AperTO - Archivio Istituzionale Open Access dell'Università di Torino

Soluble cyanine dye/ β -cyclodextrin derivatives: Potential carriers for drug delivery and optical imaging

This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/1527534> since 2015-11-02T11:05:15Z

Published version:

DOI:10.1016/j.dyepig.2014.11.014

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

UNIVERSITÀ DEGLI STUDI DI TORINO

This is an author version of the contribution published on:

Questa è la versione dell'autore dell'opera:

[Dyes and Pigments, 2015, DOI: 10.1016/j.dyepig.2014.11.014]

*ovvero [T. Carmona, G. Marcelo, L. Rinaldi, K. Martina, G. Cravotto, F. Mendicuti,
114, Elsevier, 2015, pagg.204-2014]*

The definitive version is available at:

La versione definitiva è disponibile alla URL:

[<http://www.sciencedirect.com/science/article/pii/S014372081400446X>]

Soluble cyanine dye/ β -cyclodextrin derivatives: potential carriers for drug delivery and optical imaging

T. Carmona[†], G. Marcelo[†], L. Rinaldi^{††}, K. Martina^{††}, G. Cravotto^{††*}, F. Mendicuti^{†*}

[†]Departamento de Química Analítica, Química Física e Ingeniería Química, Universidad de Alcalá, 28871 Alcalá de Henares, Madrid, Spain.

^{††} Dipartimento di Scienza e Tecnologia del Farmaco and Interdepartmental Centre

“Nanostructured Interfaces and Surfaces” – NIS, University of Turin, Via P. Giuria, 9, 10125 Turin, Italy.

* Corresponding authors

Phone number: 34-91-8854672

Fax number: 34-91-8854763

E-mail: francisco.mendicuti@uah.es

ABSTRACT

Two soluble cyanine/ β -cyclodextrin derivatives have been synthesized under simultaneous ultrasound/microwave irradiation. UV-Vis, steady-state, time-resolved fluorescence and circular dichroism spectroscopies were used to evaluate their photophysical properties, as well as to study their complexation with the anticancer drug doxorubicin. Titration experiments were performed by monitoring corrected emission intensity. The analysis of fluorescence data provided stability constants for doxorubicin complexes with cyanine/ β -cyclodextrins which are 4 orders of magnitude greater than those reported for its complexation with native β -cyclodextrin and one order greater than its association with DNA. The complexation has also been studied using Molecular Mechanics and Molecular Dynamics simulations. Both electrostatic and van de Waals binding energy contributions are important to system stabilization. The potential use of these systems as carriers has been evaluated via *in vitro* experiments on HeLa cells and by monitoring cell entrance via confocal laser scanning microscopy.

KEYWORDS

Cyanines, cyclodextrins, doxorubicin, fluorescence, molecular dynamics, imaging

1. Introduction

Cyanine dyes are well-known compounds which display colorimetric and fluorescent properties and whose structure is based on two aromatic or heterocyclic rings linked via a polymethine chain with conjugated carbon-carbon double bonds.[1, 2] These dyes are able to cover all the wavelengths in the visible spectrum and show narrow absorption bands and high molar absorptivity, meaning that low-concentrations can be used for detection purposes.[3] Nowadays, one of the most promising applications of cyanine dyes is their use as fluorescence probes in the field of optical imaging.[4, 5] Their water solubility can be dramatically increased by introducing highly polar functionalities, such as the sulfonic group, to their structure, thus making their biomedical applications much easier.[6]

β -cyclodextrins (β CyDs) are natural cyclic oligosaccharides, formed of (*R*-1,4)-linked *R*-D-glucopyranose units, and possess a basket-shaped topology with an inner cavity which exhibits relatively non-polar behavior. Thanks to these features, β CyDs are able to form reversible, non-covalent inclusion complexes with mainly apolar guest molecules (G) with dimensions that fit inside the cavity. [7] Various kinds of dye moiety appended CyD derivatives have been proposed as “turn on” or “turn off” fluorescent chemical sensors, in which fluorescence intensity is enhanced or decreased upon complexation with guest molecules.[8] The combination of CyD and cyanine in the same structure endows systems with great multi-functionality. Not only would they act as carrier systems but they would also allow the monitoring of cell uptake. To our knowledge only a few publications have reported the synthesis of cyanine/CyD hybrids. Reddington *et al.* in 1997 synthesized the first cyanine- β CyD derivatives to enhance dye photostability and create a photostable fluorescent labeling reagent.[9] The successful use of these compounds as spectroscopic probes which recognize colorless guest molecules, such as 1-adamantanol and vitamin B6, has recently been described.[10] Efficient selective CyD derivation is still a difficult task.[11] However, so-called “enabling techniques” such as microwave (MW) and ultrasound (US) have been successfully used to promote specific reactions on CyDs.[12, 13] The microwave (MW)-promoted Cu-catalyzed 1,3-

dipolar cycloaddition (CuAAC) between CyD monoazides and monoacetylene moieties, which results in the formation of a triazole bridge, is the most efficient way to modify the CyD surface. It is known that metallic copper efficiently catalyzes the reaction under ultrasounds (US), or simultaneous US/MW irradiation, and halts the formation of copper ion- β -CyD complexes. [14-16]

Doxorubicin (Dox) is one of the first anthracyclines. It was isolated more than 40 years ago and is extensively used for the treatment of leukemia and various solid tumors.[17, 18] Dox exhibits cytostatic but also cytotoxic side effects. Toxicity is related to the formation of reactive oxygen species in the redox reaction where a quinone group is involved.[19] To reduce the undesirable action of oxygen species, this group can be entrapped via complexation with different nanocarriers, [20] such as micelles and liposomes, [21] polymeric architectures [22, 23] and nanoparticles. [24-27] This complexation also improves the solubility, stability and bioavailability of anthracycline drugs and prevents drug self-aggregation processes that compete with DNA-drug association. In fact, it has been demonstrated that Dox has no antitumor activity in its dimeric form. [28] The stabilization of anthracycline drugs can also be achieved via their complexation with CyD carriers. [29-33] However, one of the main restrictions on the use of CyD as an anthracycline carrier is the fact that the complexes formed show lower stability constants than the drug–DNA complex. [34] CyD chemical modification, by covalently attaching the appropriate moieties, can substantially increase the stability of the CyD-drug complex and such measures have been taken as a means for making complex-controlled target drug carriers. [34-38] Additionally, the appended group contains cyanine dyes, with long wavelength colorimetric and fluorometric photoactivities, as cyanine-CyD carriers can also be useful for optical and near-infrared fluorescence imaging in living cells or biological tissues.

In this work, two new cyanine- β CyD derivatives have been efficiently synthesized using CuAAC under simultaneous US/MW irradiation.[39] The reaction was repeated with a sulfonate cyanine in order to increase the poor water solubility of the first cyanine/CyD hybrid obtained. The spectral properties of both derivatives were investigated using UV-Vis, steady-state and time resolved

fluorescence and circular dichroism spectroscopies. Their complexation with Dox was also studied and the processes rationalized by Molecular Mechanics and Molecular Dynamics simulations. Furthermore, potential cyanine β CyD derivative:Dox complex carrier properties were investigated in HeLa cells using confocal laser scanning microscopy (CLSM).

<Figure 1>

2. Materials and Methods

2.1 Synthesis of cyanine- β CyD derivatives **1** and **2**

Structures for cyanine derivatives **1** and **2** are depicted in Figure 1. Commercially available reagents and solvents were used without further purification for this synthesis. β CyD was kindly provided by Wacker Chemie. IRIS3 and IRIS5 cyanine derivatives were kindly provided by Cyanine Technologies Spa. Reactions were monitored using TLC on Merck 60 F254 (0.25 mm) plates. Spot detection was carried out via staining with 5% H_2SO_4 in ethanol. NMR spectra were recorded on a Bruker 300 Avance (300 MHz and 75 MHz for ^1H and ^{13}C , respectively) at 25 °C. Chemical shifts were calibrated to the residual proton and carbon resonances of the solvent; DMSO- d_6 ($\delta\text{H} = 2.54$, $\delta\text{C} = 39.5$), D_2O ($\delta\text{H} = 4.79$). Chemical shifts (δ) are given in ppm and coupling constants (J) in Hz. ESI-mass spectra were recorded on a Waters Micromass ZQ equipped with an ESI source. Microwave/ultrasound-promoted reactions (MW/US) were carried out in a MicroSYNTH professional oven. High-resolution mass spectrometry (HRMS) was determined using MALDI-TOF mass spectra (Bruker Ultraflex TOF mass spectrometer).

2.1.1. Preparation of **1**. Mono-6-azido- β -CyD (100mg, 1 mmol) and then cyanine IRIS3 (564,54 mg, 4 mmol) were dissolved in DMF (10 mL) in a 100 mL two-necked round-bottomed flask equipped with an optical-fiber thermometer for reactions under combined MW/US. 50 mg Cu powder (50% w/w) was added after the reagents were completely dissolved. The reaction was heated for 2 h

at 100°C (15W MW and 20W US). The catalyst was filtered off and washed with methanol and the filtrate was evaporated, dissolved in water and precipitated with acetone. The crude product was collected by filtration on a Hirsch funnel. The product was purified via reverse phase column chromatography (H₂O–CH₃OH gradient from 95:5 to methanol 100%). The desired product was recovered in a 23% yield as a purple powder. ¹H-NMR (300 MHz, DMSO-d⁶) δ = 8.05 (s, 1H, H-4 triazole), 7.8-7.1 (m, 9H, H-Ar, H-b), 6.61-6.54 (m, 1H, H-a), 5.83-5.51 (m, O(2)H, O(3)H), 4.76 (br s, 7H, H-1), 4.43 (O(6)H), 3.72-3.51 (m, overlapped with water, H-d, H-2, H-3, H-4, H-5, H-6), 2.91 (m, 2H, H-g), 1.61-1.16 (m, 16H, CH₃, H-f, H-e); ¹³C NMR (75 MHz, DMSO-d⁶). (ESI): m/z calcd for C₇₃H₁₀₆N₅O₃₄⁺ [M + 2H]²⁺ 799,33 found 799.34. m/z (MALDI-TOF MS): calcd for C₇₃H₁₀₆N₅O₃₄⁺ [M]⁺: 1596.6719, found: 1596.6717.

2.1.2. Preparation of **2**. Mono-6-azido-β-CyD (114 mg, 1 mmol) and then cyanine IRIS5 (242 mg, 4 mmol) were dissolved in H₂O (10 mL) in a 100 mL two-necked round-bottomed flask equipped with an optical-fiber thermometer for reactions under combined MW/US. 60 mg Cu powder (10% w/w) was added after the reagents were completely dissolved. The reaction was carried out for 2 h at 75°C (15W MW and 20W US). The catalyst was filtered off and washed with methanol and the filtrate was evaporated. The product was purified via reverse phase column chromatography (H₂O–CH₃OH gradient from 98:2 to methanol 100%). The desired product was recovered in a 33% yield as a blue powder. ¹H-NMR (300 MHz, D₂O) δ = 8.05 (s, 1H, H-4 triazole), 7.9-7.2 (m, 8H, H-Ar, H-b, H-d), 6.45-6.10 (m, 2H, H-a, H-e), 5.3-4.95 (br s, 7H, H-1), 4.3-4.05 (m, H-f, CH₂-CH₃, H-2, H-3, H-4, H-5, H-6), 2.75-2.55 (m, 2H, H-i), 1.96-1.51 (m, 14H, CH₃, H-h), 1.54-1.27 (m, 5H, H-g, CH₂-CH₃); ¹³C NMR (75 MHz, D₂O) δ = 174.24, 155, 151, 145-142, 126.78, 120.21, 111.20, 104.24, 103-100.91, 81.71, 73.81-70.18, 61.12-59.11, 49.16, 27.87, 27.60-26.58, 26.4-25.2, 12.07. (ESI): m/z calcd for C₇₅H₁₀₆N₅O₄₀S⁻ [M + 2H]⁻ 890,30 found 890,35. m/z (MALDI-TOF MS): calcd for C₇₃H₁₀₄KN₅O₄₀S₂ [M + H]⁺: 1794.5336, found: 1794.5337.

<SCHEME 1>

2.2 Solvents and solutions

The buffers used were either a 0.05 M Tris-HCl buffer (tris(hydroxymethyl)aminomethane hydrochloride, Trizma ® hydrochloride, for luminescence ≥ 99.0 , Sigma-Aldrich) whose pH=7.4 was adjusted using a NaOH solution, or a 0.01 M phosphate buffered saline solution (PBS) of pH=7.4 (NaCl 0.138 M; KCl - 0.0027 M, Sigma-Aldrich). The latter was used for cell imaging experiments. Deionized water (Milli-Q) was used to prepare buffer solutions. Buffers were checked for impurities by fluorescence. Cyanine/ β -CyD derivative solutions were prepared in Tris buffer via dilution from **1** and **2** stock 8.87×10^{-5} M and 9.99×10^{-5} M solutions, respectively. Doxorubicin hydrochloride, Dox (98.0-102.0 %, Sigma-Aldrich) was used as received. Its concentration in the Tris buffer for titration was 5×10^{-7} M. Rhodamine 101 (for fluorescence, Sigma-Aldrich) in methanol (spectrophotometric degree, Aldrich) was used as standard for fluorescence quantum yield measurements. [40]

2.3 Spectroscopic methods

Absorption spectra were recorded on a UV-Vis Perkin-Elmer Lambda 35 Spectrophotometer in the 200-750 nm range. Steady-state fluorescence measurements were carried out using a PTI Quanta Master spectrofluorimeter equipped with a single monochromator in the excitation and emission paths. A slit width of 6 nm was selected for both excitation and emission paths and polarizers were fixed at the “magic angle” condition. Detection was enabled by a photomultiplier which was cooled by a Peltier system. Fluorescence decay measurements were performed on a time-correlated single-photon-counting FL900 Edinburgh Instruments Spectrometer. Either a H₂ filled thyatron-gated lamp (nF900) or a monochromatic NanoLed (Horiba), which emits at 332 nm, were used as excitation sources. Concave grating monochromators were employed at both the excitation and emission paths. Photons were detected using a red sensitive cooled photomultiplier (Peltier system). Data acquisition was carried out using 1024 channels of a multichannel analyzer with a time window width of 200 ns. A total of 10,000 counts were taken at the maximum peak channel for each measurement.

Instrumental response functions were regularly achieved by measuring the scattering of a Ludox solution. Intensity fluorescence profiles were fitted to multi-exponential decay functions using the iterative deconvolution method [41]. The weighted average lifetime of a multiple-exponential decay function was then defined as:

$$\langle \tau \rangle = \frac{\sum_{i=1}^n A_i \tau_i^2}{\sum_{i=1}^n A_i \tau_i} \quad (1)$$

where A_i is the pre-exponential factor of the component with a lifetime τ_i of the multi-exponential decay function $I(t) = \sum_{i=1}^n A_i \tau_i$, [42]

The fluorescence anisotropy, r , obtained from the fluorescence polarization measurements by using the *L-format* method, [43] was defined as:

$$r = (I_{VV} - GI_{VH}) / (I_{VV} + 2GI_{VH}) \quad (2)$$

where all magnitudes are well-known.

The fluorescence intensity correction (I_{corr}) for the large absorbance of the samples and for the cell optical path length was calculated using:

$$I_{corr} = I_{obs} \text{antilog} \left(\frac{A_{ex} + A_{em}}{2} \right) \quad (3)$$

where A_{ex} and A_{em} are the absorption at the wavelength of excitation and emission respectively and I_{obs} was directly obtained at λ_{em} from the emission spectrum. [44] Fluorescence quantum yields were calculated using Rhodamine 101 in methanol ($\phi=1$) ($\phi=0.38$) as a standard [40, 45] at the excitation

wavelengths of 515 and 550 nm for **1** and **2**, respectively. Right angle geometry and rectangular 1.0 cm path cells were used for all fluorescence measurements.

Circular Dichroism spectra were obtained on a JASCO J-715 spectropolarimeter. Recorded spectra were the average of three scans taken at a speed of 50 nm min⁻¹ with a time response of 0.125 s. Measurements were performed at 25 °C. Sensitivity and resolution were set at 20 mdeg and 5 nm respectively. Quartz cells with different paths were used to maintain the optimum absorbance value (0.8 to 1.2). Solutions were prepared in 0.05 M Tris-HCl Buffer (pH=7.4).

2.4 Cell growth and imaging experiments

HeLa cells (ATCC CCL-2) were kindly provided by the Cell Culture Unit of the Universidad de Alcalá de Henares (UAH). Cells were cultured with a RPMI1640 medium containing 10% fetal bovine serum (FBS, Sigma Ref. F7524) and 10% antibiotic antimycotic solution (Sigma, Ref. A5955) at 37°C under a 5% CO₂, 95% air-humidified atmosphere. The culture media was changed every 2 days. HeLa cells were seeded into a 60μ-Dish 35mm with a glass bottom (Ibidi, Ref. 81158) at an initial cell density of 2×10⁴ cells/dish. After 48h, 100 μL of a PBS solution containing **2** (7×10⁻⁴ M) and Dox (6×10⁻⁴ M) were added and the cells were incubated for 24 hours. The cells were then imaged using a laser scanning confocal microscope (~~LSCM~~) (Leica TCS-SP5) equipped with a continuous Ar ion laser emitting at 405, 488, 514, 561 and 633 nm. Experiments were performed by the Confocal Microscopy Service and the Biomedical Networking Center (CIBER-BBN), located at the facilities of the Cell Culture Unit (www.uah.es/enlaces/investigacion.shtm).

2.5 Association constants for the Dox-CyD Complexes

For the 1:1 stoichiometry of the Dox:CyD complexation process which is described by the following equilibrium:

the association constant K can be expressed as:

$$K = \frac{[\text{Dox} : \text{CyD}]}{[\text{Dox}][\text{CyD}]} \quad (5)$$

If we assume that two fluorescent species are present at equilibrium, both the free Dox guest and the complexed form, Dox: CyD, then the binding constants can be determined from the non-linear dependence of fluorescence intensity with the total initial CyD concentration, $[\text{CyD}]_0$, according to the following expression: [46, 47]

$$I = I_0 + (I_\infty - I_0) \frac{(1 + K[\text{Dox}]_0 + K[\text{CyD}]_0) \pm \sqrt{(1 + K[\text{Dox}]_0 + K[\text{CyD}]_0)^2 - 4K^2[\text{Dox}]_0[\text{CyD}]_0}}{2K[\text{Dox}]_0} \quad (6)$$

where, I , I_0 and I_∞ are the corrected fluorescence intensities at each $[\text{CyD}]_0$, for free Dox (measured in absence of CyD) and for the complex (at $[\text{CyD}]_0 \rightarrow \infty$), respectively. $[\text{Dox}]_0$ and $[\text{CyD}]_0$ are the initial concentrations of the drug guest and the CyD cyanine derivative. Equation 6 assumes that Dox fluorescence intensity is quenched upon CyD addition, as actually occurs.

2.6 Molecular Modeling Protocols

Molecular Mechanics (MM) and Molecular Dynamics (MD) calculations were performed using Sybyl-X 2.0[48] and the Tripos Force Field [49] to study the **1**:Dox and **2**:Dox (1:1) complexes in water solution. Charges for the **1** and **2** cyanine derivatives, as well as for Dox itself, were obtained on the non-distorted macroring and most extended **1** and **2** cyanine appended group conformations and on the optimized structure for Dox, which is depicted in Figure 5S of the supplemental material. The internally stored 6-31G(d) basis set was used within the Gaussian 03 program. [50] A relative

permittivity of 1.0 was used for electrostatic interactions in the presence of water. Cut-off distances for van der Waals and electrostatic interactions were set at 12 Å. The MAXIMIN2 algorithm and the conjugate gradient termination method ($3.0 \text{ kcal}\cdot\text{mol}^{-1}\cdot\text{\AA}^{-1}$) were used for energy optimization [51] in MM calculations. Systems were solvated with water using the Molecular Silverware algorithm (MS). [52] Periodic boundary conditions (PBC) were also employed.

The strategies used for performing the Dox complexation processes by MM were similar to the ones used previously for CyD complexation with various other guests [53-57]. The centre of mass of **1** and **2** CyD macroring bridging oxygen atoms were located at the origin of a coordinate system (a scheme of the coordinate system used in the calculation is shown in the Supporting Information, Figure 5S). Dox was forced to approach and pass through the cavity with the best orientation along the x coordinate. The 1:1 complexation was emulated by making Dox approach the **1** and **2** CyD macrorings from $x=60$ (Å) to -6 (Å) at 1 Å steps. Each structure created was solvated (PBC, MS), optimized ($3.0 \text{ kcal}\cdot\text{mol}^{-1}\cdot\text{\AA}^{-1}$) and saved for further analysis. Minima binding energy (MBE) host:guest structures from the MM analysis were again optimized ($0.5 \text{ kcal}\cdot\text{mol}^{-1}\cdot\text{\AA}^{-1}$) and used as starting conformations for 2 ns MD simulations at 300K in the presence of water (PBS). Simulation characteristics were similar to those used in other complexation or association processes. [58, 59] Bonds involving H atoms were kept from vibrating, but the other conformational parameters were variable. In brief, the MD trajectories of the systems were performed from a starting point of 1 K which was increased by 20 K intervals and the whole system equilibrated at each intermediate temperature for 500 fs up to the temperature of interest. Once at this temperature, an additional equilibration period of up to 25 ps was used. The whole heating/equilibration period was discharged from the analysis. From this point, the rest of the 2 ns trajectory was simulated at 2 fs integration time steps. The momenta were reset every 10 fs. A bath temperature coupling factor of 50 was considered and velocities rescaled at 100 fs intervals. Structures obtained from the analysis of MD trajectories were saved every 250 fs, yielding 8,000 images for subsequent analysis.

<Figure 2>

3. Results and Discussion

3.1 Synthesis of Cyanine/ β -CyD derivatives

The synthesis of Cyanine/ β -CyD derivatives was carried out employing a Cu(I)-catalyzed Huisgen 1,3-dipolar cycloaddition by which monoazidoCyD derivatives were efficiently linked together via a 1,2,3-triazole moiety using metallic copper under sonochemical conditions (Scheme 1). 6^I-Azido-6^I-deoxy- β CyD was subjected to cycloaddition in the presence of an excess of cyanine IRIS3 in order to obtain the monosubstituted β CyD **1**. The product was obtained in a 23% yield but very poor solubility was observed. A sulfonate cyanine (IRIS5) was reacted with 6-monoazido-6-monodeoxy- β CyD to obtain product **2**, in a 33% yield, as a means to increase solubility and to avoid dye aggregation. All products were isolated as solids and chemical structures were confirmed by ¹H NMR, ¹³C NMR and mass spectroscopy (see Supporting Information).

3.2 Absorption and fluorescence

Figure 2 shows absorption spectra for **1** and **2**/tris-HCl buffer solutions at 25°C. The spectrum for **1** exhibited a relatively strong absorption band at 545 nm ($\epsilon \approx 23,000 \text{ M}^{-1}\text{cm}^{-1}$) and less intense ones below 300 nm, centered at 279 nm ($\sim 6,100 \text{ M}^{-1}\text{cm}^{-1}$) and around 215 nm ($\sim 11,000 \text{ M}^{-1}\text{cm}^{-1}$). Intense low energy bands, responsible for the red color, are attributed to electronic transitions involving π electrons along the polymethine chain whose placements are strongly dependent on its length. A solution of **2**, however, displayed a much more intense absorption spectrum than that of **1**, especially in the low energy region ($\times 10$). Moreover, the shape of the bands which appeared in the spectrum of **2** seemed to match those obtained for **1**, but shifted to longer wavelengths by about ~ 100 - 110 nm.

Thus these bands appeared at 650 nm, blue color ($\epsilon \approx 200,000 \text{ M}^{-1}\text{cm}^{-1}$), 385 nm ($\sim 2,900 \text{ M}^{-1}\text{cm}^{-1}$) and 333 nm ($\sim 10,200 \text{ M}^{-1}\text{cm}^{-1}$). Additionally, other more energetic bands centered at 288 nm ($\sim 9,700 \text{ M}^{-1}\text{cm}^{-1}$) and 215 nm ($\sim 23,800 \text{ M}^{-1}\text{cm}^{-1}$) were also observed in the spectrum.

Bathochromic displacements are usually attributed to improved electronic delocalization caused by the fact that the polymethine chain was two members longer for **2** than **1**. No evidence of the presence of aggregates was found in the spectra analysis at the concentrations used in our experiments. Linear Beer-Lambert dependence was observed at these concentrations (except for the saturation of the UV-Vis spectrophotometer at 650 nm for the most concentrated solutions of **2**).

<Figure 3>

Steady-state fluorescence emission spectra for **1** (or **2**) solutions were performed upon excitation at the shoulder placed at 515 nm (at 600 nm) to monitor the entire emission spectra. Figure 3 (a) and (b) depict fluorescence emission spectra at 25 °C for **1** and **2** Tris-buffer solutions at different dye concentrations. The emission spectra of **1** exhibited a broad band whose maximum was located at $\sim 558 \text{ nm}$ and which was accompanied by a shoulder at $\sim 600 \text{ nm}$ for the most dilute solutions. A monotonic 10 nm slight bathochromic displacement from 558 to 568 nm and obviously an increase in the intensity was observed when increasing the concentration. However, a slight decrease in the fluorescence occurred for the large concentrations. This must be due to a decrease in the excitation intensity at the center of the 10 mm path cell caused by the *inner filter effect*. [23] The fluorescence intensities, corrected using eq. 3 as a function of the concentration of **1** at different temperatures which is linear in the whole range of concentrations, are superimposed in Figure 3 (a). A decrease in the fluorescence intensity was observed with temperature. Spectra for solutions of **2** presented a unique emission band whose maximum underwent a large bathochromic displacement from $\sim 662 \text{ nm}$ to $\sim 694 \text{ nm}$ with [2]. The intensity initially increased almost linearly with concentration for the diluted solutions, but drastically decreased at concentrations larger than $\sim 6 \times 10^{-6} \text{ M}$. In addition to the *inner*

filter effect, the large molar absorptivities of the low energy band and its overlap with emission maxima, as depicted in Figure 6S of the supporting information, can result in a quenching of the fluorescence at the overlapping region (the shorter wavelengths) via emission self-absorption. Subsequently, a remarkable emission band displacement and an intensity decrease were observed with increasing [2]. This also occurred in a small extension for the cyanine derivative **1**. Once corrected using eq. 3, superimposed in Figure 3(b), emission intensity became linear with concentration in the whole range of [2]. An increase in temperature, not shown, also caused a decrease in fluorescence intensity.

Fluorescence quantum yields of approximately 0.09 and 0.08 were obtained in Tris-buffer solutions at 25°C for **1** and **2** cyanine β CyD derivatives at 515 nm and 550 nm, respectively. Table 1 provides a summary of some of the photophysical parameters for **1** and **2** CyD derivatives.

<Table 1>

Fluorescence intensity profiles were also monitored at 25°C for **1**/ and **2**/Tris-buffer solutions of varying concentration. Excitation wavelengths were 515 nm for **1** and either 580 nm (upper limit for excitation with the H₂ lamp) or 332 nm (monochromatic emitter NanoLed) for **2**. Emission was monitored at the maxima (or near) of their respective bands, 560 nm and 665 nm, respectively. Decay profiles for **1** were mono-exponential and showed very short lifetime values which were in the 0.3-0.5 ns range and no apparent special trend was observed with varying concentration. Profiles for solution **2**, however, were more complex and were fitted to bi-exponential decay functions. When exciting with a 332 nm NanoLed, one fast component with a lifetime of around 1.0-1.1 ns, which contributes 85-90 % to the total fluorescence decay was detected and a slower component with a lifetime of 11-15 ns, whose contribution was 10-15 %, was also observed. The weighted average lifetime $\langle\tau\rangle$ was almost constant (2.3-2.8 ns) in the whole range of dye concentrations used. Rather

similar lifetime components of 1.2 and 10.3 ns, which provide 90.4 and 9.6 % contributions respectively, and $\langle\tau\rangle\approx 2.2$ ns were obtained using a low intensity hydrogen pulsed lamp at 580 nm.

Anisotropies (r) were obtained from fluorescence depolarization measurements, using eq. 2, under $\lambda_{\text{ex}} = 515$ nm (600 nm) by recording the emission at 560 nm (at 665 nm) for **1** (or **2**) cyanine derivative Tris-buffer solutions. As Figure 7S (supporting information) depicts, a rather concentration insensitive and constant value of $r=0.246\pm 0.004$ was obtained for **1** at 25 °C. However, anisotropies for **2** decreased with [2] from an initial value of $r=0.156\pm 0.004$ for the most diluted solution to 0.08-0.12 for the most concentrated ones. The large anisotropy values for both cyanines are caused by the low rotational diffusion of a large chromophore attached to a large macroring. The relative values of r for the chromophores of both **1** and **2** can give us some information about differences in the excited state lifetimes, of which that for **2** is larger, and probably about the self-absorption processes at the emission wavelengths of **2**, which also depolarizes the fluorescence at the highest dye concentration (see Figure 6S). In addition, larger τ values mean higher rotational diffusion during excited state lifetimes.

3.3 Circular Dichroism spectra

A chiral macroring, like β CyD, can induce a circular dichroism signal (Cotton effect) on an achiral chromophore (cyanine substituents for **1** or **2**), independently of whether it is bound to the cyclodextrin or not. The occurrence of an induced Circular Dichroism (ICD) spectrum can provide information on the location of the appended cyanine relative to the CyD cavity, as the sign and intensity of the ICD signal is related to its distance and orientation relative to the n -fold CyD axis. [60-63] On the other hand, exciton coupling (EC) is produced by the presence of two or more chromophores, which are close enough in space and at least one of which displays a large molar absorptivity, that couple their electric transition moments. This interaction can also occur intramolecularly between two

chromophore groups in a single molecule. As a result, a typical bisignate Cotton effect appears in the ICD spectrum.[64]

<Figure 4>

Figure 4 (a) and (b) depict the CyD spectra for **1** and **2** solutions that were recorded in the 215-600 nm and 450-750 nm ranges, respectively. Dye **1** did not present any induced signal at any of the concentrations measured. Apparently, the cavity-chromophore or appended chromophore–chromophore distances are not short enough to produce any interaction capable of providing an ICD signal at the concentrations used. However, **2** presented a very complex induced spectrum and a relatively low intensity positive signal at ~650 nm as well as two intense bands; a negative one located at 288 nm and a positive one at 222 nm. These signals would seem to correspond to the interaction of the cyanine appended chromophore with the β CyD cavity. The intensity of these bands, not shown, varied linearly with [**2**], indicating that the interaction was intramolecular. In fact, molar ellipticity values at 223 and 280 nm are independent of [**2**]. In addition, the ICD spectrum for the absorption band centered at ~333 nm splits into very weak intensity bands of opposite sign in what looks like an exciton coupling bisignal. This effect is probably due to the intramolecular interaction of two portions of the appended chromophore. Interestingly however, neither absorption, emission, ICD spectra nor fluorescence depolarization measurements revealed the presence of any significant dye aggregation at the concentrations used in our experiments.

Evidence of the interaction between the cyanine moiety and the CyD cavity was also sought in the NOESY spectrum of **2**. As depicted in the spectrum, the experimental data (Figure 4S of the supporting information) confirmed the interaction between the CyD macroring and cyanine appended group. NOEs were observed from the β CyD to both the aromatic, polymethine chain (Ha and Hb), and methyl of cyanine dye.

<Figure 5>

3.4 Interaction of **1** and **2** with Doxorubicin (Dox)

Dox was dissolved in a 0.05 M Tris-HCl buffer of pH=7.4 in order to achieve a concentration of 5×10^{-7} M. At this concentration, Dox dimer species are negligible. [20] 10 μ L aliquots of solutions of **1** (or **2**) were added to the 10 mm quartz cell containing 2 mL of the above Dox solution for titration and stirred during temperature equilibration (10 min) before the emission spectrum was recorded. At the end of the titration, the Dox/**1** or **2** molar ratios reached values of approximately 1:2. The effect of Dox dilution upon the addition of each aliquot never gave a Dox concentration error of more than 7%. Nevertheless, fluorescence emission intensities were corrected for this dilution.

Emission spectra for the isolated Dox solution upon $\lambda_{\text{ex}}=470$ nm exhibit, as depicted in Figure 5, two maxima centered at ~ 560 nm and ~ 590 nm, the second one being a little more pronounced, whereas **1** and **2** showed maxima emission bands centered at ~ 560 nm and ~ 665 nm respectively, *i.e.* at the emission maxima for Dox or near to it. As a consequence, the emission intensity of Dox during titrations with derivatives of **1** (or **2**) was corrected to remove their fluorescence and/or to take into account the fact that a fraction of the incident light is absorbed by β CyD derivatives at the Dox excitation wavelength of (470 nm).

Figure 5 (left) depicts an increase in emission spectra upon the addition of **1** to the Dox solution. Although Dox's complexation with native β CyD usually causes a decrease in intensity with $[\beta\text{CyD}]$, [29], an increase takes place in this case as a consequence of the overlap of the emission spectra of Dox and **1**. It was thus necessary to perform a correction which was carried out using a fraction of intensities, $F_{\text{Dox+1}} / F_1$ (measured as the area under the emission spectra), for the Dox solution in the presence of **1** ($F_{\text{Dox+1}}$) and for the **1** solution in the absence of Dox at the same dye concentration (F_1). The top of Figure 6 shows the representation of $F_{\text{Dox+1}} / F_1$ versus [**1**] which monotonically decreases.

<Figure 6>

Dox emission spectra, carried out in the presence of **2**, exhibited a band above 660 nm due to the fluorescence of **2** in addition to the Dox emission, upon exciting mainly Dox (470 nm). As seen in Figure 6 (right), some bands increase (670 nm) with [**2**], while others decrease (~560 nm and ~590 nm). However, a fraction of the incident light is absorbed by **2** at the excitation wavelength of 470 nm. Hence, to avoid this effect during titration, fluorescence intensities ($I_{\text{Dox}+\mathbf{2}}$), at 560 nm (where only Dox emits) for Dox solutions in the presence of varying concentrations of **2**, were corrected by measuring the ratio of this intensity and the fraction of the total light absorbed by **2** at 470 nm. If A_2 is the absorbance for **2** for each Dox+**2** solution at 470 nm, this fraction is $(1 - 10^{-A_2})$. The right side of Figure 6 depicts the $I_{\text{Dox}+\mathbf{2}} / (1 - 10^{-A_2})$ ratios as a function of [**2**], which also monotonically decreases.

Adjustments to the experimental data in equation 6 give association constants for the 1:1 stoichiometry Dox:**1** and Dox:**2** complexes of $(3.3 \pm 0.2) \times 10^6$ and $(12.8 \pm 1.5) \times 10^6 \text{ M}^{-1}$, respectively. These values are four orders of magnitude larger than those for the complexation of native βCyD with Dox, for which a value of $2.1 \times 10^2 \text{ M}^{-1}$ has been reported [29], and even larger still than values for the association of Dox to DNA ($5.4 \times 10^5 \text{ M}^{-1}$). [33] Swiech et al., have recently studied the Dox complexation abilities of several CyD derivatives which contain aromatic appended moieties connected via a triazole group to the macrorings. They reported binding constants in the 10^3 - 10^4 M^{-1} order of magnitude which decrease at acidic pH values which cause the protonation of the triazole group. [38] Nevertheless, the same authors studied Dox complexation with other similar CyD derivatives in both aqueous and aqueous-DMSO solutions and some of the formation constants were in the 10^5 M^{-1} order. [37] Ideal carrier systems should release the drug into the cell interior in order to avoid the unspecific loss of the therapeutic molecule before it reaches its target and also ensure the stability of the drug during transport. In general, CyD drug inclusion complexes rapidly dissociate to

free both CyDs and drug after parenteral administration because of their weak interaction.[65] Importantly, Stella et al. state that CyD:drug association constants of more than 10^4 – 10^5 M⁻¹ are required to maintain a stable complex *in vivo*. [65, 66] Therefore, the constants for Dox complexation with **1** and **2** fully satisfy the required values for successful cell target delivery without previous dissociation.

<Figure 7>

3.5 Molecular Modeling

The calculation of the binding energy upon the approach of Dox to **1** and **2** (Figure 5S of the supporting information), which was carried out according to previously described protocol, showed that the Dox:**1** and Dox:**2** inclusion processes take place across some barriers of potential, which nevertheless appear to be easily surmountable, to reach the stable 1:1 minima binding energy (MBE) complexes depicted in Figure 8S of the supporting information. In these structures, which are rather similar, Dox totally penetrates the CyD cavities. Both electrostatic and van der Waals contributions to binding energy are important to the complexation. The optimized ($0.5 \text{ kcal mol}^{-1}\text{\AA}^{-1}$) MBE structures, depicted in Figure 8S, were employed as starting conformations for the 2 ns MD simulations. Figure 7 shows the binding energy histories and contributions from the MD simulation analysis for Dox:**1** and Dox:**2** structures, whose values are favorable throughout the trajectories. The MD simulations reinforce the results, obtained from MM, which show that both electrostatic and van de Waals contributions are important to the stabilization of the system. The distances between the Dox center and the center of mass of the **1** and **2** β CyD macrorings are quite short and hardly change during the MD; average values were $0.81\pm0.17 \text{ \AA}$ and $1.35\pm0.22 \text{ \AA}$, respectively. These distances show that Dox is completely inside the **1** and **2** macrocycles. Both structures were apparently stable during the MD trajectory and, as a consequence, large association constants, such as those found experimentally, are expected.

3.6 Imaging experiments in HeLa cells

The different photophysical characteristics for cyanine from derivative **2** and Dox chromophores allows us to study the internalization of the Dox:**2** complex via confocal laser scanning microscopy (LSCM) and to obtain information about the drug and the carrier uptake separately. The cellular uptake of the **2**-Dox complex was studied using HeLa cells. Experiments were conducted at a [CD]/[Dox] molar ratio of 100. The majority of the Dox is in the form complexed with **2** at this molar ratio.

<Figure 8>

According to the parameters shown in Table 1 and Figure 2, two excitation wavelengths, 488 and 633 nm, were selected for recording the fluorescence images. Dox was principally excited at 488 nm, hence most of the detected fluorescence was ascribed to Dox fluorescence emission which showed its distribution in the cytoplasm, as depicted in Figure 8 (B), in green. However, only **2** was excited at 633 nm and the cyanine fluorescence was also observed in the cytoplasm in red, as illustrated in Figure 8 (C). Information on **2** and Dox colocalization inside the cell, which is the result of merging fluorescence cell images obtained after excitation at both wavelengths, is shown in Figure 8 (D). After 24 h of incubation Dox and **2** were distributed along the cytoplasm. Moreover, the emission of **2** is associated to that of Dox in many areas of the image. We might conclude that both Dox and **2** are taken simultaneously towards the HeLa cytoplasm cells in the supramolecular **2**-Dox complex form without apparently reaching the nuclei.

The internalization of only the drug was also studied for a Dox solution ($4.8 \times 10^{-7} \text{M}$) (Supporting Information Figure 9S). Dox presented a high degree of internalization. [67] The fluorescence of Dox was observed in the cytoplasm, as well as in the nuclei of the HeLa cells after 240 min of incubation.

The absence of Dox emission from the nuclei in our experiments was ascribed to either the strong Dox-**2** binding affinity or to a different mechanism of internalization in the cells. **4. Conclusions**

We have designed and synthesized, via CuAAC under simultaneous US/MW irradiation, two water soluble cyanine/ β CyD derivatives (**1** and **2**) which have been used as versatile carriers for drug delivery and optical imaging. Dyes **1** and **2** present reasonable fluorescence properties and form strong inclusion complexes with doxorubicin (Dox) in a Tris-HCl buffer at pH=7.4. Their association constants are four orders of magnitude greater than those obtained for Dox complexation with native β CyDs and one order greater than those obtained for the complexation of Dox with DNA. Molecular Modeling analysis confirms the stability of complexes of **1** and **2** with doxorubicin. The ability of **2** to act as a Dox carrier through the formation of a **2**-Dox complex has been studied with HeLa cells by LSCM, Dox and **2** are simultaneously up-taken by the HeLa cells and localized in the cytoplasm. Unlike, only Dox, in the absence of carrier, presents a high degree of internalization reaching the nuclei of the HeLa cells.

Acknowledgements. This work was funded by the Compagnia di San Paolo - University of Turin (Progetti di Ricerca di Ateneo 2011, grant D15E11001710003). G.M would like to thank the Universidad de Alcalá for her postdoctoral contract. Authors are grateful to Isabel Trabado for her support with cell growth and imaging experiments.

Appendix A. Supporting Information

NMR spectra and some additional figures are presented. This material is available at <http://>

References

- [1] Panigrahi M, Dash S, Patel S, Mishra BK. Syntheses of Cyanines: A Review. *Tetrahedron*. 2012;68:781-805.
- [2] Streckowski L. *Heterocyclic Polymethine Dyes: Synthesis, Properties and Applications*. Berlin Springer-Verlag, 2008.
- [3] Henary M, Levitz A. Synthesis and applications of unsymmetrical carbocyanine dyes. *Dyes Pigments*. 2013;99(3):1107-016.
- [4] Behnke T, E. Mathejczyk JE, Brehma R, Würtha C, Ramos Gomes F, Dullinc C, et al. Target-specific nanoparticles containing a broad band emissive NIR dye for the sensitive detection and characterization of tumor development. *Biomaterials*. 2013;34:160-70.
- [5] Huang C-S, George S, Lu M, Chaudhery V, Tan R, Zangar RC, et al. Application of photonic crystal enhanced fluorescence to cancer biomarker microarrays. *Anal Chem* 2011; 83:1425-30.
- [6] Sameiro M, Gonçalves T. Fluorescent Labeling of Biomolecules with Organic Probes. *Chem Rev*. 2009;109:190-212.
- [7] Szejtli J. *Cyclodextrins and Their Inclusion Complexes*. Budapest: Akademiai Kiado, 1982.
- [8] Ogoshi T, Harada A. Chemical Sensors Based on Cyclodextrin Derivatives. *Sensors*. 2008;8(8):4961-82.
- [9] Guether R, Reddington MV. Photostable cyanine dye β -cyclodextrin conjugates. *Tetrahedron Lett*. 1997;38:6167-70.
- [10] Zhao J-L, Lv Y, Ren H-J, Sun W, Liu Q, Fu Y-L, et al. Synthesis, spectral properties of cyanine dyes- β -cyclodextrin and their application as the supramolecular host with spectroscopic probe. *Dyes Pigments*. 2013;96:180-8.
- [11] Szejtli J. Topics in Inclusion Science. In: Davies JED, editor. *Cyclodextrin Technology*. Dordrecht Klunwer; 1988.
- [12] Cravotto G, Nano GM, Palmisano GJ. A Sonochemical Protocol to the Synthesis of Permodified Cyclodextrins. *J Carbohyd Chem*. 2001;20(6):495-501.
- [13] Trotta F, Martina K, Robaldo B, Barge A, Cravotto G. Recent advances in the synthesis of cyclodextrin derivatives under microwaves and power ultrasound. *J Incl Phenom Macrocycl Chem*. 2007;57(1-4):3-7.
- [14] Martina K, Caporaso M, Tagliapietra S, Heropoulos G, Rosati O, Cravotto G. Synthesis of water-soluble multidentate aminoalcohol β -cyclodextrin derivatives via epoxide opening. *Carbohydr Res*. 2011;346:2677-82.
- [15] Boffa L, Gaudino EC, Martina K, Jicsinszky L, Cravotto G. A new class of cationic cyclodextrins: synthesis and chemico-physical properties. *New J Chem*. 2010;34(9):2013-9.
- [16] Cintas P, Barge A, Tagliapietra, Boffa L, Cravotto G. Alkyne-azide click reaction catalyzed by metallic copper under ultrasound. *Nat Protoc*. 2010;5(3):607-16.
- [17] Ben DD, Palumbo M, Zagotto G, Capranico G, Moro S. DNA topoisomerase II structures and anthracycline activity: insights into ternary complex formation. *Curr Pharm Des*. 2007;13:2766-80.
- [18] Minotti G, Menna P, Salvatorelli E, Cairo G, Gianni L. Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. *Pharmacol Rev*. 2004;56:185-9.
- [19] Nawara K, McCracken JL, Krysinski P, Blanchard GJ. Structure-Dependent Complexation of Fe³⁺ by Anthracyclines. 1. The Importance of Pendant Hydroxyl Functionality. *J Phys Chem B*. 2013;117:6859–67.
- [20] Anand R, Ottani S, Manoli F, Manet I, Monti S. A close-up on doxorubicin binding to γ -cyclodextrin: an elucidating spectroscopic, photophysical and conformational study. *RSC Advances*. 2012;2:2346-57.
- [21] Cukierman E, Khan DR, 762–770. The benefits and challenges associated with the use of drug delivery systems in cancer therapy. *Biochem Pharmacol*. 2010;80(5):762-70.
- [22] Yokoyama M. Polymeric micelles as a new drug carrier system and their required considerations for clinical trials. *Expert Opin Drug Deliv* 2010;7:145-58.
- [23] Lammers T. Improving the efficacy of combined modality anticancer therapy using HPMA copolymer-based nanomedicine formulation. *Adv Drug Delivery Rev*. 2010;62:203-30.
- [24] Betancourt T, Brown B, Brannon-Peppas L. Doxorubicin-loaded PLGA nanoparticles by nanoprecipitation: preparation, characterization and in vitro evaluation. *Nanomedicine*. 2007;2:219-32.
- [25] Yallapu MM, Foy SP, Jain TK, Labhasetwar V. *Pharm Res Chem Intermed*. 2010;27:2283-95.

- [26] Horcajada P, Chalati T, Serre C, Gillet B, Sebrie C, Baati T, et al. Porous metal-organic-framework nanoscale carriers as a potential platform for drug delivery and imaging. *Nat Mater*. 2010; 9 172-8.
- [27] Davis ME, Chen Z, D. M. Shin DM. Nanoparticle therapeutics: an emerging treatment modality for cancer. *Nat Rev Drug Discovery*. 2008; 7:771-82.
- [28] Nakanishi T, Fukushima S, Okamoto K, Suzuki M, Matsumura Y, Yokoyama M, et al. Development of the polymer micelle carrier system for doxorubicin. *J Control Release*. 2001;74:295-302.
- [29] Husain N, Ndou TT, Muñoz De La Peña A, Warner IM. Complexation of doxorubicin with β - and γ -cyclodextrins. *Appl Spectrosc* 1992;46:652-8.
- [30] Uekama K, Hirayama F, Irie T. Cyclodextrin Drug Carrier Systems. *Chem Rev*. 1998;98:2045-76.
- [31] Bekers O, Beijnen JH, Vis BJ, Suenaga A, Otagiri M, Bult A, et al. Effect of cyclodextrin complexation on the chemical stability of doxorubicin and daunorubicin in aqueous solutions. *Int J Pharm*. 1991;72:123-30.
- [32] Bekers O, Beijnen JH, Otagiri M, Bult A, W. J. M. Underberg WJM. Inclusion complexation of doxorubicin and daunorubicin with cyclodextrins. *J Pharm Biomed Anal*. 1990;8:671-4.
- [33] Bekers O, Van Den Bosch Kettenes JJ, Van helden SP, Seijkens D, Beijnen JH, Bult A, et al. Inclusion complex formation of anthracycline antibiotics with cyclodextrins; a proton nuclear magnetic resonance and molecular modeling study. *J Inclusion Phenom Mol Recognit Chem*. 1991;11:185-93.
- [34] Schneider YJ, Baurain R, Zenebergh A, Trouet A. DNABinding Parameters of Daunorubicin and Doxorubicin in the Conditions Used for Studying the Interaction of Anthracycline-DNA Complexes with Cells in Vitro. *Cancer Chemoth Pharm*. 1979;2:7-10.
- [35] Thiele C, Auerbach D, Joung G, Wenz G. Inclusion of chemotherapeutic agents in substituted β -cyclodextrin derivatives. *J Incl Phenom Macrocycl Chem*. 2011;69:303-7.
- [36] Yamanoi T, Kobayashi N, Takahashi K, Hattori K. Preparation of a cyclodextrin conjugated with two arbutin moieties as a drug-carrier model, and its high ability for the inclusion of doxorubicin. *Lett Drug Des Discov*. 2006;3: 188-91.
- [37] Swiech O, Mieczkowska A, Chmurski K, Bilewicz R. Intermolecular Interactions between Doxorubicin and β -Cyclodextrin 4-Methoxyphenol Conjugates. *J Phys Chem B* 2012;116:1765-71.
- [38] Swiech O, Dutkiewicz P, Wójciuk K, Chmurski K, Kruszewski M, Bilewicz R. Cyclodextrin Derivatives Conjugated with Aromatic Moieties as pH responsive Drug Carriers for Anthracycline. *J Phys Chem B* 2013;117:13444-50.
- [39] Cravotto G, Fokin VV, Garella D, Binello A, Boffa L, Barge A. Ultrasound-Promoted Copper-Catalyzed Azide-Alkyne Cycloaddition. *J Comb Chem*. 2010;12(1):13-5.
- [40] Brouwer AM. Standards for photoluminescence quantum yield measurements in solution *Pure Appl Chem* 2011; 83(12):2213-28.
- [41] O'Connor DV, Ware WR, Andre JC. Deconvolution of fluorescence decay curves. A critical comparison of techniques. *J Phys Chem*. 1979;83(10):1333-43.
- [42] Lakowicz JR. Time-Domain Lifetime Measurements. *Principles of Fluorescence Spectroscopy*. 3rd ed. New York: Springer; 2006. p. 97.
- [43] Lakowicz JR. Fluorescence Anisotropy. In: Lakowicz JR, editor. *Principles of Fluorescence Spectroscopy*. 3rd ed. New York: Springer; 2006. p. 354.
- [44] Lakowicz JR. Instrumentation for Fluorescence Spectroscopy. *Principles of Fluorescence Spectroscopy*. 3rd ed. New York: Springer; 2006. p. 56.
- [45] Lakowicz JR. Instrumentation for Fluorescence Spectroscopy. In: Lakowicz JR, editor. *Principles of Fluorescence Spectroscopy*. 3rd ed. New York: Springer; 2006. p. 54.
- [46] González-Álvarez MJ, Carmona T, Evren D, Mendicuti F. Binding of a neutral guest to cucurbiturils: photophysics, thermodynamics and molecular modelling. *Supramol Chem*. 2014;26(5-6):414-26.
- [47] Park JW. Fluorescence Methods for Studies of Cyclodextrins Inclusion Complexation and Excitation Transfer in Cyclodextrin Complexes. In: Douhal A, editor. *Cyclodextrins Material, Photochemistry, Photophysics and Photobiology*. Amsterdam: Elsevier; 2006. p. 11.
- [48] St Louis, MO, 63144 USA: SybylX 2.0, Tripos International, 1699 S Hanley Road; 2013.
- [49] Clark M, Cramer RD, III, Van ON. Validation of the general purpose Tripos 5.2 force field. *J Comput Chem*. 1989;10(8):982-1012.
- [50] Frisch MJ, Trucks GW, Schlegel HB, Scuseria GE, Robb MA, Cheeseman JR, et al. Gaussian 09. Revision A.1 ed. Wallingford, CT, USA,: Gaussian, Inc.; 2009.
- [51] Brunel Y, Faucher H, Gagnaire D, Rassat A. Program of minimization of the empirical energy of a molecule by a simple method. *Tetrahedron*. 1975;31(8):1075-91.

- [52] Blanco M. Molecular Silverware. I. General solutions to excluded volume constrained problems. *J Comput Chem.* 1991;12(2):237-47, 2 plates.
- [53] Di Marino A, Mendicuti F. Rationalizing some experimental facts on the complexation of 2-methyl naphthalenecarboxylate and hydroxypropylated cyclodextrins by molecular mechanics and molecular dynamics. *J Incl Phenom Macro Chem.* 2007;58(3-4):295-305.
- [54] González-Álvarez MJ, Di Marino A, Mendicuti F. Fluorescence, Induced Circular Dichroism and Molecular Mechanics of 1-Methyl Naphthalenecarboxylate Complexes with 2-Hydroxypropyl Cyclodextrins. *J Fluoresc.* 2009;19(3):449-62.
- [55] Usero R, Alvariza C, González-Álvarez MJ, Mendicuti F. Complexation of Dimethyl 2,3-Naphthalenedicarboxylate with 2-hydroxypropyl- α -, - β - and - γ -cyclodextrins in Aqueous Solution by Fluorescence, Circular Dichroism and Molecular Mechanics. *J Fluoresc.* 2008;18(6):1103-14.
- [56] Di Marino A, Mendicuti F. Thermodynamics of complexation of dimethyl esters of tere-, iso-, and phthalic acids with α - and β -cyclodextrins. *Appl Spectrosc.* 2004;58(7):823-30.
- [57] Alvariza C, Usero R, Mendicuti F. Binding of dimethyl 2,3-naphthalenedicarboxylate with α -, β - and γ -cyclodextrins in aqueous solution. *Spectrosc Acta Pt A-Molec Biomolec Spectr.* 2007;67A(2):420-9.
- [58] González-Álvarez MJ, Balbuena P, Ortiz Mellet C, García Fernández JM, Mendicuti F. Study of the Conformational and Self-Aggregation Properties of 2I,3I-O-(o-Xylylene)-per-O-Me- α - and - β -cyclodextrins by Fluorescence and Molecular Modeling. *J Phys Chem B.* 2008;112(44):13717-29.
- [59] González-Álvarez MJ, Vicente J, Ortiz Mellet C, Fernández García JM, Mendicuti F. Thermodynamics of the Dimer Formation of 2I,3I-O-(o-Xylylene)-per-O-Me- γ -cyclodextrin: Fluorescence, Molecular Mechanics and Molecular Dynamics. *J Fluoresc.* 2009;19(6):975-88.
- [60] Kodaka M, Fukaya T. Induced circular dichroism spectrum of a β -cyclodextrin complex with heptylviologen. *Bull Chem Soc Jpn.* 1989;62(4):1154-7.
- [61] Kodaka M, Fukaya T. Complex formation between heptylviologen and cyclodextrin. *Bull Chem Soc Jpn.* 1986;59(6):2032-4.
- [62] Kodaka M. Sign of circular dichroism induced by β -cyclodextrin. *J Phys Chem.* 1991;95(6):2110-12.
- [63] Kodaka M. A general rule for circular dichroism induced by a chiral macrocycle. *J Am Chem Soc* 1993;115(9):3702-5.
- [64] Berova N, Nakanishi K. Exciton Chirality Methods: Principles and Application. In: Berova N, Nakanishi K, Woody RW, editors. *Circular Dichroism: Principles and Applications.* New York: Wiley-VCH; 2000. p. 337-82.
- [65] Stella VJ, He Q. Cyclodextrins. *Toxicol Pathol.* 2008;36:30-42.
- [66] Stella VJ, Rao VM, Zannou EA, Zia VV. Mechanisms of drug release from cyclodextrin complexes. *Adv Drug Delivery Rev* 1999;36(1):3-16.
- [67] Amiji MM. *Nanotechnology for Cancer Therapy.* first ed. Boca Raton, FL CRC Press; 2006.

Scheme Legends

Scheme 1. Synthesis of **1** and **2** cyanine derivatives. *Reagents and conditions* a) for product 1: Cu powder, MW/US, DMF, 100°C, 2h; for product 2: Cu powder, MW/US, H₂O, 75 °C, 2h.

Figure Legends

Figure 1. Structures for **1** and **2** cyanine β CyD derivatives.

Figure 2. Absorption spectra at 25°C for **1** (----) and **2** (—) 1.8×10^{-5} M and 1.0×10^{-5} M in 0.05 M Tris-HCl buffer solutions, respectively and for a $\sim 2 \times 10^{-5}$ M Dox/Tris-HCl buffer solution (---). The structure for the Dox drug is superimposed.

Figure 3. Fluorescence emission spectra for **1** (a) and **2** (b) buffer solutions of varying concentration at 25°C; [**1**]=0.89, 1.77, 3.54, 5.32, 7.10, 8.88, 17.74, 35.48, 53.21 and 70.95 μ M; [**2**]=0.99, 1.99, 3.99, 5.99, 7.99, 9.99, 19.97, 39.95, 59.92, 79.89 and 99, 87 μ M. Superimposed are the corrected fluorescence intensities according to eq. 3, at several temperatures 5°C (■), 25°C (●) and 45°C (▲).

Figure 4. Circular dichroism (ICD) spectra at 25°C for **1** and **2**/Tris-buffer solutions recorded in the (a) 215- 600 nm and (b) 450-750 nm ranges respectively.

Figure 5. Emission spectra for Dox tris-buffer solutions during titration with **1** and **2** CyDs at 25°C upon excitation at 470 nm. (Left) Emission for Dox upon addition of **1**, both spectra (Dox and **1**) are superimposed; (right) bands to the blue that decrease with [**2**] are due to Dox; the peak to the red, which increases in intensity, is due to emission from **2**.

Figure 6. (left) Ratios of the fluorescence intensities (F) (measured as the area under the emission spectra, upon $\lambda_{\text{ex}}=470\text{nm}$) for Dox (5×10^{-7} M) buffer solutions in the presence of varying concentrations of **1** and in the absence of Dox at the same concentrations of **1** to avoid the effect of **1**'s emission on the emission spectra. (right) Ratios of the fluorescence intensity at 560 nm (almost only emitted Dox) for Dox (5×10^{-7} M) buffer solutions in the presence of different concentrations of

2, $I_{\text{Dox}+\mathbf{2}}$, and the fraction of light absorbed by **2**, to avoid the effect of a fraction of the incident light which was absorbed by **2**.

Figure 7. (upper part) Total binding energy histories (black), electrostatics (dark gray) and van der Waals (gray) contributions, as well as (bottom) the Dox-host distances during the 2 ns MD trajectories for **1** and **2** cyanine derivative complexes with Dox in water. Superimposed are the MBE structures for the 1:1 stoichiometry complexes.

Figure 8. LSCM of HeLa cells incubated with [**2**-Dox] for 24 h: A) Bright-field image, B) and C) Fluorescence intensity images upon excitation at 486 and 633 nm respectively; D) The constructive image obtained by overlapping the corresponding cell images upon excitation at 486 and 633 nm.