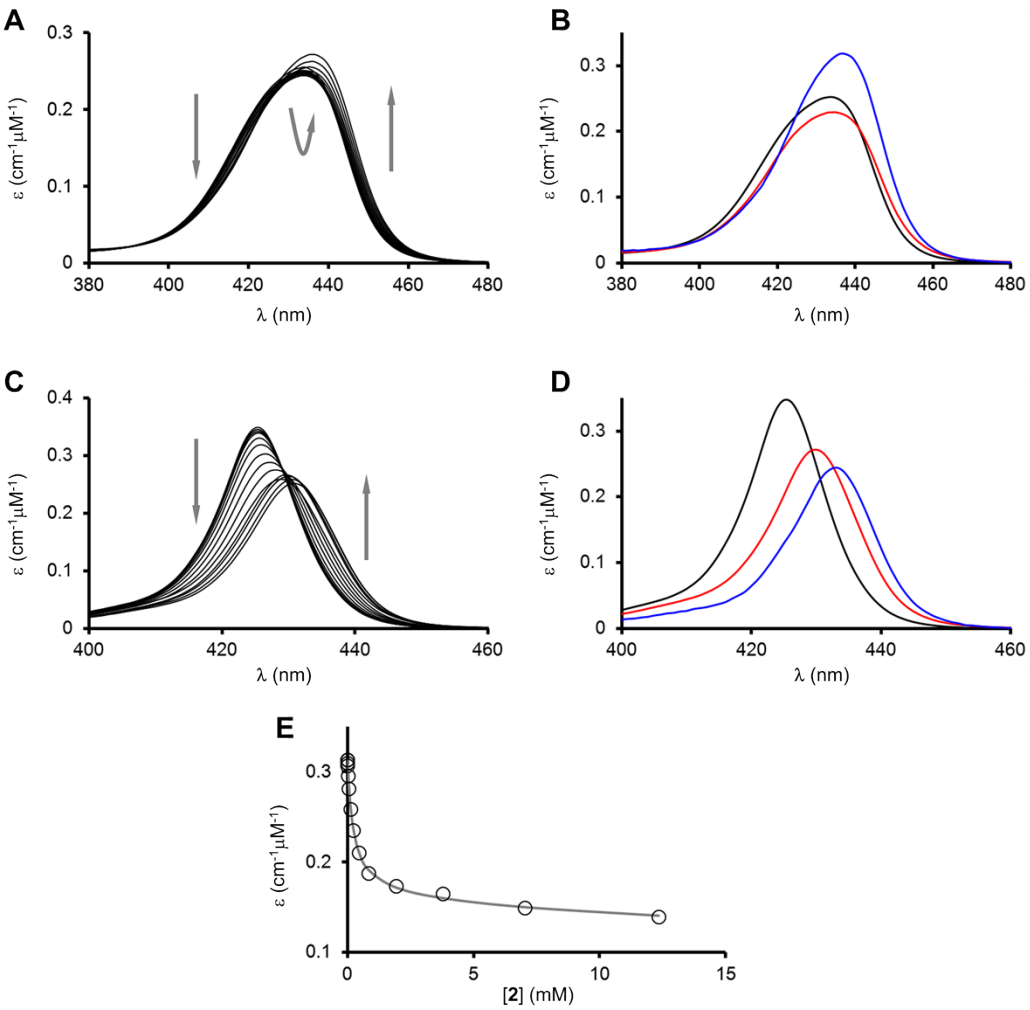
$$[2]_0 = [2] + [1 \cdot 2] + 2[1 \cdot 2_2] \qquad \text{(Supplementary Equation 7)}$$

$$A_o = \varepsilon_0[1] + \varepsilon_1[1 \cdot 2] + \varepsilon_2[1 \cdot 2_2] \qquad \text{(Supplementary Equation 8)}$$

Where ε_0, ε_1 and ε_2 are the extinction coefficient of receptor **1** and complexes **1•2** and **1•2**$_2$ respectively and [**1**]$_0$ and [**2**]$_0$ are the total concentrations of **1** and **2** respectively. This system of equations cannot be resolved analytically. Instead, a minimization routine is needed to find the fitting parameters. We use the program Specfit 3.0 (see ref 15 in main text) for this purpose (see below). For reference porphyrin **1R** we use the same model, replacing **1** for **1R** in all cases

Binding constant determination. In a typical experiment, 14 samples of 1.000 mL volume each containing amphiphile **1** 6 μM were prepared by evaporating the appropriate amount of methanolic stock solution of **1** and re-dissolving it in the working buffer. A solution of 2 (2.50 mM) was prepared by evaporating the appropriate amount of methanolic stock and re-dissolving in the working buffer. The appropriate amount of this solution was added to 13 of the 14 samples, so the concentration of **2** ranged from 2 to 235 μM. After equilibrating at room temperature for 1 hour the UV spectrum of each of the 14 samples was recorded, and again 5 hours later. The spectra recorded in either time were identical, showing that equilibration was achieved during the first hour. For titration of **1R** a similar procedure was applied, using a solution of **1R** 3 μM in the working buffer and concentration of **2** ranging from 2 μM to 12.3 mM. For each spectra, the baseline drift was removed by subtracting the value of absorbance at 500 nm (were the chromophore does not absorb). The corrected spectra of the Soret band region of the porphyrin moiety (380 to 480 nm) were used to calculate the binding constants, using the program Specfit 3.0. Specfit uses a Newton-Raphson method to determine the speciation, the stability constants and the extinction coefficient of the pure species. We judged a

fitting acceptable if the program was able to find a global minimum during the fitting procedure and if the standard deviation of logarithmic form of the constant was lower than 0.1, i.e., less 20% of the stability constant. The experimental data did not fit well to a model considering the formation of a unique complex **1•2** with 1 to 1 stoichiometry (i.e., the standard deviation of the fitting was >50%), but the fitting was excellent for a model that considered the formation of two different complexes with stoichiometry 1 to 1 and 1 to 2 (i.e., complexes **1•2** and **1•2**$_2$ and **1R•2** and **1R•2**$_2$, Supplementary Equations 1-8). For **1**, Figure 2C in main text shows the fitting of the experimental data to the model, Supplementary Figure 4 A the overall spectral changes in the soret band region of the UV-Visible spectrum and Supplementary Figure 4B the extrapolated extinction coefficients for the pure species in the same region of the spectrum. Supplementary Table 1 shows the calculated binding constants. For **1R**, Supplementary Figure 4 C-D shows the data and the fitting of the model and the numerical results for the binding constants are also in Supplementary Table 1.



Supplementary Figure 4. A. Changes in the Soret band region of the spectrum of **1** upon addition of increasing amounts of **2** (see Figure 2C in main text for the fitting of the variations at 415 and 428 nm to the binding model defined by Supplementary Equations 1-4). B. Soret band region of the spectra of pure **1** (black trace), pure complex **1•2** (red trace) and pure complex **1•2$_2$** (blue trace) as extrapolated from the fitting of the experimental data with Specfit 3.0. C. Changes in the Soret band region of the spectrum of **1R** upon addition of increasing amounts of **2.** B. Soret band region of the spectra of pure **1R** (black trace) pure complex **1R•2** (red trace) and pure complex **1R•2$_2$** (blue trace) as extrapolated from the fitting of the experimental data with Specfit 3.0. Fitting of the changes at 424 nm to the binding model defined by Supplementary Equations 5-8).

Supplementary Table 1. Binding constants with ligand **2**[a]

	K_1	K_2
1	38000 ± 7500	4500 ± 1100
1R	4500 ± 350	50 ± 4.9

a. The units are M^{-1} in all cases. The error quoted is twice the standard deviation of 3 measures. Values of extinction coefficient for all the species present (free receptors **1** and **1R**, complexes **1•2** and **1•2**$_2$ and **1R•2** and **1R•2**$_2$) derived from the fitting of the experimental data are graphically represented in Supplementary Figure 4B and 4D.

TEM experiments*.* For negative stain EM experiments, a solution of **1** 280 μM (for nanoparticle samples only) or of **1** 280 μM and 2 560 μM (for drug-loaded nanoparticles) in the working buffer was diluted 10 fold (using the same buffer) and 5μL of the resulting solution was applied to a carbon-coated, glow discharged, 300-mesh copper grid and blotted after 30 seconds. The grids were stained twice with 10 μL of 2% (w/v) uranyl acetate, blotted after 30 seconds and allow to air-dry. Images were collected using minimal electron dose at a nominal magnification of 52000x in a Tecnai 12 microscope (FEI, Eindhoven, NL) with a tungsten filament operating at 120 kv. Images were recorded with a Gatan 1K Ultrascan camera (Gatan, USA) between 1.2-2.0 μm underfocus.

Bacterial killing experiments*.* Five types of bacterial killing experiments were performed: (a) using **1** only, (b) **1R** only, (c), **1** with **2**, (d) **1R** with **2** and (e) **2** only. In a typical experiment, a sample containing **1** or **1R** 280μM, and/or **2** 560μM (1 mL) was prepared in methanol and incubated for 18 hours in the dark at room temperature, after which time the methanol was removed under reduced pressure for 30 minutes. The samples were then re-dissolved in 1 mL of NaCl buffer (pH 5.6, 0.1M), and used immediately. The sample was serially diluted in NaCl buffer, yielding 5 samples ranging from 280 to 17.5 μM of **1**/**1R** (and/or 580 to 35 μM of **2**) and then 20 μl of each were added in triplicate to each well of two separate 96 well microtitre plate. For the control sample, 20 μL of buffer without test material (**1**, **1R**, or **2**) were added in triplicate to each of the plates. A five-day-old culture of *M. fortuitum* was taken and diluted into

Middlebrook 7H9 broth to 10^7 cfu/mL and 2μL was added to each well making sure the bacterial culture was mixed well with the test material. The microtitre plates were then incubated in a humidified chamber at 37^0C without shaking for 24 hours. After this time, both plates were removed from the chamber, but only one was exposed to light from an LED at an energy dose of 75 J/cm^2 after which, both plates were incubated in the humidified chamber for a further 4 days. After this time, 15 μL was removed from each well and added to 1 mL of Middlebrook 7H9 broth and incubated at 37^0C with shaking at 200 rpm for a further 4 days. The bacterial growth was quantified measuring the optical density at 600 nm, comparing with the appropriate blank samples. To do the measurements, 500 μL of each sample was removed and added to 500 μL of 4% gluteraldehyde (v/v in 100mM NaCl, pH 5.6). The blanks for the control samples (bacteria alone without test material) consisted of 15μl of NaCl in 500μl of Middlebrook 7H9 broth and 500μl of 4% gluteraldehyde. The blanks for the samples treated with **1** or **1R** alone were prepared by adding 15μl of **1** or **1R** at the appropriate concentration (diluted in 100mM NaCl, pH 5.6) to 500μl of Middlebrook 7H9 broth and 500μl 4% gluteraldehyde. The blanks for the samples treated with **2** alone were prepared by adding 15μl of **2** at the appropriate concentration (diluted in 100mM NaCl, pH 5.6) to 500μl of Middlebrook 7H9 broth and 500μl 4% gluteraldehyde. The blanks for the samples treated with **1** and **2** (or **1R** and **2**) were prepared by adding 15μl of **1** (or **1R**) in the presence of **2** at the appropriate concentration (diluted in 100mM NaCl, pH 5.6) to 500μl of Middlebrook 7H9 broth and 500μl 4% gluteraldehyde.

Bacterial strain and maintenance. *Mycobacterium fortuitum* (patient isolate) was maintained on Middlebrook agar and a single colony was inoculated into Middlebrook broth and incubated at 37^0C for five days with shaking at 150 rpm before using in subsequent experiments.

Supplementary Table 2. Summary of cell survival data with **1** alone[a]

1 (μM)	**1** (μg/mL)	**1**	**1+LED**
280	374	51.1 ± 13.4	45.7 ± 9.6
140	187	47.9 ± 12.9	34.2 ± 19.3
70	94	43.5 ± 6.9	37.2 ± 8.0
35	48	49.2 ± 22.0	44.7 ± 14.6
17.5	23	71.1 ± 31.7	40.3 ± 16.8

a. The units are percentages referred to the control sample in all cases. The error quoted is twice the standard deviation of 3 measures.

Supplementary Table 3. Summary of cell survival data with **1** in presence of **2**[a]

1(**2**) [**2**] (μM)	**1**(**2**) [**2**] (μg/mL)	**1 wit 2**	**1 wit 2 +LED**
280 (370) [560]	374 (77) [116]	0 ± 2.9	0 ± 6.5
140 (160) [280]	187 (33) [58]	19.1 ± 8.3	0 ± 9.1
70 (67) [140]	94 (14) [29]	27.7 ± 4.3	0 ± 11.1
35 (26) [70]	48 (5.4) [14.5]	50.9 ± 4.1	0 ± 4.4
17.5 (9.5) [35]	23 (2.0) [7.3]	51.8 ± 5.7	34.0 ± 3.3

a. The units are percentages referred to the control sample in all cases. The error quoted is twice the standard deviation of 3 measures. The number in parenthesis refers to the concentration of **2** bound to the nanoparticle calculated using the binding constants reported in Supplementary Table 1. The number in brackets refers to the total concentration of drug **2**.