β-Cyclodextrin polymer nanoparticles as carriers for Doxorubicin and Artemisinin: a spectroscopic and photophysical study

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Supplementary Information

(SI-1) Synthesis and characterization of β-CyD polymer

(SI-2) Dimerization of DOX

**(SI-3) Titration of DOX with β-CyD monitored with circular dichroism.
Effect of addition of pβ-CyD on DOX UV-Vis absorption.**

**(SI-4) Global analysis of equilibrium spectroscopic data with Singular Value Decomposition
(SVD) and non linear regression modelling**

(SI-1) Synthesis and characterization of β-CyD polymer

β-CyD polymer was prepared by reacting β-cyclodextrin (β-CyD) with epichlorohydrin (EP) under strong alkaline conditions as previously described[1] (Scheme S1). Typically, 100 g of anhydrous β-CyD were dissolved in 160 mL NaOH 33% w/w aqueous solution and left under mechanical stirring overnight. Then, 81.52 g of EP (molar ratio β-CyD/ EP = 10) were rapidly added to the solution heated to 30°C. In order to obtain a high molecular weight polymer, the reaction was stopped in the vicinity of the gelation point by addition of acetone. The aqueous phase was heated to 50°C overnight, neutralized with 6N HCl and ultrafiltered using membranes with a cut-off of 30,000 g/mol. The β-CyD polymer, which has a branched structure as shown in scheme S1, was finally recovered by freeze-drying.

The β-CyD polymer was characterized by a β-CyD weight ratio of ~70% (w/w), which was determined by ^{1}H NMR in deuterated water, from the integration of the anomeric proton of the β-CyD and the integration of the hydrogen atoms of epichlorohydrin and the protons of the pyranose ring (Bruker 300 MHz spectrometer).

The average molecular weight of the polymer, 2.1×10^5 g/mol, was determined by size exclusion chromatography using pullulan standards. The chromatographic analysis was performed on a chromatograph equipped with a pump P100 (Spectra physics), a Rheodyme injector, and a set of two columns TSK-gel Type SW 4000-3000 (Tosoh Bioscience). A MiniDawn light scattering detector (with a semi conductor diode laser, λ=690 nm) was connected at the end of the columns, and the scattered light was measured at three angles (45°, 90° and 135°). The analysis was done in aqueous 0.1 M^{-1} $LiNO_3$ at a polymer concentration of 10 mg/mL. The flow rate of the mobile phase was 1.0 mL/min.
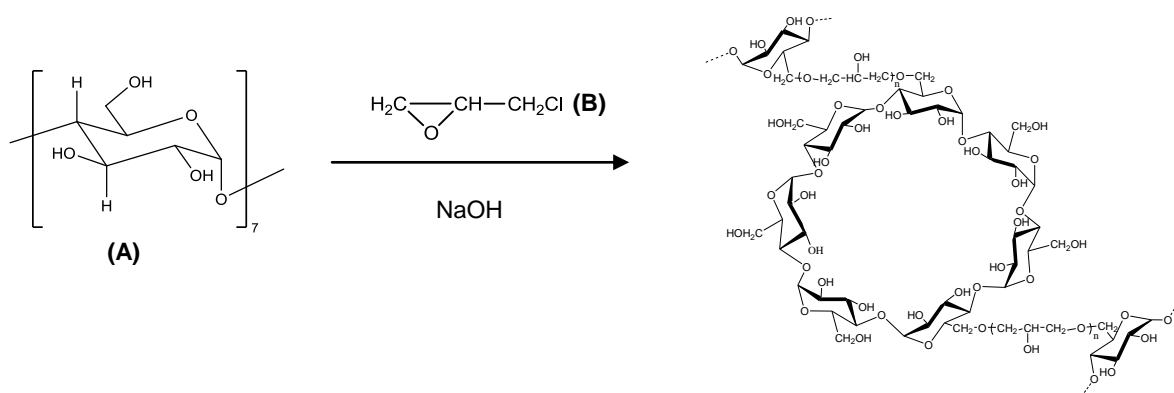
Scheme S1. Synthesis of pβ-CyD. The pβ-CyD with a branched structure was obtained by polycondensation of β-CyD (A) with epichlorohydrin (B) under strong alkaline conditions.

(SI-2) Dimerization of DOX at 22 °C

A set of absorption spectra was obtained upon DOX dilution in the range 5.0×10^{-5} M $- 1.0 \times 10^{-7}$ M (Fig. S1). The whole set of spectra was globally analysed adopting a dimerization equilibrium model with the program SPECFIT/32 based on Singular Value Decomposition (SVD) and non linear regression methods (see SI-4 below). According to literature [1, 2] the experimental absorption spectrum at concentration $\sim 1 \times 10^{-6}$ M (λ_{max} = 500 nm, $\varepsilon \sim 12000$ M^{-1} cm^{-1}) was assigned to the monomer. This spectrum was fixed in the calculation. A dimerization constant log (K_d/M^{-1}) = 4.8±0.1 was determined. The spectra of the DOX dimer was also extracted and is reported together with that of monomer in the inset of Fig. S1.
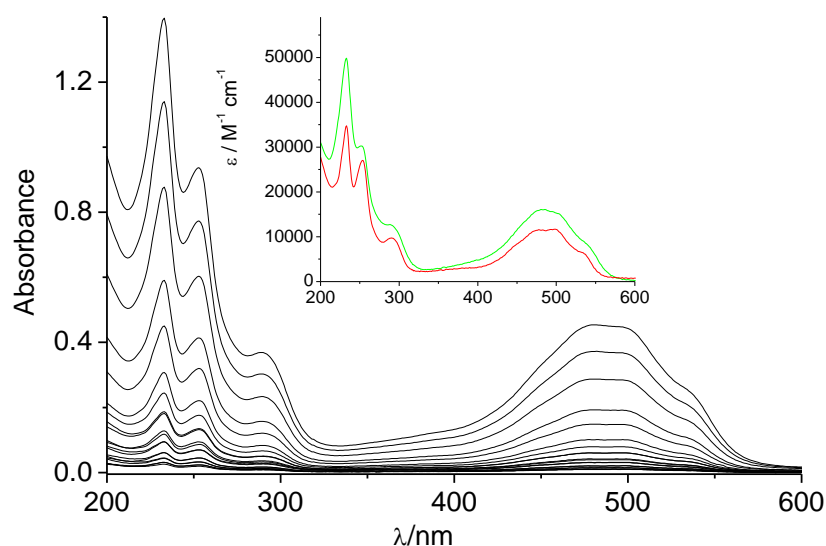

Fig. S1- Absorption spectra upon DOX dilution in the range 5.0×10^{-5} M $- 1.0 \times 10^{-7}$ M, in 0.01 M phosphate buffer at pH 7.4, at 22 °C. Cells of different pathlengths were used to register the spectra that are represented after being normalized to cell pathlength of 1 cm. Inset: absolute spectra of DOX monomer (red) and dimer (green).

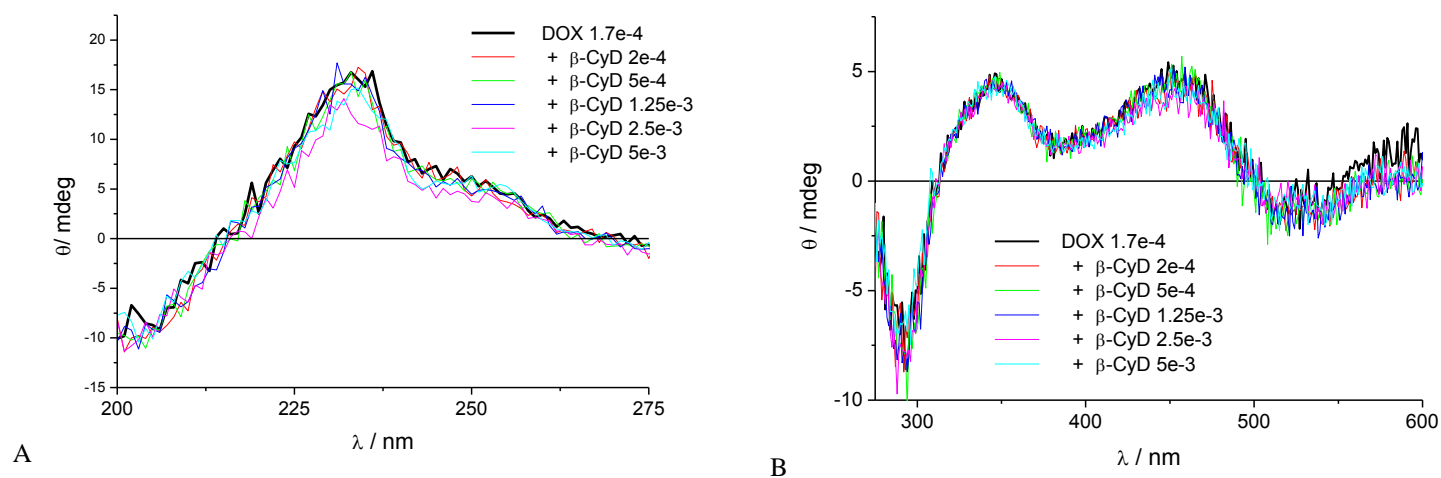## (SI-3) Titration of DOX with β-CyD monitored with circular dichroism

Fig. S2. Ellipticity of DOX 1.7×10^{-4} M in phosphate buffer 0.01M at pH 7.4 at 22 °C upon titration with β-CyD from 2.0×10^{-4} M up to 5×10^{-3} M. A-cell pathlength 0.2 cm; B-cell pathlength 0.5 cm

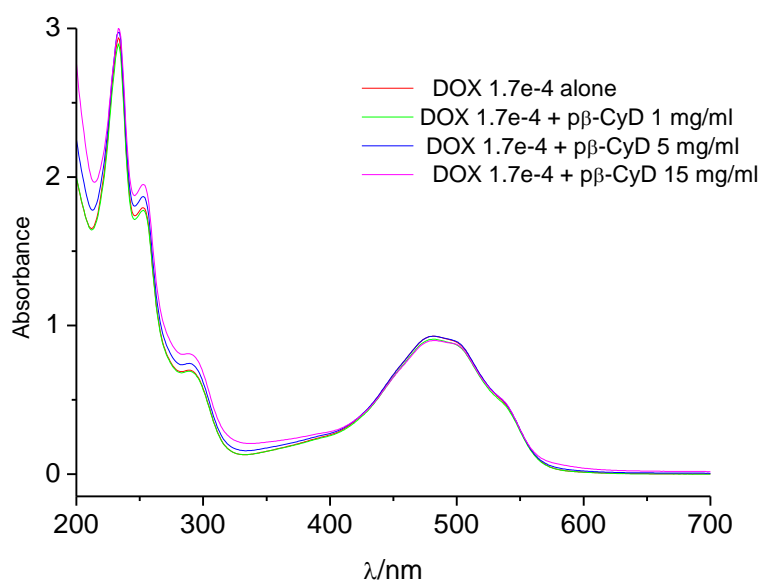## Titration of DOX with pβ-CyD monitored with UV-Vis absorption

Fig. S3. Titration of DOX 1.7×10^{-4} M in Tris buffer 0.01 M at pH 7.4 at 22 °C upon titration with pβ-CyD from 1 to 15 mg/mL. Cell pathlength 0.5 cm.

(SI-4) Global analysis of equilibrium spectroscopic data with Singular Value Decomposition (SVD) and non linear regression modelling

This application was performed using the commercial SPECFIT/32 program, based on the publications of A. Zuberbühler et al..[3, 4] Multiwavelength spectroscopic data sets (absorbances, ellipticities, fluorescence intensities) are arranged in matrix form $\mathbf{Y}$, where a number Nw of wavelengths and a number Nm of corresponding measured spectroscopic signals are ordered in columns, whereas ligand and receptor concentrations are inserted in rows. Thus each element of the data matrix Y_{ij} corresponds to a wavelength j and an experimental quantity (absorbance, circular dichroism, fluorescence intensity) for a given couple of concentrations i of ligand and receptor (typically in our experiments one of them is kept constant). A least square best estimator $\mathbf{Y'}$ of the original data $\mathbf{Y}$ is reconstructed as the eigenvector representation $\mathbf{Y'} = \mathbf{U} \times \mathbf{S} \times \mathbf{V}$, where $\mathbf{S}$ is a vector that contains the relative weights of the significant eigenvectors (Ne, number of significant eigenvectors), $\mathbf{U}$ is a matrix (Nm×Ne) of concentration eigenvectors ($U^T \times U = 1$, orthonormal) and $\mathbf{V}$ (Ne×Nw) is a matrix of spectroscopic eigenvectors ($V \times V^T$, orthonormal). This $\mathbf{Y'}$ matrix contains less noise than $\mathbf{Y}$ because the SVD procedure can factor random noise from the principal components. This reconstructed data matrix $\mathbf{Y'}$ is utilized in the global fitting instead of the original data matrix $\mathbf{Y}$. Complexation equilibria are solved assuming a complexation model (i.e. contemporary presence of a number of complexes of given stoichiometries in equilibrium with free species in solution) and optimizing the numeric combination of all the spectroscopic contributions to best reproduce the $\mathbf{Y'}$ signals. The analysis relies mainly to absorption data but also fluorescence data may be analysed, provided they are relevant to optically thin samples (linear dependence of fluorescence signal on concentration for all the species involved). Given the direct linearity between absorbance or CD and concentration and the relation that must exist between the concentrations of the various species in the postulated simultaneous equilibria, the program calculates the conditional association constants and the spectra of the complexes based on a non linear least square fit, using the Levenberg-Marquardt algorithm, to best reproduce the experimental

data for all the explored wavelengths and ligand-receptor concentration couples. The quality of the fits were evaluated on the basis of their Durbin-Watson (DW) factor and the relative error of fit. The DW test is very useful to check for the presence of auto-correlation in the residuals. This method is recommended for systematic misfit errors that can arise in titration experiments. It examines the tendency of successive residual errors to be correlated. The Durbin-Watson statistics ranges from 0.0 to 4.0, with an optimal mid-point value of 2.0 for uncorrelated residuals (i.e. no systematic misfit). In contrast to the χ^2 (Chi-squared) statistics, which requires the noise in the experimental data is random and normally distributed, the DW factor is meaningful even when the noise level in the data set is low. Since the factorized data usually have a significantly lower noise level than the original data, DW test is ideal for the present type of data.

We applied this method to analyze CD titration experiments. Below as an example we describe the analysis of the experiment in Figure 1B of the main article, i.e the titration of DOX with pβ-CyD with CD monitoring in the region 250-600 nm.

```
 [FACTOR ANALYSIS]
Tolerance = 1.000E-09
Max.Factors = 10
Num.Factors = 5
Significant = 2
Eigen Noise = 3.220E-01
Exp't Noise = 1.610E-01
 #  Eigenvalue  Square Sum  Residual    Prediction
 1   1.383E+05   3.115E+03   1.217E+00   Data Vector
 2   2.897E+03   2.181E+02   3.220E-01   Data Vector
 3   1.532E+02   6.488E+01   1.756E-01   Possibly Data
 4   3.258E+01   3.230E+01   1.240E-01   Probably Noise
 5   1.741E+01   1.489E+01   8.418E-02   Probably Noise
```

```
[MODEL]
Species = 4
Parameters = 4
```

[SPECIES]	[COLORED]	[FIXED]	[SPECTRUM]	Notes
1 0 0	False	False		CyD unit
0 1 0	True	True	FIXED	DOX monomer
1 1 0	True	False		1:1 complex
0 2 0	True	False		DOX Dimer

[SPECIES]	[FIXED]	[PARAMETER]		[ERROR]
1 0 0	True	0.00000E+00	+/-	0.00000E+00
0 1 0	True	0.00000E+00	+/-	0.00000E+00
1 1 0	False	2.25747E+00	+/-	1.54188E-01
0 2 0	True	4.84000E+00	+/-	0.00000E+00

[CONVERGENCE]
Iterations = 3
Sum(Y-y)^2 Residuals = 1.71089E+01
Std. Deviation of Fit(Y) = 9.01540E-02

[STATISTICS]
Experimental Noise = 9.754E-02
Relative Error Of Fit = 2.7188%
Durbin-Watson Factor = 2.7187
Goodness Of Fit, Chi^2 = 8.542E-01

[COVARIANCE]
1.817E-01

[CORRELATION]
1.000E+00

Bibliography

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