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Interaction of 6-*p*-toluidinylnaphthalene-2-sulphonate with β-cyclodextrin

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Abstract

The present study examines the complexation of β-cyclodextrin (β-CD) with 6-*p*-toluidinylnaphthalene-2-sulfonate (TNS), a fluorescent probe for exploring hydrophobic regions of several biological substances. The complexation was monitored in aqueous solution by ultraviolet spectrophotometry. At first, the stoichiometry of the formed complex was examined by applying the continuous variation (Job plot) method. Next, the kinetic of the complex formation as well as the determination of the stability constant were calculated by monitoring the spectrophotometric properties of TNS in the presence of increasing concentrations of β-CD applying both linear and nonlinear models. The results suggested that TNS forms a stable complex with β-CD with a stability constant of 1109 M^{-1} and 1:1 molar ratio, at least at the examined concentrations.

Keywords: Beta-cyclodextrin; Inclusion complex; UV-spectrophotometry; 6-*p*-toluidinylnaphthalene-2-sulphonate; Stability constant

1. Introduction

6-*p*-toluidinylnaphthalene-2-sulfonate (TNS) (Scheme 1) is a fluorescent probe for exploring hydrophobic regions and has been used for clarifying the structures and functions of biological substances [1]. The fluorescence of TNS is practically quenched in water, but in hydrophobic environment, such as a proteinic cavity, it is significantly increased. Also, it has been reported that TNS fluorescence increases rapidly when a cyclodextrin is added to the aqueous solution, enabling the use of TNS in aqueous environment [2] for fluorometric determination.

Cyclodextrins (CDs) [3] are cyclic oligosaccharides made up of glucopyranose units bonded together via α(1,4)-linkages. The most common CDs are α, β and γCD, containing six, seven and eight units, respectively. CDs are torus-shaped molecules and the inside of the CD cavities is relatively hydrophobic, consisting of a circular configuration of hydrogen atoms and glycoside oxygen atoms, while all hydroxyl groups are outside the molecule. Due to this configuration the CDs are able to form inclusion complexes with various molecules and ions [4]. The fit of the

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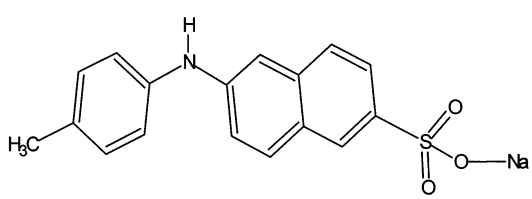
entire or at least part of the guest molecules in the CD (host) cavity determines the stability of the inclusion complexes and the selectivity of the complexation process.

The present study describes a step-by-step spectrophotometric procedure for the examination of complexation with cyclodextrins, from the kinetic of complex formation up to the determination of stability constant applying improved nonlinear models, which avoid known assumptions of the linear ones. To this end, the complexation of TNS with β-CD was examined spectrophotometrically and the changes in TNS absorbance, due to hydrophobic forces, when β-CD is added, were used for the examination of complexation as follows: First, the complex formation rate constant at different concentrations of β-CD and the molar ratio were calculated. Next, the complex stability constant K_{st} was calculated using both linear and improved nonlinear models. The last were solved by nonlinear least-squares regression analysis, applying an iteration procedure. Nonlinear models do not take into account approximations, like, for instance, the concentration approximation that the known linear models employ. Linear methods are solved mainly graphically, using the linear least-squares regression analysis applied to known mathematical models such as Benesi–Hildebrand, Scatchard etc. As most of these models suffer from theoretical and practical drawbacks [5] Diederich [6] suggests nonlinear procedures which are free of assumptions, have much broader applicability and are likely to displace the evaluations done according to Benesi–Hildebrand or Scatchard linear models.



Scheme 1. Molecular structure of TNS.

2. Experimental

2.1. Materials and methods

TNS was supplied by Sigma (St. Louis, MO), β-CD (MW=1135) was obtained from Wacker Chemie. Water was de-ionized and doubly distilled using a Millipore Milli-Q Plus System. The analysis was carried out by ultraviolet spectroscopy using a Perkin-Elmer Lambda 7 UV/Vis Spectrophotometer with an attached thermostated system for keeping temperature constant (25 ± 0.1°C). Complex formation between TNS and β-CD in aqueous solutions was investigated according to the spectral shift method.

2.2. Determination of the time needed for the formation of the TNS-β-CD inclusion complex

A TNS stock solution (1×10^{-4} mol l^{-1}) was prepared and preserved at a temperature of 4°C protected from light since TNS is a photosensitive molecule. 40 ml of this stock solution were diluted to 100 ml with water (4×10^{-5} M, solution **A**). At t=0, aliquots of 5 ml of solution **A** were pipetted into 10 ml calibrated flasks and diluted to volume with water ([TNS] = 2×10^{-5} mol l^{-1}). The contents were quickly mixed and transferred into a cuvette of 1 cm pathlength. The solutions were analyzed against a blank reference cell, containing water. The same experiment repeated at times 2, 5, 7, 10, 15 and 25 min, where aliquots of 5 ml of A were pipetted into 10 ml calibrated flasks ($[TNS]_t$=2×10^{-5} mol l^{-1}) containing 2×10^{-4} mol l^{-1} of aqueous solution of β-CD in a molar ratio 1:10 ([TNS]:[CD]=1:10). The contents were mixed, stirred periodically, left protected from light and transferred into a cuvette of 1 cm pathlength. The solutions were analyzed against a blank reference cell, containing β-CD at the same concentration, in order the same refractive index to be achieved for both cells. Fig. 1 demonstrates that the maximum difference in absorbance (ΔA), which corresponds to the formation of the TNS-β-CD complex, was reached after 7 min. From Fig. 1 it is also obvious that this difference was stabilized for the rest of the time intervals.

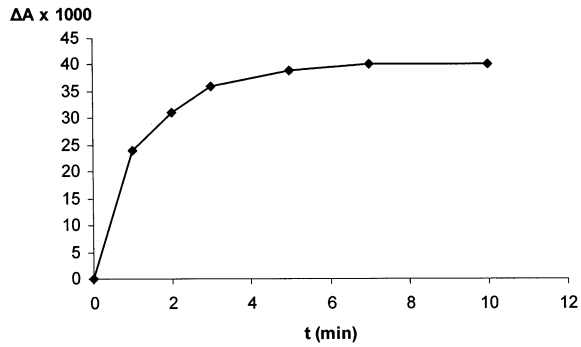


Fig. 1. Plot of the difference in TNS absorbance (ΔA) versus time.

In order to examine if there is any relationship between the rate of complex formation and the TNS-β-CD molar ratio the same experiment was repeated where the molar ratio TNS:β-CD varied from 1:0.5 to 1:50. Aliquots of 5 ml of solution **A** of TNS were pipetted into 10 ml calibrated flasks ($[TNS] = 2 \times 10^{-5}$ mol l^{-1}), containing increasing amounts of aqueous β-CD standard solutions and diluted to volume with water. The contents were mixed, stirred for 7 min and analyzed against a blank reference cell, containing β-CD at the same concentration.

2.3. Determination of stoichiometry by continuous variation method

A reliable determination of the complex stoichiometries can be provided by the continuous variation technique (Job plot) [7], based on the difference in absorbance $\Delta A (\Delta A = A_0 - A)$ of TNS in the presence of β-CD. ΔA values were calculated by measuring the absorbance of TNS in the absence (A_0) and presence (A) of the corresponding concentration of the CD. The inclusion complex of TNS (2.2×10^{-5} mol l^{-1}) with β-CD was prepared by mixing aqueous solutions of TNS and β-CD resulting to certain molar ratios in the final standard volumes in order that the total concentration remained constant ($[TNS]_t + [\beta\text{-CD}]_t = M$). Subsequently, $\Delta A[TNS]_t$ for β-CD was plotted against r, $\{r = [TNS]_t/([TNS]_t + [CD]_t)\}$. The quantities $\Delta A[TNS]_t$ are proportional to the concentrations of the complexes. The concentrations of free TNS and β-CD in a 1:n inclusion complex TNS-CDn can be expressed as follows:

$$[TNS] = rM - [TNS\text{-}\beta\text{-CD}],$$

$$[CD] = M(1 - r) - n[TNS\text{-}\beta\text{-CD}].$$

For a given value of r, the concentration of the complex TNS-β-CD will reach a maximum, corresponding to the point where the derivative d[TNS-CD]/dr=0. Derivatization of the above two equations according to r gives $d[TNS]/dr = M$ and $d[CD]/dr = -M$. Transformation of the above equations results to a single solution: the maximum absolute complex concentration is reached for $r = (n+1)^{-1}$ and does not depend on M or the binding constant [8].

2.4. Determination of stability constant

The molar absorptivity (ε) of any 'guest' molecule (drug to be complexed) changes depending on binding to cyclodextrins [9]. This may happen due to changes in the environmental polarity of the guest's chromophore, when this moves from the polar aqueous media to the apolar cyclodextrin cavity. According to Beer's law, the absorbance A_0 of a solution of TNS, with a total concentration G_t (or TNS_t), is equal to:

$$A_0 = \varepsilon_G b G_t. \quad (1)$$

In this solution, a known amount of cyclodextrin (β-CD in the present study) was added at a total concentration of CD_t. Thus, the absorbance A of the resulting solution becomes:

$$A = \varepsilon_G b G + \varepsilon_{CD} b CD + \varepsilon_{CD:G} b CD{:}G, \quad (2)$$

where G, CD and CD:G are the concentrations of the free guest (TNS), the free cyclodextrin (β-CD) and the 1:1 formed complex respectively. Substituting G and CD in Eq. (2) with their equivalents from the mass balance equations ($G_t = G + CD{:}G$ and $CD_t = CD + CD{:}G$), and placing the cyclodextrin absorbance ($\varepsilon_{CD} b CD$) equal to zero (since cyclodextrins do not absorb in the UV area), Eq. (2) becomes:

$$A = \varepsilon_G b G_t + \Delta\varepsilon_{1:1} b CD{:}G, \quad (3)$$

where $\Delta\varepsilon_{1:1} = \varepsilon_{1:1} - \varepsilon_G - \varepsilon_{CD}$Combination of Eq. (3) with the binding constant definition ($K_{1:1} =$ CD:G/CD:G), results in:

$$\frac{\Delta A}{b} = \frac{G_t K_{1:1} \Delta\varepsilon_{1:1} \mathrm{CD}}{1 + K_{1:1}\mathrm{CD}}, \quad (4)$$

where $\Delta A = A - A_0$.

Eq. (4) describes the binding isotherm of the formed 1:1 complex and shows the hyperbolic dependence of $K_{1:1}$ on the free, non-complexed cyclodextrin concentration (CD). At this point, the assumption that $\mathrm{CD} = \mathrm{CD}_t$ is employed (when a high excess of CD_t is being used compared to G_t) and through this, Eq. (4) is transformed into a double reciprocal linear equation (Eq. (5)), known as Benesi–Hildebrand [10].

$$\frac{1}{\Delta A} = \frac{1}{G_t K_{1:1} \Delta\varepsilon_{11} \mathrm{CD}} + \frac{1}{G_t \Delta\varepsilon_{1:1}}. \quad (5)$$

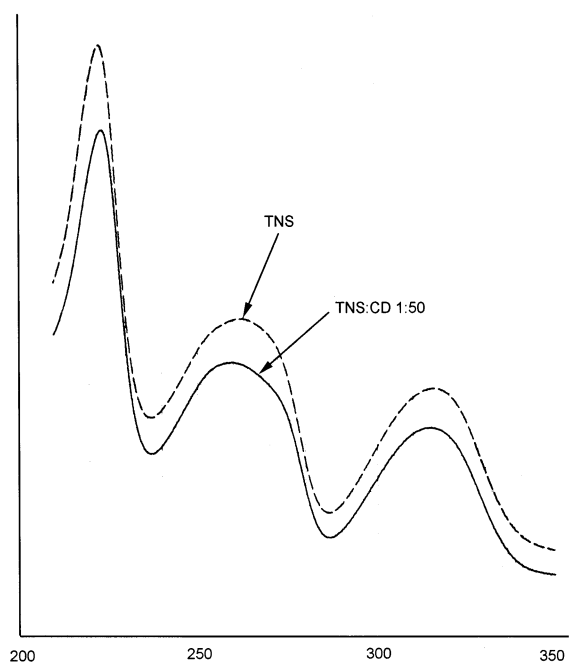
Avoiding the above assumption ($\mathrm{CD} = \mathrm{CD}_t$), different processes were followed resulting in a mathematical model, which does not involve any approximation. More specifically, substitution of CD:G in Eq. (3) with its equal from the cyclodextrin mass balance equation, results in:

$$\mathrm{CD} = \mathrm{CD}_t - \left(\frac{\Delta A}{\Delta\varepsilon_{1:1}}\right). \quad (6)$$

Substitution of CD from Eq. (6) to Eq. (4) gives after consecutive transformations:

$$\Delta A = \frac{G_t K_{1:1}(\Delta\varepsilon_{1:1}\mathrm{CD}_t - \Delta A)}{\Delta\varepsilon_{1:1} + K_{1:1}(\Delta\varepsilon_{1:1}\mathrm{CD}_t - \Delta A)}\Delta\varepsilon_{1:1}. \quad (7)$$

Eq. (7) involves neither limitations nor assumptions and correlates the difference in the absorbance (ΔA) with the initial total concentrations of the guest (G_t) and the cyclodextrin (CD_t). The unknown parameters $K_{1:1}$ and $\Delta\varepsilon_{1:1}$ can be calculated by the nonlinear least-squares regression analysis (see later).



Scheme 2. UV spectra of TNS in the absence and presence of β-CD in 1:50 molar ratio.

3. Results and discussion

The spectrophotometric properties of TNS were modified in the presence of increasing concentrations of β-CD. The measurements were carried out by scanning the wavelengths between 200 and 400 nm, using equimolar aqueous solution of β-CD as a blank to take into account its absorbance. Three absorption maximum wavelengths were observed at 223, 262 and 316 nm (Scheme 2), which were changed due to β-CD presence. These changes were intensified as the concentrations of β-CD increased. These concentrations ranged from 0.5 to 50 times higher than that of TNS. The effects of β-CD on the absorption spectra of TNS are characteristic.

The hypsochromic effects at the absorption maximum wavelengths of TNS were intense in the presence of β-CD suggesting that TNS forms stable complex with β-CD, thus expecting large stability constant K_{st} for TNS-β-CD complex ($\geq$ 1000 M^{-1}). By adding β-CD, there was a slight bathochromic shift for the first absorption maximum, reaching 224 nm at the maximum concentration of β-CD used. As for the second peak, a hypsochromic shift was observed in the presence of β-CD, reaching 259 nm. Finally, the last peak demonstrated a hypsochromic shift (314 nm).

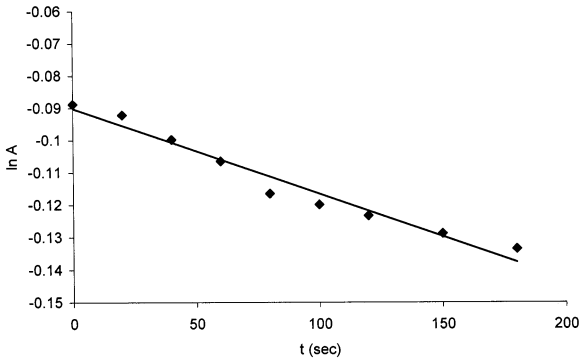


Fig. 2. Linear transformation of the decrease in TNS absorbance versus time.

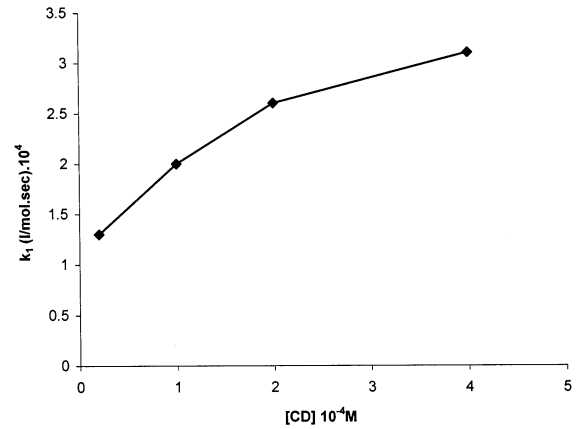
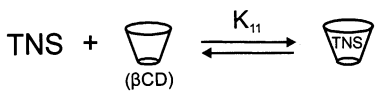


Fig. 3. Plot of k_1 versus β-CD concentrations denoting that as the concentration of β-CD increases the formation rate constant increases also.

3.1. Determination of the association rate constant

The equilibrium state of a 1:1 host: guest system is presented graphically in the following scheme:



The stability constant K_{st} (in the scheme K_{11}) could be expressed by the following equation $K_{st} = [\text{TNS-β-CD}]/[\text{β-CD}][\text{TNS}] = k_1/k_2$, where k_1 is the formation (association) rate constant and k_2 is the dissociation rate constant. It was noticed that the absorbance (A) of TNS in the presence of various concentrations of β-CD versus time (t), could be expressed as follows: $A = A_0 e^{-k_1 t}$, which could be easily transformed to

$$\ln A = \ln A_0 - k_1 t, \quad (8)$$

where A_0 is the absorbance of TNS in the absence of β-CD. The examined proportions of TNS:β-CD concentrations were 1:1, 1:5, 1:10 and 1:20. Graphical representation of Eq. (8) for the 1:10 molar ratio is presented in Fig. 2. Furthermore, in Fig. 3 the calculated k_1 values were plotted towards the concentrations of β-CD.

3.2. Determination of complex stoichiometries

If a physical parameter directly related to the concentration of the complex (e.g. absorbance) can be measured under these conditions, and is then plotted as a function of r, the maximum value for this parameter will occur at $r = m/(m+n)$, where m and n are TNS and β-CD proportions in the complex, respectively ($TNS_m - CD_n$) [11]. This means that if the complex stoichiometry is 1:1 ($m = 1$, $n = 1$), the maximum value for the examined parameter will be reached at $r = 0.5$ or almost 0.5. ΔA values were calculated by measuring the absorbance of TNS solutions in the presence and absence of β-CD. In these standard solutions the total concentration M of the two species maintained constant, $M = 2.2 \times 10^{-5}$ mol/l, but the ratio of the initial concentrations, expressed by r, (see Experimental) varied between 0 ([TNS]:[β-CD] = 0:10) and 1 ([TNS]:[β-CD] = 10:0). Since the standard solution was prepared, a 7 min time period of continuous stirring was taking place, before its absorbance was monitored. The method was repeated, but instead of aqueous solutions of β-CD it was used water at the same volume proportion. The solutions were analyzed against a blank reference cell containing water. The resulting continuous variation plots (Fig. 4) demonstrate that since the $\Delta A[\text{TNS}]_t$ maximum has an r value of 0.5, TNS-β-CD complex has 1:1 stoichiometry.

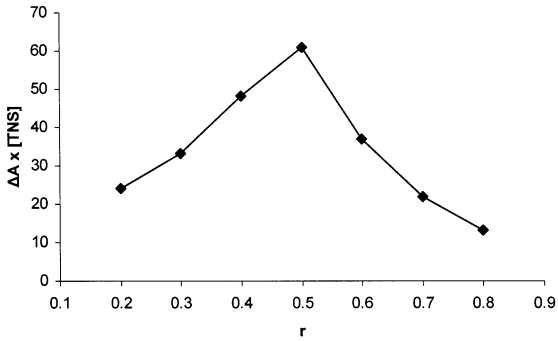


Fig. 4. Continuous variation plot (Job plot) of TNS-β-CD inclusion complex.

3.3. Determination of stability constant

The stability constant of this complex was determined by UV spectrophotometry, based on the decrease in absorbance (ΔA), due to the complexation. The measurement took place at 223 nm, the main of the three absorption maximum wavelengths, in order to achieve more certain values of A. The stability constant was calculated from the linear plot, resulting from the following linear model solved by linear regression analysis:

$$1/\Delta A = 5.23 \times 10^{-3} \ (\pm 3.78 \times 10^{-4})1/[\mathrm{CD}] + 5.765(\pm 0.690)$$

$$F = 191.4, \ P = 0.0008, \ R = 0.992$$

Graphical solution of the above linear model (Benesi–Hildebrand) is shown in Fig. 5 where the value of K_{st} was calculated to be 1109 M^{-1} for TNS-β-CD complex.

The binding constant for TNS-β-CD complex was calculated also using the above described nonlinear model by monitoring the changes in TNS absorbance in the presence of increasing concentrations of β-CD. The nonlinear estimation of the parameters was based on a iteration procedure following the Marquardt iterative algorithm by which the estimates are evaluated against a set of control criteria at every iteration. If successive iterations fail to change the sum of squares of the convergence criterion, the procedure stops. In standard multiple regression, the regression coefficients were estimated by 'finding' those coefficients that minimize the residual variance (sum of squared residuals) around the regression line. Any deviation of an observed score from a predicted score signifies some loss in the accuracy of our prediction, for example, due to random noise (error). Therefore, it can be said that the goal of the least-squares estimation is to minimize a loss function; specifically, this loss function is defined as the sum of the squared deviation about the predicted values. To minimize the loss function (to find the best fitting set of parameters) and to estimate the standard errors of the parameters estimates, a very efficient algorithm was used (quasi-Newton) that approximates the second-order derivatives of the loss function and guide the search for the minimum.

By the proposed nonlinear method resulted a K_{st} value of 1275 M^{-1} and so it could be concluded that both the examined models provided comparable values for the K_{st}. The deviation in

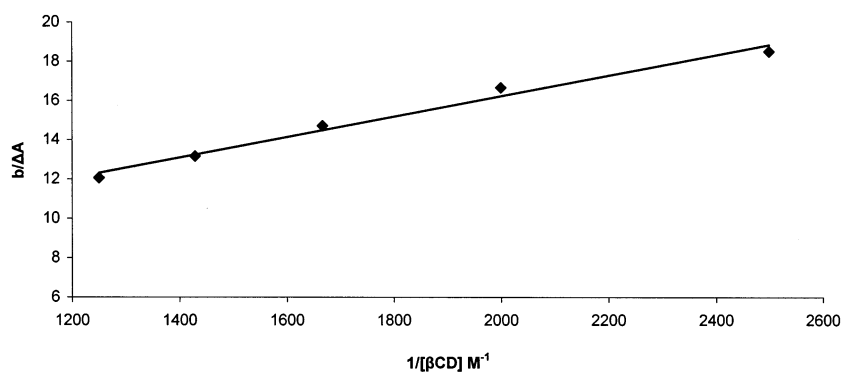


Fig. 5. Benesi–Hildebrand plot for the effect of β-CD on the TNS absorbance at 223 nm.

values is in the acceptable range and it is a common phenomenon in literature [12]. In studies dealing with the calculation of stability constant it is essential to distinguish between methods in which the values are estimated graphically (from the slope and intercept of the resulting lines), like the Benesi–Hildebrand models, and those in which the values are calculated statistically (least-squares regression). The main objection for using the graphical solution is that this does not estimate the parameter variances. To overcome this problem the use of a weighted linear or even better the nonlinear least-squares regression could be applied.

In conclusion, the present study proposed a spectrophotometric study for the complexation of TNS with β-CD as well as an alternative mathematical model for calculating the binding constant iteratively. The iteration solution of this model is based on the 'true' values of the parameters and its use appears to be as simple as the common linear ones. This model requires only the initial concentrations of the free species (TNS and β-CD) without any limitations, therefore, experimental and theoretical shortcomings could be avoided.

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