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جامعة الإمارات العربية المتحدة
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College of Science

Department of Chemistry

SEQUESTRATION EFFECT ON THE OPEN-CYCLIC
SWITCHABLE PROPERTY OF WARFARIN INDUCED BY
CYCLODEXTRIN: TIME-RESOLVED FLUORESCENCE STUDY

Naji Saleh Ahmed Al-Dubaili

This thesis is submitted in partial fulfilment of the requirements for the degree of
Master of Science in Chemistry

Under the Supervision of Dr. Na'il Saleh Ibrahim

November 2018

Declaration of Original Work

I, Naji Saleh Ahmed Al-Dubaili, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled "*Sequestration Effect on the Open-Cyclic Switchable Property of Warfarin Induced by Cyclodextrin: Time-Resolved Fluorescence Study*", hereby, solemnly declare that this thesis is my own original research work that has been done and prepared by me under the supervision of Dr. Na'il Saleh Ibrahim, in the College of Science at UAEU. This work has not previously been presented or published or formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

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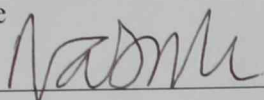
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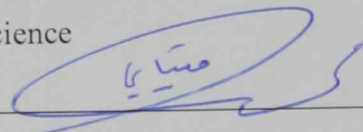
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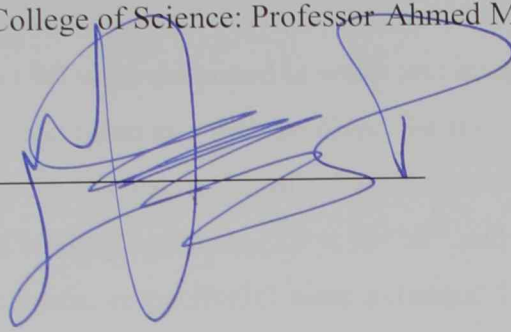
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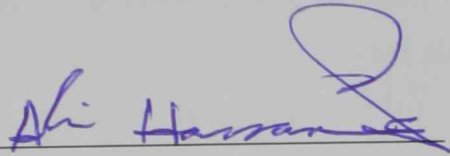


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Abstract

In this work, the photophysical properties of the fluorescent anticoagulant drug warfarin (W) were examined in water and inside methyl- β -cyclodextrins (Me- β -CD) through absorption and time-resolved fluorescence measurements at pH 3 and 9 and upon the selective excitation of different isomers of warfarin at 280 and 320 nm. The values of binding constants ($2.9 \times 10^3 \text{ M}^{-1}$ and $4.2 \times 10^2 \text{ M}^{-1}$ for protonated and deprotonated forms, respectively) were extracted from the spectrophotometric data. Both absorption and time-resolved fluorescence results established that the interior of the macromolecular host binds preferentially the open protonated form, red shifts the maximum of its absorption of light at $\sim 305 \text{ nm}$, extends its excited-state lifetime, and decreases its emission quantum yield (Φ_F). Collectively, sequestration of the open guest molecules decreases markedly their radiative rate constants (k_r), likely due to formation of hydrogen-bonded complexes in both the ground and excited states. Due to lack of interactions, no change was observed in the excited-state lifetime of the cyclic form in the presence of Me- β -CD. The host also increases the excited-state lifetime and Φ_F of the drug deprotonated form (W^-). These later findings could be attributed to the increased rigidity inside the cavity of Me- β -CD. The pK_a values extracted from the variations of the UV-visible absorption spectra of W versus the pH of aqueous solution showed that the open isomer is more acidic in both ground and excited states. The positive shifts in pK_a values induced by three derivatives of cyclodextrins: HE- β -CD, Ac- β -CD, and Me- β -CD supported the preferential binding of these hosts to open isomers over cyclic.

Keywords: open-cyclic tautomers, molecular switching, decay-associated spectra, warfarin, excited-state lifetime, cyclodextrins.

Title and Abstract (in Arabic)

تأثير تكوين معقدات داخل أوعية السيكلودكسترين على خاصية الانفتاح والانغلاق في دواء الوارفارين

الملخص

في هذا العمل ، تم فحص الخواص الفيزيائية الضوئية لعقار الوارفارين المضاد للتجلط الدموي في الماء وداخل الأوعية الجزئية للميثيل-سيكلودكسترين (Me- β -CD) من خلال قياسات الامتصاص والانبعث عند الرقم الهيدروجيني 3 و 9 الخاصة بايزومرات مختلفة من الوارفارين. تم استخلاص معاملات الارتباط (2.9×10^3 متر⁻¹ و 4.2×10^2 متر⁻¹) لأشكال حمضية وقاعدية مختلفة للعقار. أثبتت كل من نتائج الامتصاص والانبعثات الحركية أن الجزء الداخلي من المضيف يفضل الارتباط بالشكل الحمضي المفتوح ، مسببا امتصاص العقار لطاقة ضوئية أقل عند 305 نانومتر ، وإطالة العمر الزمني للحالة المثارة ، كما يقلل من نسبة الانبعث (Φ_F). وبشكل عام إن الارتباط بواسطة جزيئات الضيف بالشكل ذو خاصية الانفتاح تقلل بشكل ملحوظ من معاملات الإشعاع (k_T) للدواء، ويرجع ذلك إلى تكوين روابط هيدروجينية في كل من الحالة المستقرة و المثارة.

مفاهيم البحث الرئيسية: التحول الجزيئي، الأطياف الحركية، زمن الحالة المثارة، أوعية السيكلودكسترين، وارفارين.

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I also owe a great debt of gratitude to the master program coordinator Professor Abbas Khaleel for his guidance, support, and assistance throughout my preparation of this thesis. It is a pleasure as well to thank all members of the Department of Chemistry at the United Arab Emirates University for assisting me all over my studies and research.

Finally, special deep appreciation is given to my family for providing me with unfailing support and encouragement that lead to this accomplishment.

Dedication

To my beloved parents and family

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List of Abbreviations

Ac- β -CD	Acetyl- β -cyclodextrins
DAS	Decay-associated spectra
G	Guest
H	Host
HE- β -CD	(2-Hydroxyethyl)- β -cyclodextrins
Me- β -CD	Methyl- β -cyclodextrins
TCSPC	Time-Correlated Single Photon Counting
W	Warfarin
α -CD	Alpha-cyclodextrins
β -CD	Beta-cyclodextrins
γ -CD	Gamma -cyclodextrins

Chapter 1: Introduction

1.1 Relevant Literature

1.1.1 Supramolecular Chemistry as a Science

Since its first development in 1987 by Lehn, Cram and Pedesen [1], supramolecular chemistry has been expanding into various applications, including biomedical applications [2], [3]. The host-guest concept involves non-covalent interaction of a guest (G) molecule inside the cavity of a host (H) molecule (Figure 1). These interactions include hydrogen-bonding interaction, π - π stacking interaction, electrostatic interaction, van der Waals force, and hydrophobic/hydrophilic attraction [4].

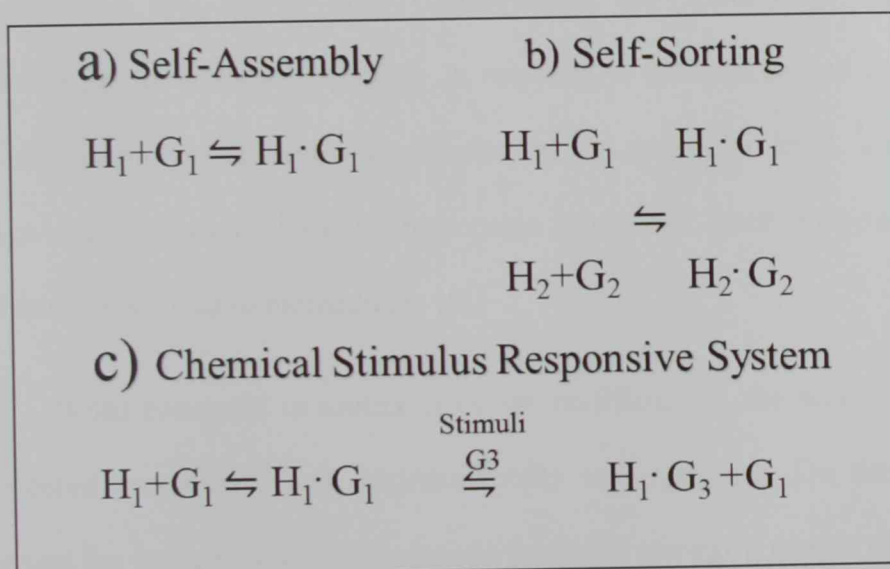


Figure 1: Self-assembly (a), self-sorting (b) and stimuli-responsiveness of supramolecular systems (c)

Figure 1 illustrates two important features of those non-covalent interactions: reversibility and responsiveness to external stimuli, such as pH, light, electrical

signal, heat, chemical competitor, etc. While self-assembly process (Figure 1a) describes transformation of small building blocks into one defined aggregate under certain conditions, self-sorting (Figure 1b) process is simultaneous occurrence of multiple self-assemblies into multiple well-defined aggregates under similar conditions. If building blocks of those self-assembling or self-sorting systems re-configured in responding to certain stimuli, the systems said to be stimuli-responsive systems (Figure 1c).

Supramolecular materials are naturally composed of two or more components that intrinsically self-assembled by non-covalent interactions. Such weak interactions inherently endow the materials with reversible, spontaneous assembly or disassembly feature, allowing convenient dissociation and reconstruction of the supramolecular systems at a low energy cost. Consequently, the “bottom-up” supramolecular approach has an adaptive capability in response to external stimuli that can trigger the self-assembly/disassembly equilibrium at the molecular level, leading into the design and fabrication of stimuli-responsive functional, smart materials for various applications, such as in biomedicine [5].

When compared to routine covalent modifications, the non-covalent method considered inexpensive and environmentally safe approach. On the one hand, it bypasses the burdens in multiple organic synthesis steps [6], and on the other it may combine already existing products in the market, whose safety and low-toxicity have been already confirmed [7], [8]. The low toxicity, in particular, motivated more researchers to utilize supramolecular systems in biological field as bio-inspired materials [9], [10].

1.1.2 Types of Molecular Containers (Hosts)

There have been several host-guest systems reported in aqueous solution for biomedical applications with stimuli-responsive abilities [11]. Cyclodextrins (CDs) [12] and Cucurbit[n]uril (CBs) [13] are examples of hosts that bind with the guest by noncovalent interactions as illustrated in Figure 2.

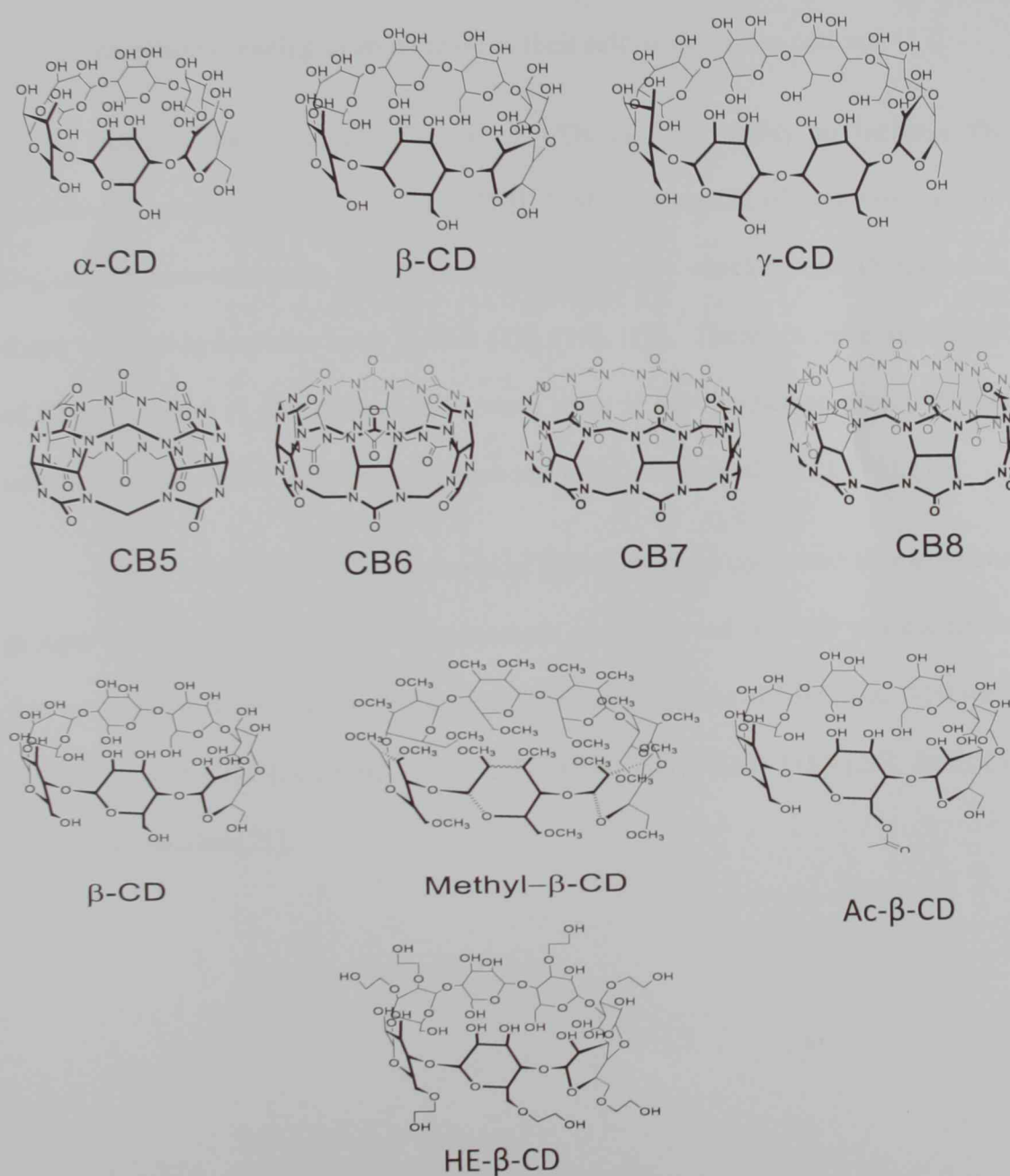


Figure 2: Chemical structures of various derivatives of cyclodextrins (CDs) and cucurbiturils (CBs) molecular containers

Table 1 summarizes some features that distinguish the two type of containers CDs and CBs. For example, CBs have much higher affinity to bind guest molecules [2]. For instance, the binding constant (K) for CDs-based host–guest complexes can reach 10^4 M^{-1} [12], [14], [15], and that for CBs-based host–guest complexes can be as high as 10^{15} M^{-1} [16]. Another important feature is the distinct carbonyl group, which is responsible for the binding between CBs and cationic guests through ion-dipole interactions, leading to an increase in their acid-dissociation constant [17].

However the important feature of CDs is their ability to increase the solubility of poorly soluble drugs [12], [14], [15]. CDs consist of (α -1,4-)-linked α -D-glucopyranose units with a hydrophobic central cavity which gives CDs the cones shape with the hydrophilic outer surface [12], [14], [15]. There are three main types of CDs (α , β and γ), the difference between them is the number of glucopyranose units in their structures which is six, seven and eight, respectively [12], [14], [15].

On the other hand, CBs composed of glycoluril units connected by methylene groups. The isolation of major CBs members was achieved only 80 years after its first synthesis in acidic media. By the year 2000, all members of CBs from 1-10 were isolated except CB9 (i.e., CB5, CB6, CB7, CB8, and CB10) [18]–[20]. In 2015, CB14 was isolated [21].

Table 1: Major differences between CBs and CDs molecular containers

	CBs	CDs
Binding constant	10^{15} M^{-1}	10^4 M^{-1}
Features	carbonyl group and ion-dipole interactions (increase pK_a of guests)	increase the solubility of insoluble guests
Structure	composed of glycoluril units connected by methylene groups with a hydrophobic cavity	Composed of glucopyranose units with a hydrophobic cavity
Types	CB5, CB6, CB7, CB8, CB10, and CB14	α , β and γ and derivatives thereof

1.1.3 pK_a of other drugs inside CBs and CDs

In general, inclusion of guest molecules inside CDs and CBs changes their physical and chemical properties. Also, CDs prefer to bind the neutral forms of guests over their cationic forms, consequently, this will induce negative pK_a shift of guest molecules [2], [22]–[24]. On the other hand, CBs will induce positive pK_a shift of guest molecules because it favors binding the protonated form rather than the neutral form [25]–[27]. The host-induced pK_a shift was related to the carbonyl groups of CBs that are partially negatively charged [2], [25], [28]–[31].

For example, Saleh *et al.* studied the encapsulation of benzimidazoles [26], [32] which are commercial fungicides [33], [34] with CB7. The inclusion affects the pK_a of the benzimidazoles and leads to positive pK_a shift. This is equivalent to say that, CB7 has stabilized the protonated form of benzimidazole guest molecule,

increasing its solubility [26]. In another example, the interaction of CB7 with tropicamide which is an ocular drug; whose pK_a is 5.4 was also examined [35], [36]. Encapsulation of tropicamide with CB7 and CB8 increases the pK_a by (0.5 and 1.5) units respectively. As a result, the ionization degree of the drug was enhanced, which is of relevant to drug bioavailability [35].

From a different prospective, inclusion of drugs inside CDs and CBs has not only induced pK_a shift but also increased the fluorescence of these drugs [31], [35]. Koner *et al.* studied the encapsulation of dapoxyl sodium sulfonate (DSS) with α - and β -cyclodextrins. Not only a 0.8 negative pK_a shift was noticed due to encapsulation of DSS inside β -cyclodextrins, but also the emission of the DSS was consequently increased significantly [37].

1.1.4 Biochemistry of Warfarin and its Importance

Warfarin is an oral anticoagulant drug that is widely used for a patient who has cardiovascular disease [38]. The mechanism of how warfarin prevents blood from clotting is shown in Figure 3 [39], which illustrates Vitamin K cycle. Warfarin reduces the production of clotting factors II, VII, IX and X, and the anticoagulant proteins C and S. This could happen by inhibiting the enzyme, Vitamin K epoxide reductase, that is required to reproduce vitamin K epoxide in the reduced form. Reduced vitamin K_1 (vitamin K_1H_2) is required as cofactor for γ -carboxylase to converts glutamic acid (Glu) residues to γ -carboxyglutamic acid (Gla) by adding CO_2 , which is needed to induce blood clotting [39].

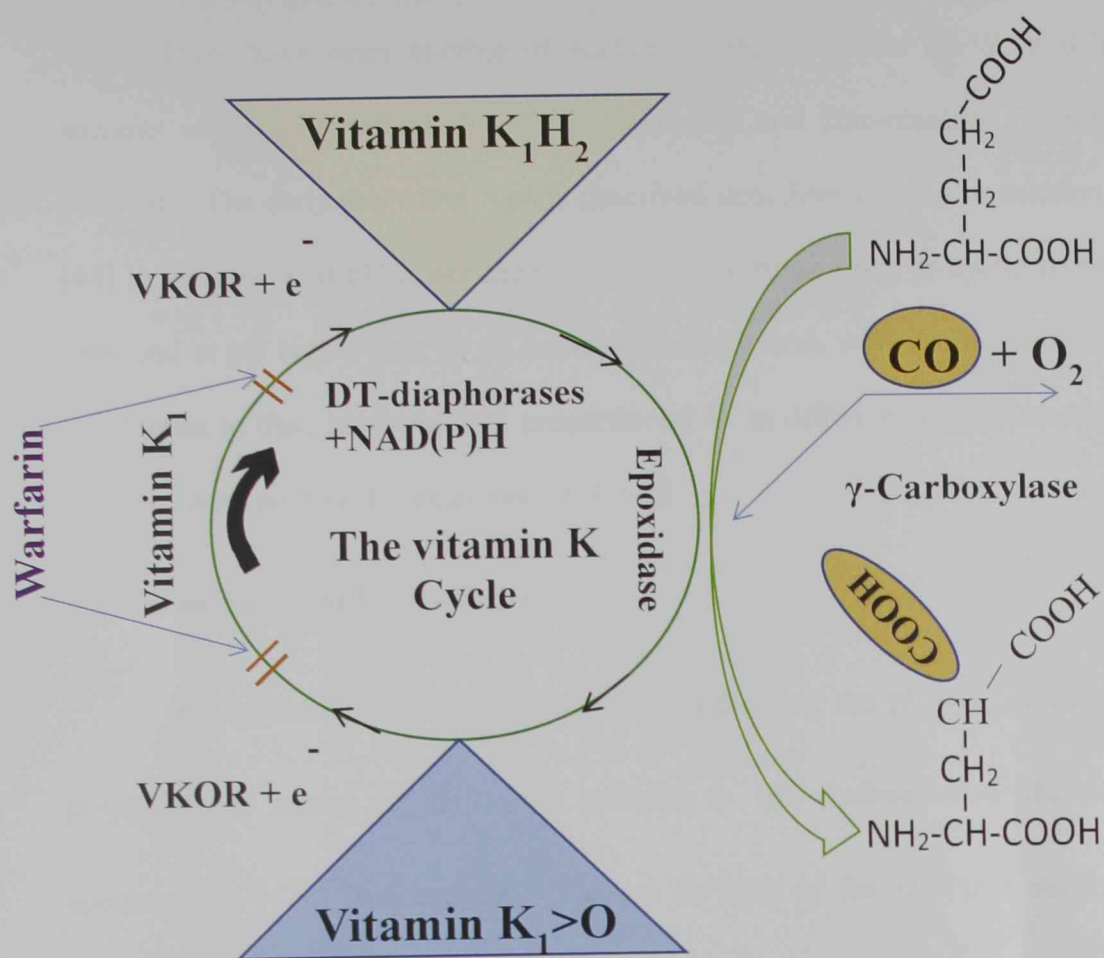


Figure 3: Vitamin K cycle

1.1.5 Warfarin as Fluorescent Probe

The fluorescence properties of warfarin which is a coumarin derivative [40] have attracted attention for several decades. Like other fluorescent probes, coumarins display photophysical properties that are sensitive to their local environments, such as supramolecular host cavities [41]. Within this context, supramolecular host-guest approach alongside fluorescence behavior of new coumarin derivative was exploited by Saleh *et al.* towards molecular recognition of optically inactive analytes [42].

There have been number of studies on the structures of W in different solvents and at different pH by NMR, absorption and time-resolved fluorescence [43]–[46]. The early precedent reports described structures of drug in solution [43], [44] to be present at pH lower than its $pK_a \sim 5$ as either open or cyclic protonated form and at pH higher than its pK_a as deprotonated form, whose side chain is open. In addition to that, photophysical properties of W in different solvents and solvent mixtures were profoundly examined [45], [46].

1.1.6 Time-Resolved Fluorescence

One of the most powerful tools for investigating the physical and chemical properties of matter or biological systems is the time-resolved fluorescence spectroscopy [47]. This becomes essential in light of the fact that steady state emission spectra sometimes are unspecified because the overlap emission profile of different fluorophores. In addition, steady-state fluorescence spectroscopy is not quantitative due to bleaching effects and gives no information in relation to the rate of deactivation process of an excited state of a drug molecule, as summarized by the Jablonski diagram in Figure 4 [48].

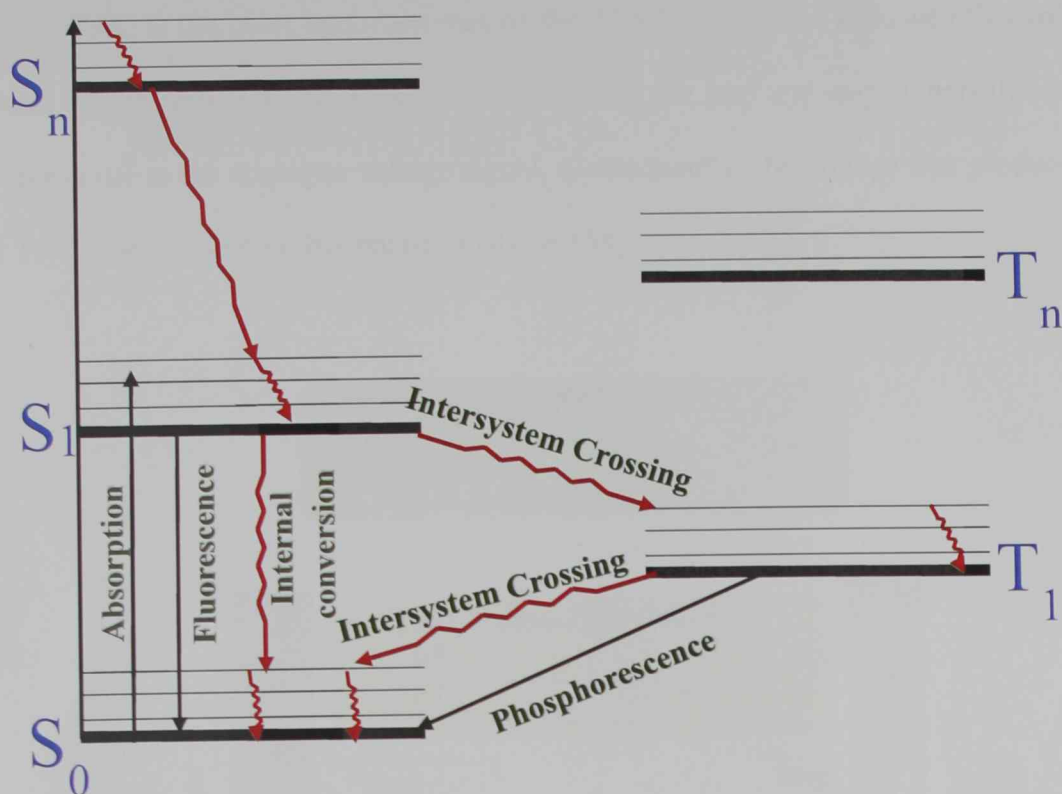


Figure 4: Representative Jablonski diagram

1.1.6.1 Time-Correlated Single Photon Counting (TCSPC)

One of the most popular ways of measuring time-resolved fluorescence is (TCSPC). The principle of this method is illustrated in Figure 5 [48]. A molecule will be excited by a laser pulse, a beam splitter is used to split part of the laser light which will reach to the photodiode. The photodiode is responsible for forming an electrical pulse that will be converted to 'start' signal by the discriminator. However, the stop signal is produced from the photon emitted by the sample that is recorded by a photomultiplier. The start signal is then required to start the time-to-amplitude converter (TAC).

TAC is the most important part of the TCSPC technique because of its role, which is measuring the difference in time between the start and stop signals that are proportional to the analogue voltage signal. Consequently, the voltage that produced by TAC is stored in a multi-channel analyzer [48].

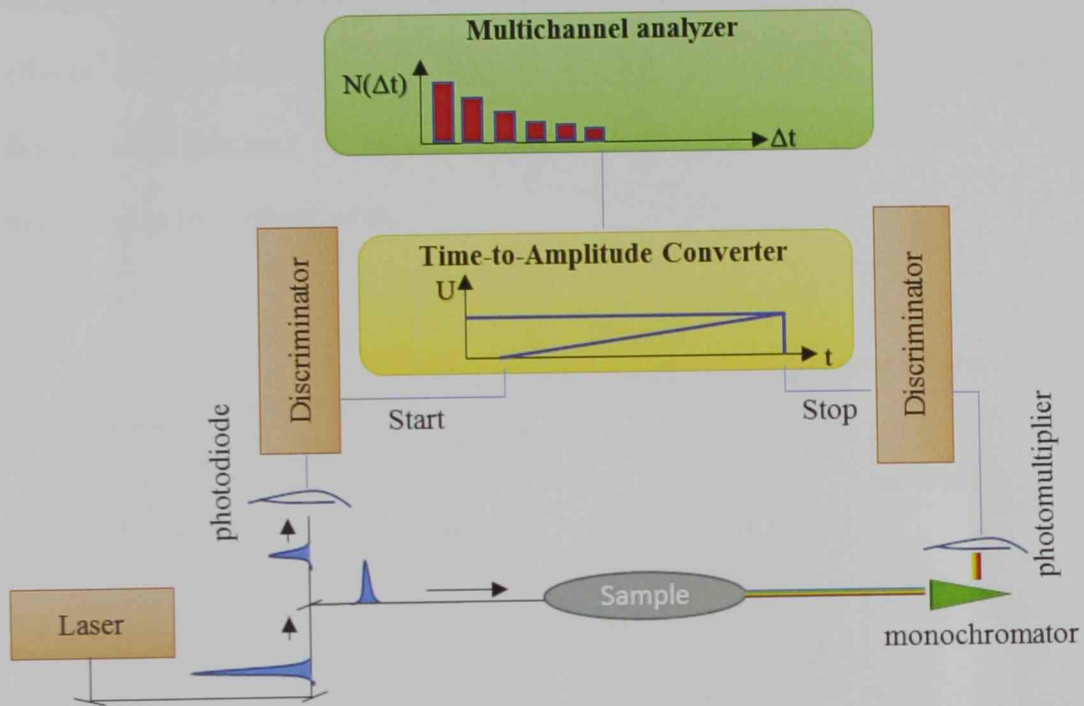


Figure 5: Time-correlated single photon counting (TCSPC) diagram

1.2 Aim of This Study

Our goal is to characterize the photophysical behaviors of warfarin (W) inside cyclodextrin macrocycles as shown in (Figure 6). In previous work, steady-state fluorescence measurements were studied. However, to understand the effect of encapsulation of warfarin on emission, a more quantitative tool for examining the effects of supramolecular cavities is needed. Accordingly, measurements of fluorescence lifetimes of W in presence and absence of cyclodextrins are highly motivated in the present work.

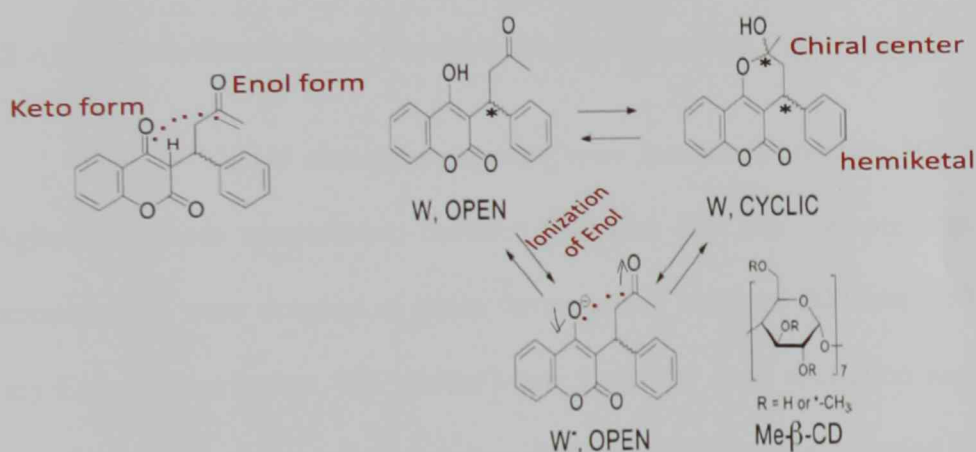


Figure 6: Structural formulas of protonated warfarin (W): Keto-Enol tautomerism, deprotonated warfarin (W⁻), and methyl-β-cyclodextrins (Me-β-CD) molecular container

Chapter 2: Experimental Work

2.1 Reagents

Wafarin (W), Acetyl- β -cyclodextrins (Ac- β -CD), Methyl- β -cyclodextrins (Me- β -CD), and (2-Hydroxyethyl)- β -cyclodextrins (HE- β -CD) were purchased from Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany (purity 99 %). D₂O, DCl and NaOD were also purchased from Sigma-Aldrich. Millipore water had conductivity less than 0.05 μ S. The pH values of the solutions were adjusted (\pm 0.2 units) by adding adequate amounts of HCl or NaOH.

2.2 Absorption/Steady-State Fluorescence Spectroscopy.

The UV/Visible absorption spectra were measured on Cary-300 instrument (Agilent) at room temperature, between 200 and 500 nm. Fluorescence spectra measurements were scanned at room temperature, between 300 and 550 nm on a Cary-Eclipse fluorimeter. Slit widths were 5 nm for both excitation and emission monochromators, unless otherwise specified. pH values were recorded using a pH meter (WTW 330i equipped with a WTW SenTix Mic glass electrode). Quartz cuvettes (1.0 cm, 3.0 mL) were used in all spectroscopic measurements and were obtained from Starna Cells Inc. (Atascadero, CA).

2.3 pH-Titration Studies

The pH titration by UV-Visible absorption spectroscopic method was accomplished by measuring the pH values of about 3 ml solutions contained in a rectangular quartz cuvette with 1 cm optical path length, and the absorption spectra were then recorded. To adjust pH, microliter volumes from 0.01 and 0.1M NaOH solution were pipetted consecutively to achieve the indicated pH values. The pK_a value was finally determined from fitting the titration data at a selected wavelength to sigmoidal formula derived from Henderson-Hasselbalch and Beer-Lambert laws. The fitting algorithm was provided by SigmaPlot's software (version 6.1; SPCC, Inc., Chicago, Illinois, USA).

2.4 Steady-State Binding Titration Studies

In the titration experiment, the total concentrations of the W were kept constant and that of the host was gradually increased. The pH of a certain volume of H₂O was first adjusted to either ~3 or ~9 in which a stock solution of W was prepared to give a final concentration of ~25 μ M. A calculated weight of β -CD derivatives was added to the same solution of W to prepare the stock solution of the complex (~ 3.5 mM). The solutions with the final concentration of β -CD were prepared by gradually adding increment volumes of the complex's stock solution to 2.4 ml of the free W directly in the quartz cuvettes. The absorption or fluorescence spectra were measured for each solution. The signals at certain wavelength were plotted as a function of host's total concentrations. The intermolecular interaction between β -CD and W may be quantified by the affinity constant referred to as the association equilibrium (K):



$$K = \frac{[W\text{-}\beta\text{-CD}]}{[W][\beta\text{-CD}]} \quad (2)$$

$$C_W = [W] + [W\text{-}\beta\text{-CD}] \quad (3)$$

$$C_{\beta\text{-CD}} = [\beta\text{-CD}] + [W\text{-}\beta\text{-CD}] \quad (4)$$

Where C_W and $C_{\beta\text{-CD}}$ mean the total concentrations of W and $\beta\text{-CD}$, respectively. It can be written that:

$$Y \text{ (Reading at certain } \lambda) = \text{constant 1} * [W] + \text{constant 2} * [W\text{-}\beta\text{-CD}] \quad (5)$$

Using Eqs.(2)-(5), we obtain

$$\Delta Y = \frac{\Delta(\text{constant})C_{\beta\text{-CD}}}{\frac{2}{KC_W - 1 - KC_{\beta\text{-CD}} + \sqrt{(1 - KC_W + KC_{\beta\text{-CD}})^2 + 4KC_W}} + 1} \quad (6)$$

Where ΔY = optical changes at a given λ ; $\Delta(\text{constant})$ = the difference between the molar absorptivity of free and $\beta\text{-CD}$ -complexed W in the case of absorption titration, and K = binding constant. The binding constants (K) were then evaluated by using the nonlinear formula of Eq. (6). Constant 2 was left as a floating parameter in the analysis by Levenberg-Marquardt algorithm, which was provided by SigmaPlot's software.

2.5 Time-Resolved Fluorescence Measurements

The emission decays of W in absence and presence of Me- β -CD at different pH values were collected using LifeSpec II spectrometer that is based on TCSPC method with excitation at 280 and 320 nm using two Edinburgh diode lasers with repetition rate at 20 MHz, time resolution of 90 ps, and a red-sensitive high-speed PMT detector (Hamamatsu, H5773-04). Emission decay were collected every 10 nm over the entire emission spectra of W and W- β -CD complex in aqueous solution with a dwell time of 50 s at each wavelength. The data were globally fitted to mono-exponential and bi-exponential model functions depending on the tested sample, then convoluted with instrument response function (IRF) of \sim 90 ps. The time-resolved data were specifically analyzed using Edinburgh FAST software (Appendix) in which decay-associated spectra (DAS) were constructed from the extracted intensity-contribution fraction (f_i) calculated from the pre-exponential amplitudes (B_i), as follows:

$$I(t) = \sum_i B_i \exp(-t/\tau_i) \quad (7)$$

$$f_i = \frac{B_i \tau_i}{\sum_j B_j \tau_j} \quad (8)$$

τ : lifetime for each electronic state (species)

Target analysis utilizing Glotaran software¹¹ were performed to confirm the kinetic expression for the population transfer of the two excited states that belong to free and CD-complexed W. Results indicated two mono-exponentially in parallel, which validates the meaning of DAS as described in text.

Chapter 3: Results & Discussion

3.1 Preliminary Investigation

At the beginning of this work, the establishment of reversible and stimuli-responsiveness host-guest complexes to calcium ions in aqueous solutions was pursued. The reversibility in drug retention and release can be achieved in response to the added calcium ions to different host-guest complexes of warfarin with cyclodextrins. While the drug causes blood bleeding, addition of calcium would then replace the drug from the cavity of host, inducing blood clotting [39]. Our envisaged results are inspired by recent reports [26], [37] on the possibility of reversibly tune the binding affinity of the guest to different hosts utilizing host-induced pK_a shifts which is accompanied by modulation of fluorescence signal.

Collectively, our focus was on creating a balance between blood bleeding and blood clotting that could be utilized in blood cells in the future while being monitored online by emission signal. The inspiration specifically comes from the ability of carbonyl-containing containers (see Ac- β -CD Figure 2) to tune the pK_a values of the embedded guests due to ion-dipole interaction.

Unfortunately, as shown in Figures 7-10 the pK_a shift is less than 1 unit between Warfarin and CDs. As a result, monitoring the stimuli responsiveness to calcium becomes impractical under these conditions. Alternatively, we opted to pursue the photochemistry of warfarin in water and inside CD with a similar aim towards establishing stimuli-responsive, reversible system yet due to the ability of host to induce molecular switching instead of pK_a shifts.

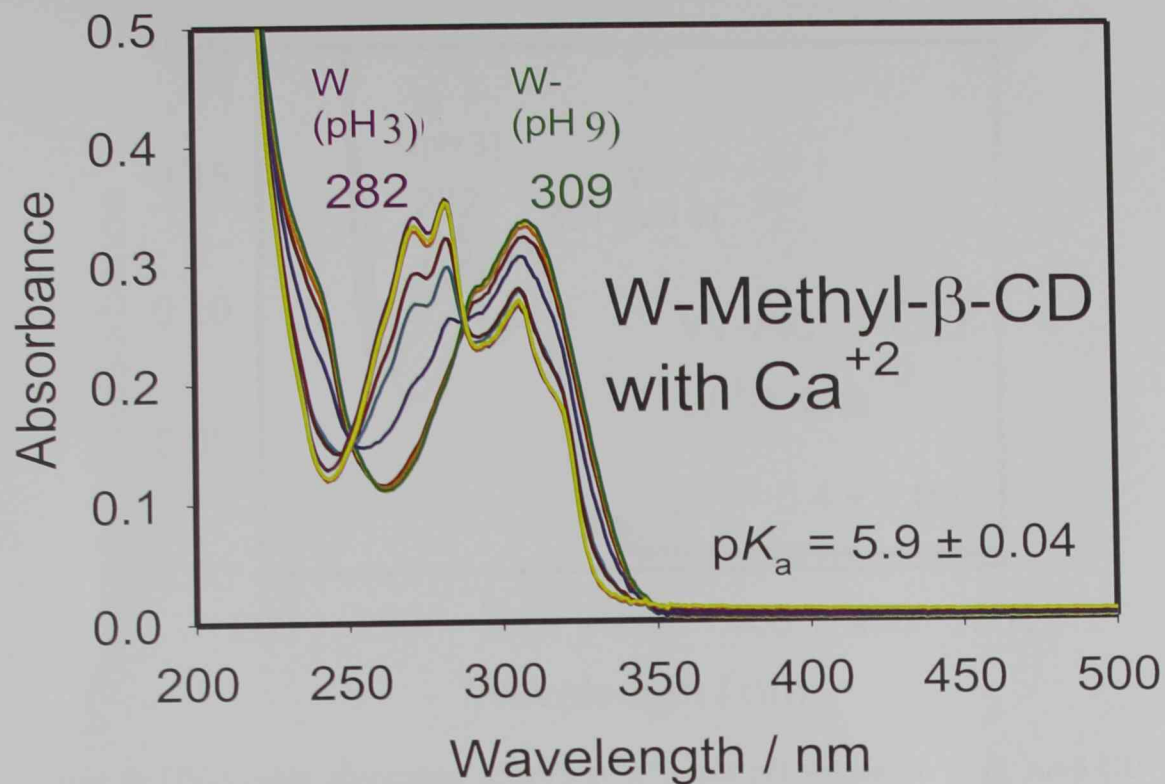


Figure 7: UV-Visible absorption spectra at different pH values of W in Me- β -CD with Ca^{+2}

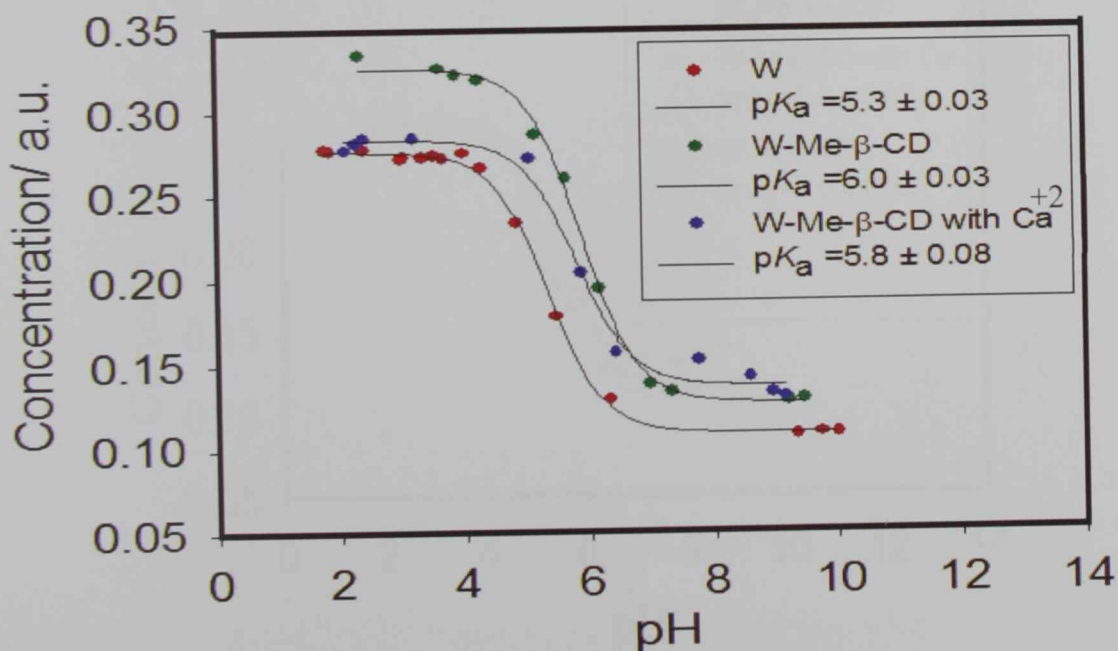


Figure 8: UV-Visible absorption spectra of W in aqueous solutions at different pH values from 2-10 in water and inside Me- β -CD. The experimental fitting error for the indicated pK_a values and the corresponding spectra are shown in the insets

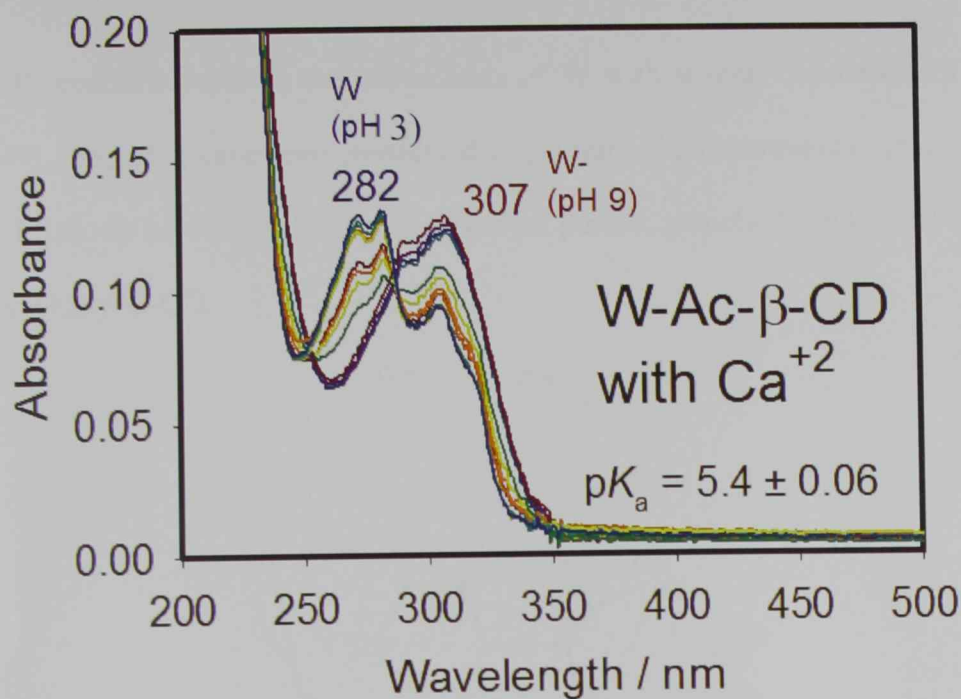


Figure 9: UV-Visible absorption spectra at different pH values of W in Ac-β-CD with Ca²⁺

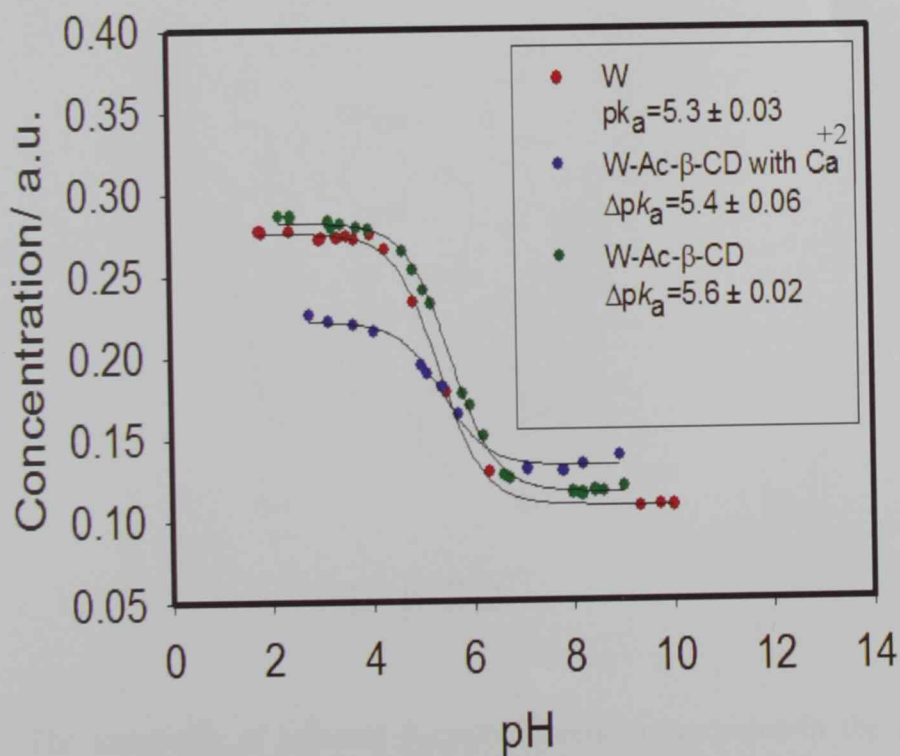
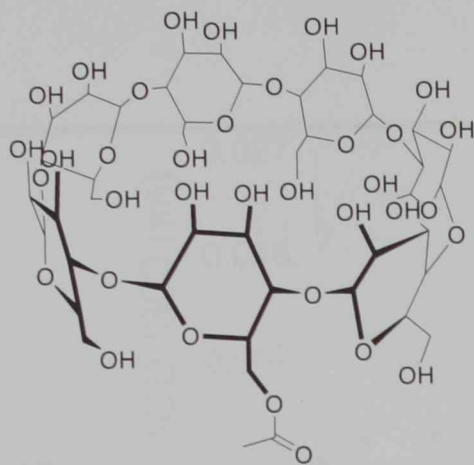


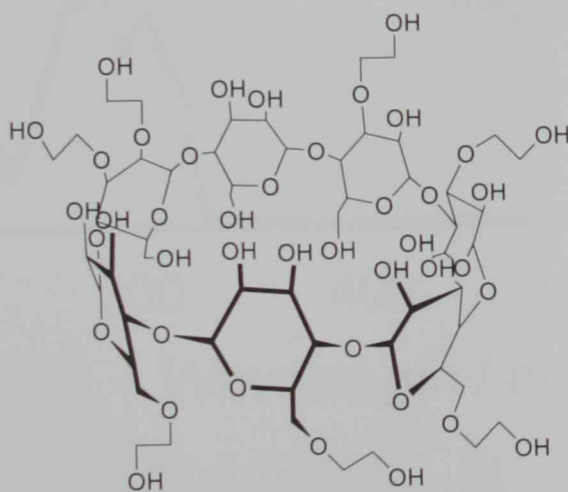
Figure 10: UV-Visible absorption spectra of W in aqueous solutions at different pH values from 2-10 in water and inside Ac-β-CD. The experimental fitting error for the indicated pK_a values and the corresponding spectra are shown in the insets

3.2 Interactions of Warfarin with Cyclodextrins

Precedent reports on the interactions of W with several derivatives of CDs [46], [49], [38], [50] have been considered in planning the experiments in this work. Figure 11 shows new derivatives of β -CD were pursued, namely Acetyl- β -CD and 2-Hydroxyl Ethyl- β -CD.



Ac- β -CD



HE- β -CD

Figure 11: The structures of selected β -cyclodextrins macrocycles in the present work that were not studied before with W

3.2.1 Interactions of Warfarin with Ac- β -CD at pH 3

The rationale behinds our selection arises from the presence of additional hydroxyl or carbonyl functional groups that could enhance interaction with W. Unfortunately, Ac- β -CD cyclodextrin at pH 3 gave weak or no interaction with W, as monitored by UV-Visible absorption measurements in Figure 12.

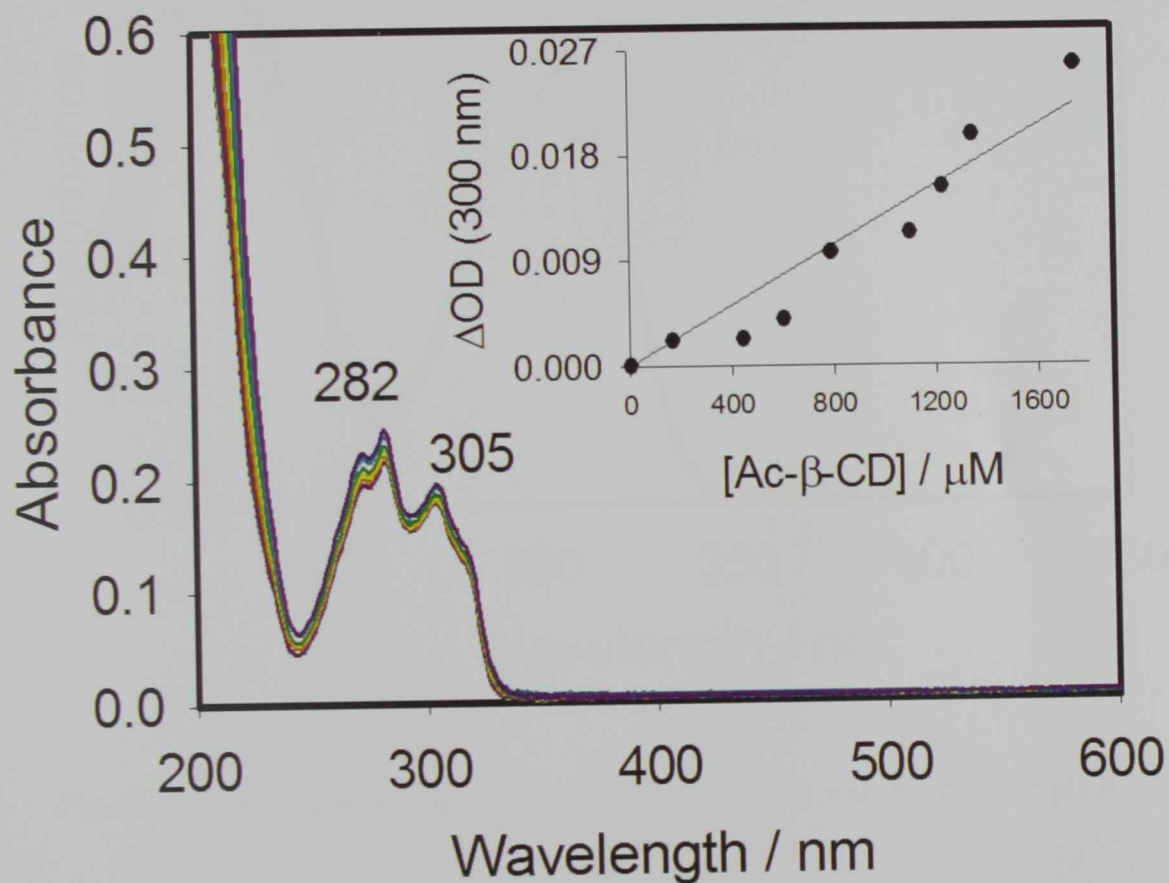


Figure 12 : UV-Visible absorption titration of W (25 μM) with Ac- β -CD at pH 3; the inset shows the corresponding titration curve and the 1:1 binding fit (solid line) with $K = (5.5 \pm 78) \text{ M}^{-1}$. The very large error in binding affinity reflects very weak binding

3.2.2 Interactions of Warfarin with Ac- β -CD at pH 9

In addition to that, even at pH 9 no isosbestic points was noticed as shown in Figure 13. Consequently, Ac- β -CD gave no interaction with warfarin at high pH.

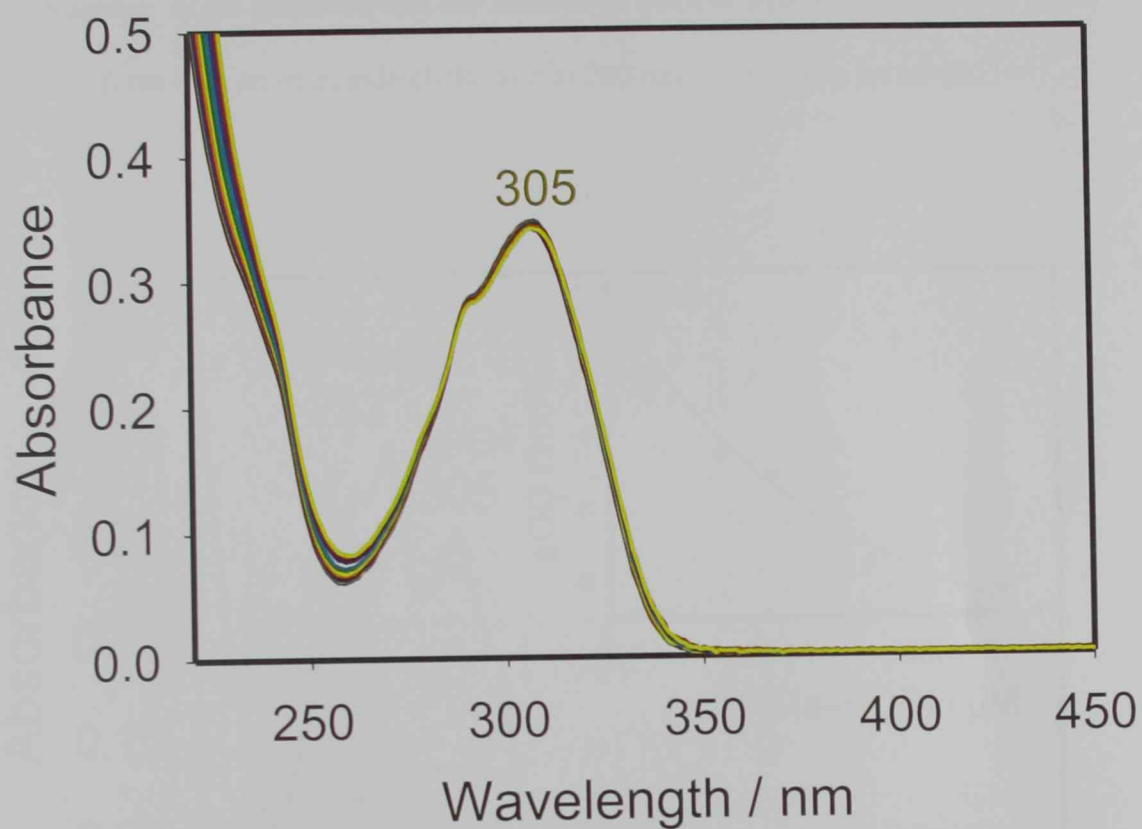


Figure 13: UV-Visible absorption titration of W (25 μ M) with Ac- β -CD at pH 9

3.2.3 Interactions of Warfarin with Me- β -CD at pH 3

However, previously examined derivative Me- β -CD gave the highest binding constants [49]. In Figure 14 at pH 3, only the open protonated form moderately reacts with the host ($K = 2,900 \text{ M}^{-1}$) because isosbestic points were only seen at 305 nm. Vasquez *et al.* indicated that the absorption peak at 310 nm is attributed to the warfarin form with an open side chain, and at 280 nm it is a cyclic hemiketal [46].

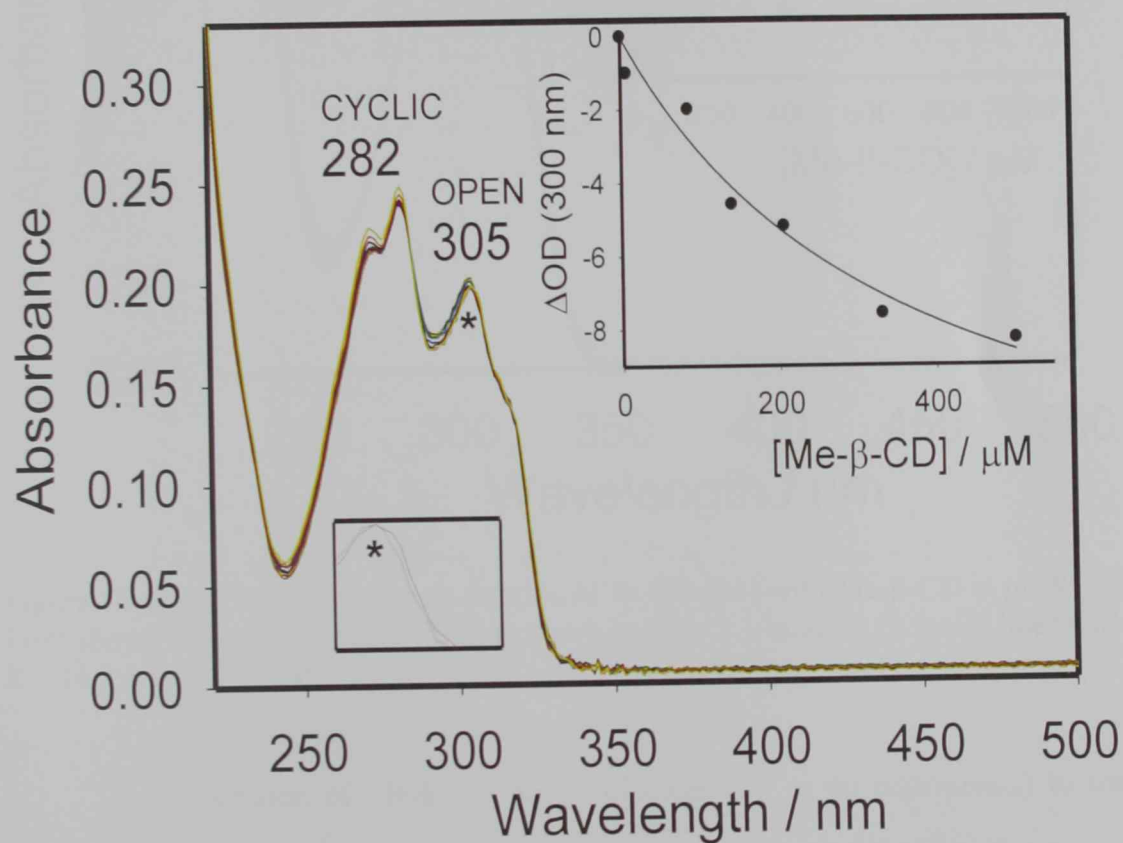


Figure 14: UV-Visible absorption titration of W (25 μM) with Me- β -CD at pH 3; the inset shows the corresponding titration curve and the 1:1 binding fit (solid line) with $K = (2.9 \pm 0.3) \times 10^3 \text{ M}^{-1}$

3.2.4 Interactions of Warfarin with Me- β -CD at pH 9

However W⁻ weakly binds CD at high pH (Figure 15) with binding constant of 420 M^{-1} in agreement with previous reports [38], [46], [50].

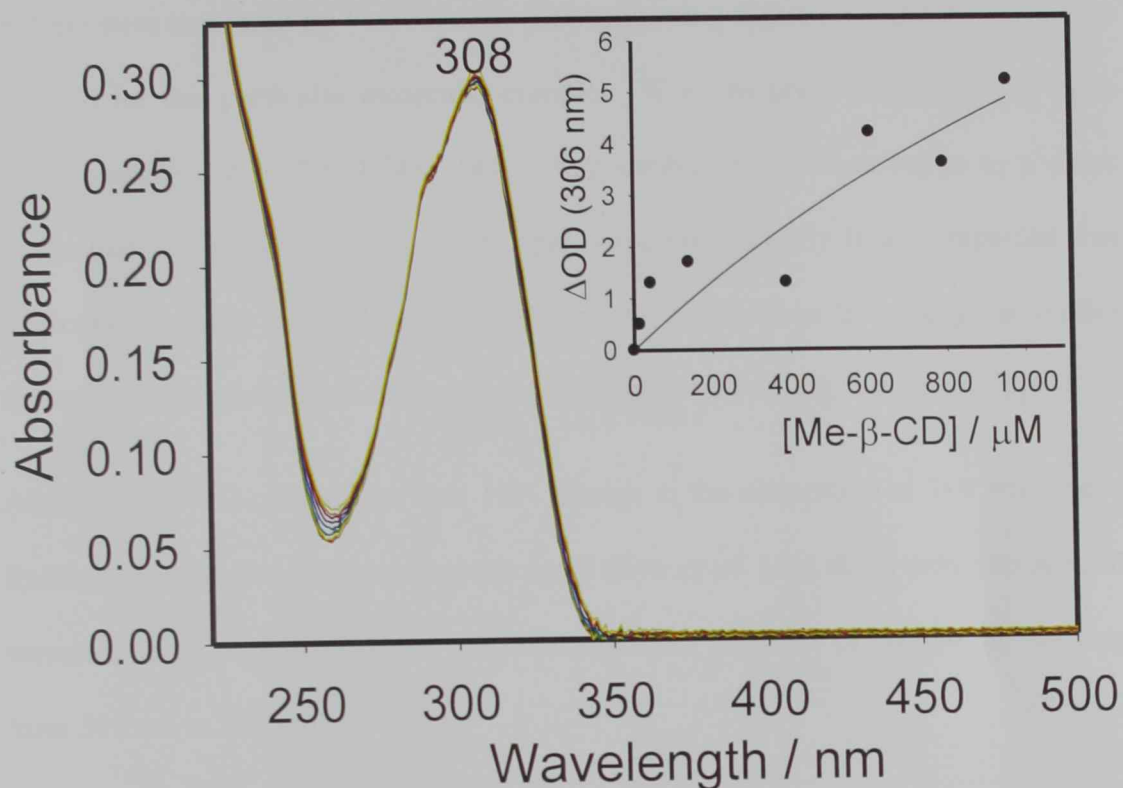


Figure 15: UV-Visible absorption titration of W (25 μ M) with Me- β -CD at pH 9; the inset shows the corresponding titration curve and the 1:1 binding fit (solid line) with $K = (4.2 \pm 1.0) \times 10^2 \text{ M}^{-1}$

Upon addition of Me- β -CD host molecules (up to 40 equivalents) to the aqueous solutions of W at pH 3 and 9 (Figure 14,15), characteristic changes in the UV-Visible absorption spectra were observed with the occurrence of several isosbestic points (311 nm, 315 nm, and 320 nm) confirming the formation of a 1:1 binding stoichiometry [46].

The corresponding binding constants between W and Me- β -CD at a given pH were derived directly from the optical titration plot at a given wavelength using the formula described in the experimental section.

Absorption spectra upon addition of β -CD to W in water at pH 7.4 (phosphate buffer) were measured by Vullve *et al.*, [46] suggesting that assumption of a 1:1 ratio is valid for this particular molecular complex. W exists predominantly in its open form when bound to β -CD [43], [44]. They attributed this observation to a steric factor that forces W to remain in its open structure. Initially it was expected that hydrophobic cavity of β -CD would stabilize the cyclic form by analogy to earlier report in other hydrophobic media such as Cytochrome P450 2C9 (CYP2C9) [51].

Addition of β -CD caused less than 10% change in the absorption at 310 nm. These findings support the conclusion made by Vullve *et al.* [46] since only the longest wavelength absorption maxima was affected upon addition of Me- β -CD, shifting from 305 nm to 306 nm at pH 3.

3.3 Optical Measurements and Host-Induced pK_a Shifts

A very recent experimental and theoretical investigations by Nowak *et al.* on several hydroxycouamrin derivatives concluded that different location of hydroxyl group should affect the value of pK_a [52]. Motivated by this work, the changes of absorption spectral profiles of W free and CD-complexes in aqueous solutions was graphed as a function of pH values as shown in Figures 16-19.

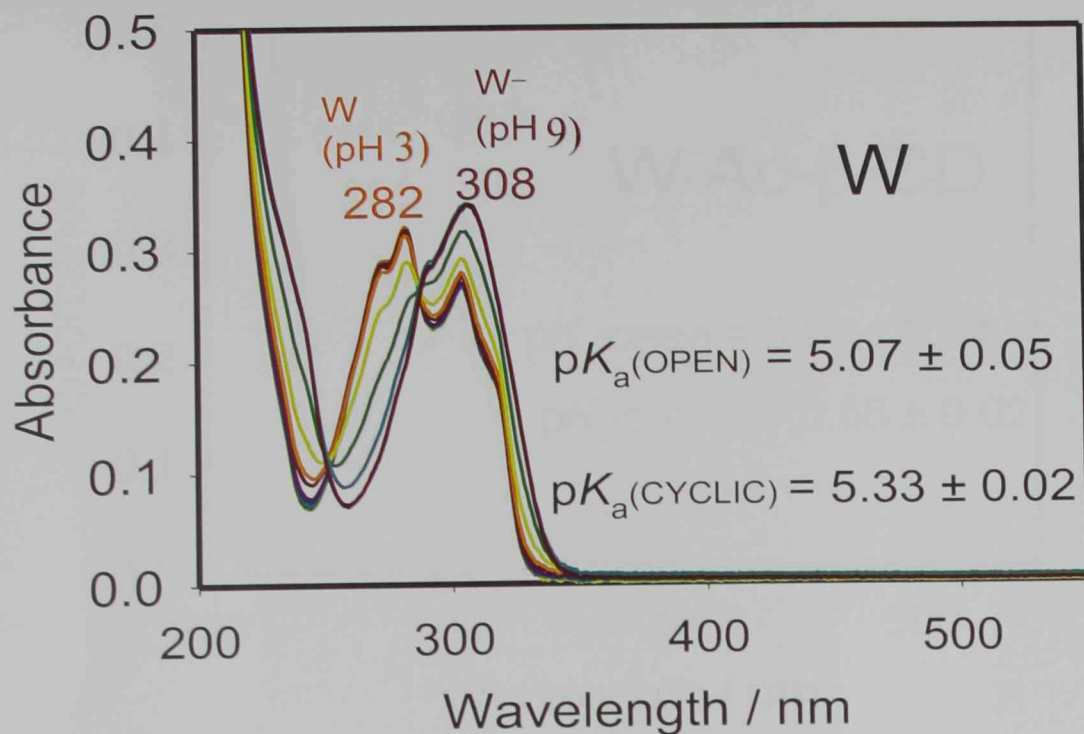


Figure 16: UV-Visible absorption spectra at different pH values of W in water. The sigmoidal fitting error for each extracted pK_a at 280 (CYCLIC) and 320 nm (OPEN) are shown in the insets

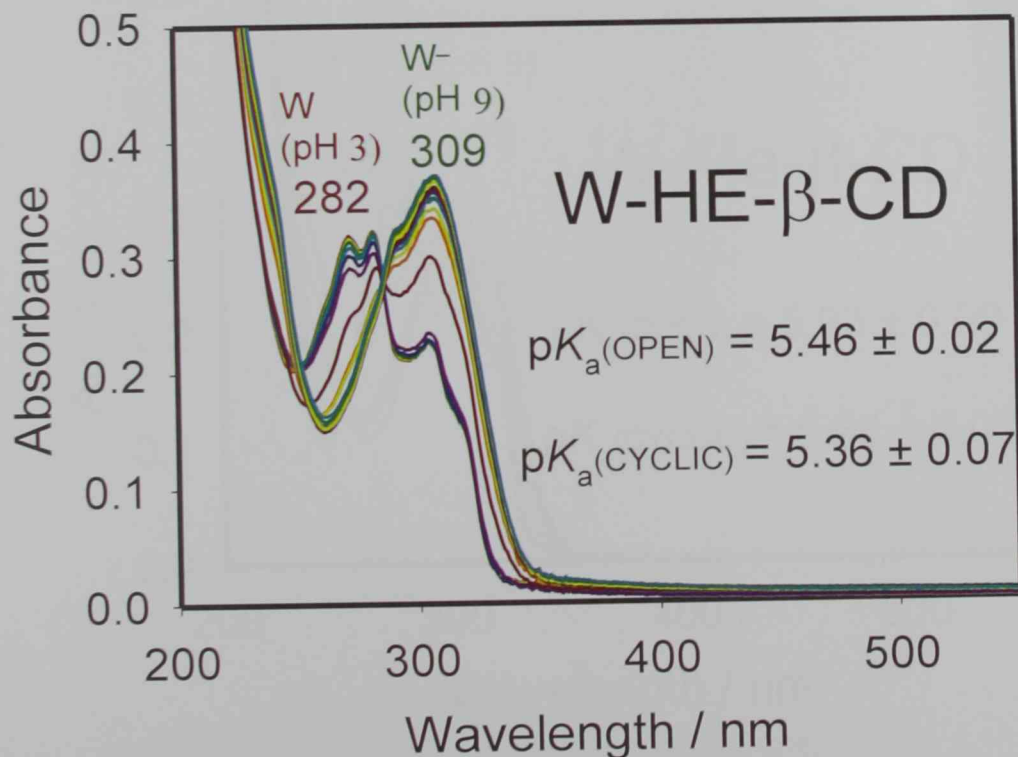


Figure 17: UV-Visible absorption spectra at different pH values of W in HE- β -CD. The sigmoidal fitting error for each extracted pK_a at 280 (CYCLIC) and 320 nm (OPEN) are shown in the insets

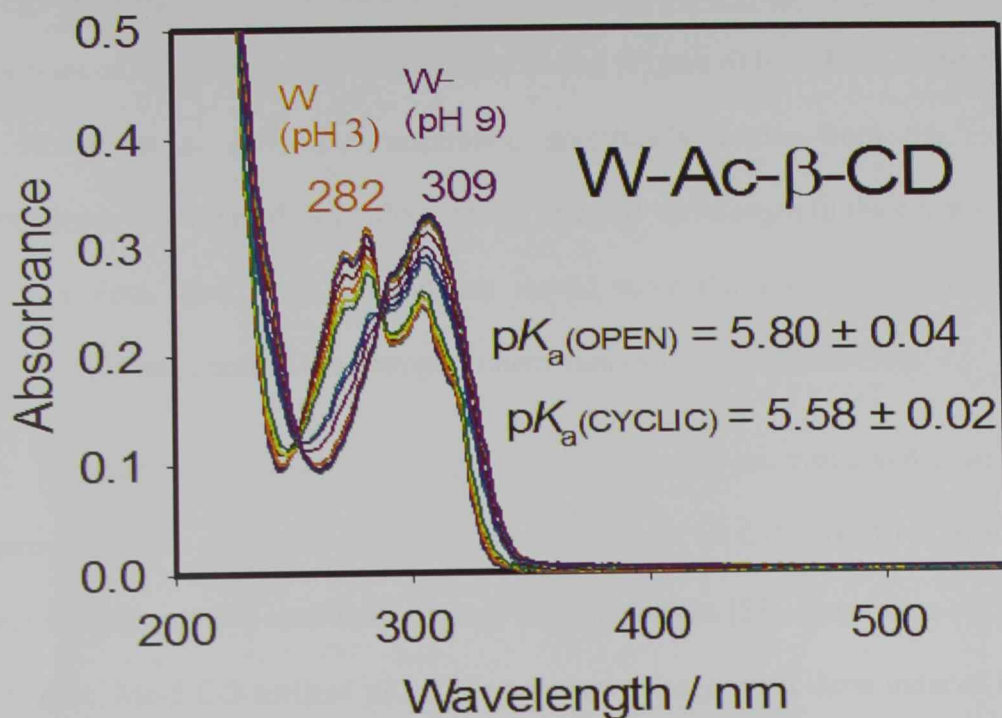


Figure 18: UV-Visible absorption spectra at different pH values of W in Ac- β -CD host. The sigmoidal fitting error for each extracted pK_a at 280 (CYCLIC) and 320 nm (OPEN) are shown in the insets

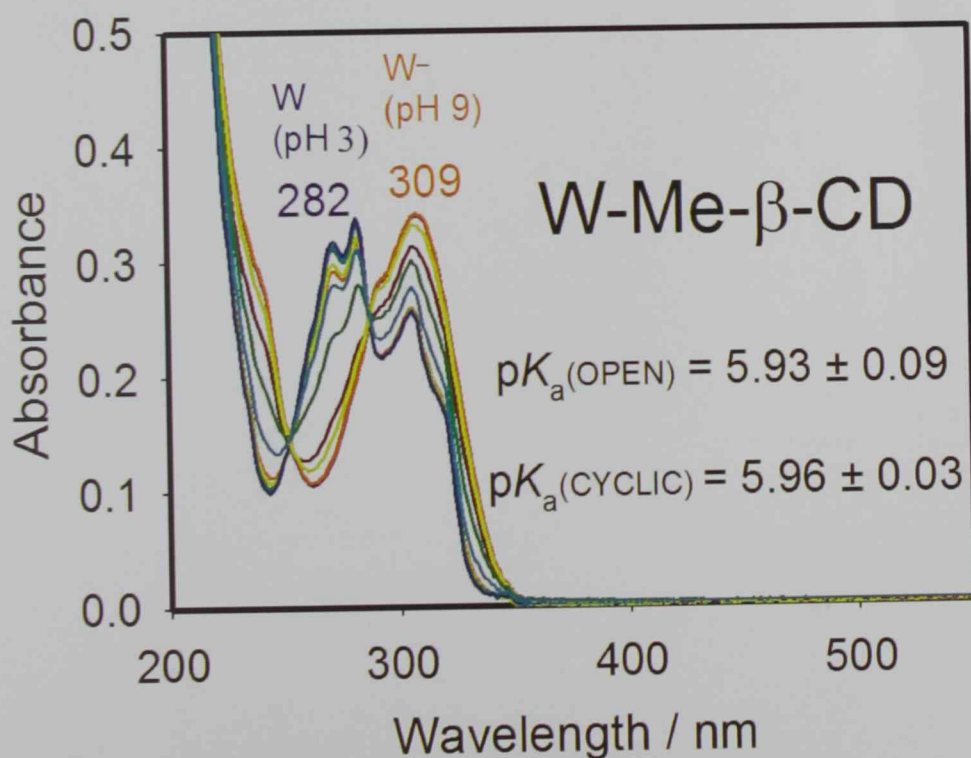


Figure 19: UV-Visible absorption spectra at different pH values of W in ME- β -CD. The sigmoidal fitting error for each extracted pK_a at 280 (CYCLIC) and 320 nm (OPEN) are shown in the insets

Although Me- β -CD-induced pK_a shifts corresponding to the deprotonation processes of the hydroxyl group in coumarin ring (Figure 6) have been studied before by Nowak *et al.* [49], our inspiration specifically comes from the expected dependence of extracted pK_a values on the selected wavelength in the corresponding titration plots. Also, these experiments should serve fluorescence lifetime data at different pH and confirm the strongest interactions with methylated CDs.

Figures 20-21 illustrated the pH titration plots for the cyclic and open forms. Positive shifts in pK_a were observed upon addition of all CD derivatives in aqueous solution under similar conditions of ionic strength effects [53]. Regardless of the type of isomer, Me- β -CD-assisted pK_a shifts are always larger than those induced by Ac- β -CD ($\Delta pK_a \sim 0.9$ vs. ~ 0.7 for open forms and ~ 0.6 vs. ~ 0.3 for cyclic forms). The HE- β -CD induced the least pK_a shifts ($\Delta pK_a \sim 0.4$ for open forms, and no shift observed in case of cyclic forms).

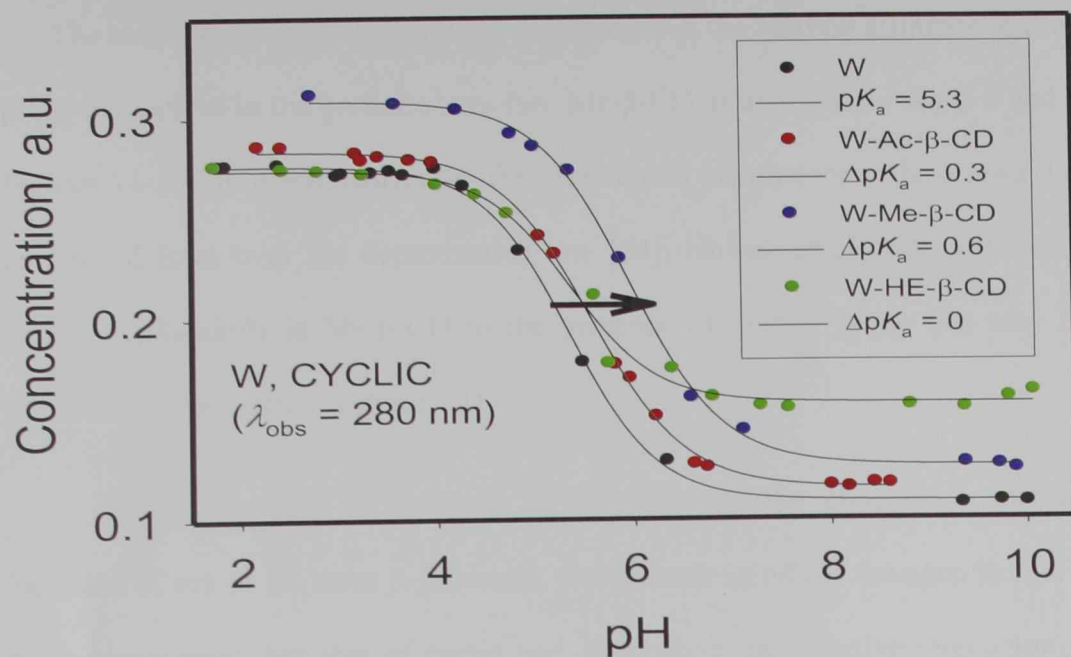


Figure 20: UV-Visible absorption spectra of W in aqueous solutions at different pH values from 2-10 at $\lambda_{\text{obs}} = 280$ nm in water and inside HE- β -CD, Ac- β -CD, and Me- β -CD. The experimental fitting error for the indicated pK_a values and the corresponding spectra are shown in Figures 16-19

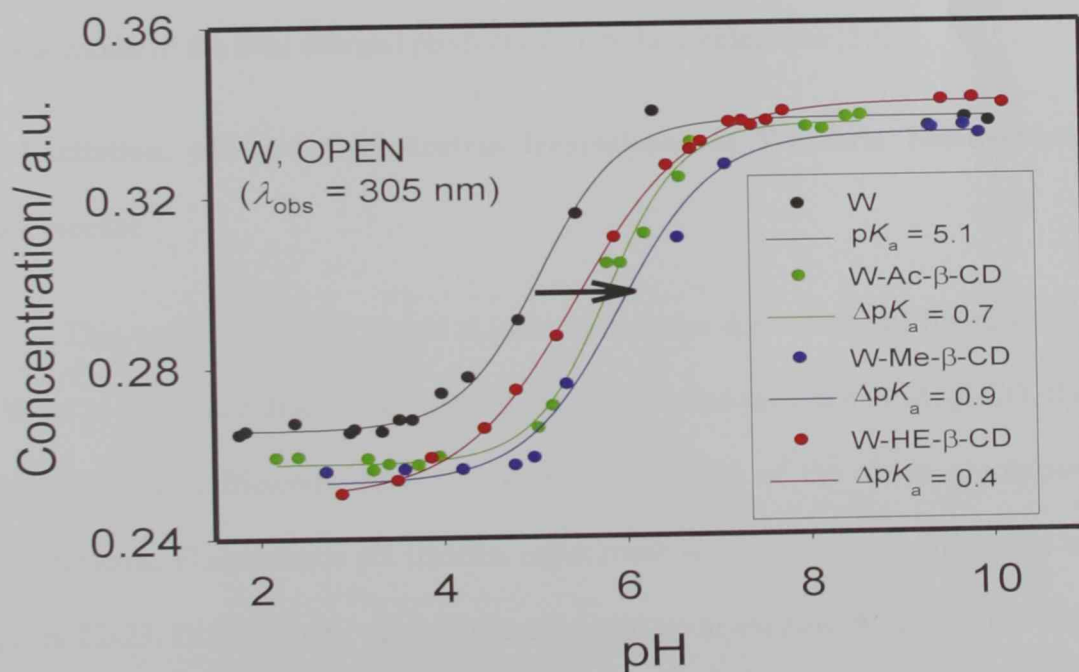


Figure 21: UV-Visible absorption spectra of W in aqueous solutions at different pH values from 2-10 $\lambda_{\text{obs}} = 305$ nm in water and inside HE- β -CD, Ac- β -CD, and Me- β -CD. The experimental fitting error for the indicated pK_a values and the corresponding spectra are shown in Figure 16-19

The host-induced pK_a shifts reflect the changes in the binding affinities of drug to β -CD derivatives in the ground states (see Me- β -CD as an example at pH 3 and 9 in Figures 14-15), thus rationalized by the preferential interactions of host towards the protonated form over the deprotonated one [54]. Nowak *et al.*, [49] attributed the strongest pK_a shifts in Me- β -CD to the presence of methyl group that may have preferentially interacted with the CD cavity.

More important to the focus of this work is the observation that upon inclusion in any of the three β -CD hosts, the increase in pK_a of the open isomer was more pronounced than that of cyclic one, supporting the selective interaction with open form over the cyclic one.

In addition to that, open form of free W appears to be more acidic in ground state (pK_a 5.1 vs. 5.3) than the cyclic one, presumably due to extending electron delocalization in the final charged product (W^-) in the former case [52].

3.4 Excitation, pH and Cyclodextrin Dependence of Warfarin Steady-State Fluorescence

This work specifically aimed at investigating the dependence of fluorescence of W on pH and excitation wavelength in the absence and presence of Me- β -CD, the host which has sufficiently interacted with W by virtue of the above absorption measurements. Fluorescence pH titration experiment was conducted as illustrated in Figures 22-23. Different pK_a values were extracted upon exciting W in water at 280 nm and 320 nm (pK_a 6.1 vs. 5.7), which is again attributed to the deprotonation of the hydroxyl proton (Figure 6) with the open form being more acidic.

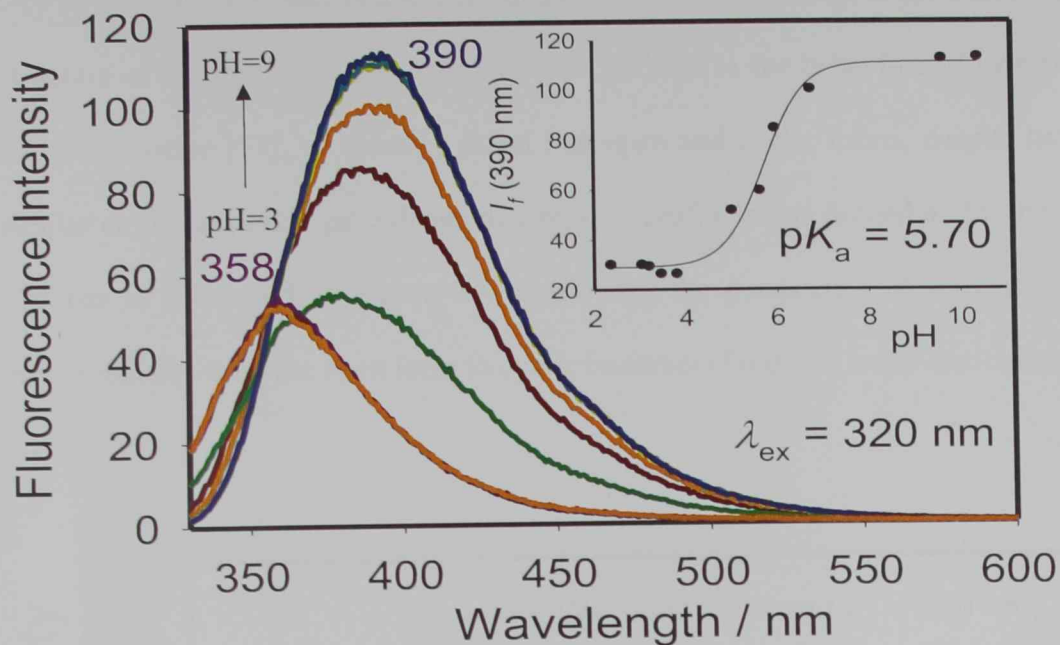


Figure 22: Fluorescence spectra of W (25 μM) at different pH values with excitation at 320 nm; the inset shows the experimental fit to a sigmoidal function (solid line), which gives $\text{p}K_a = 5.70 \pm 0.07$. Slit widths were 5 nm for excitation and emission

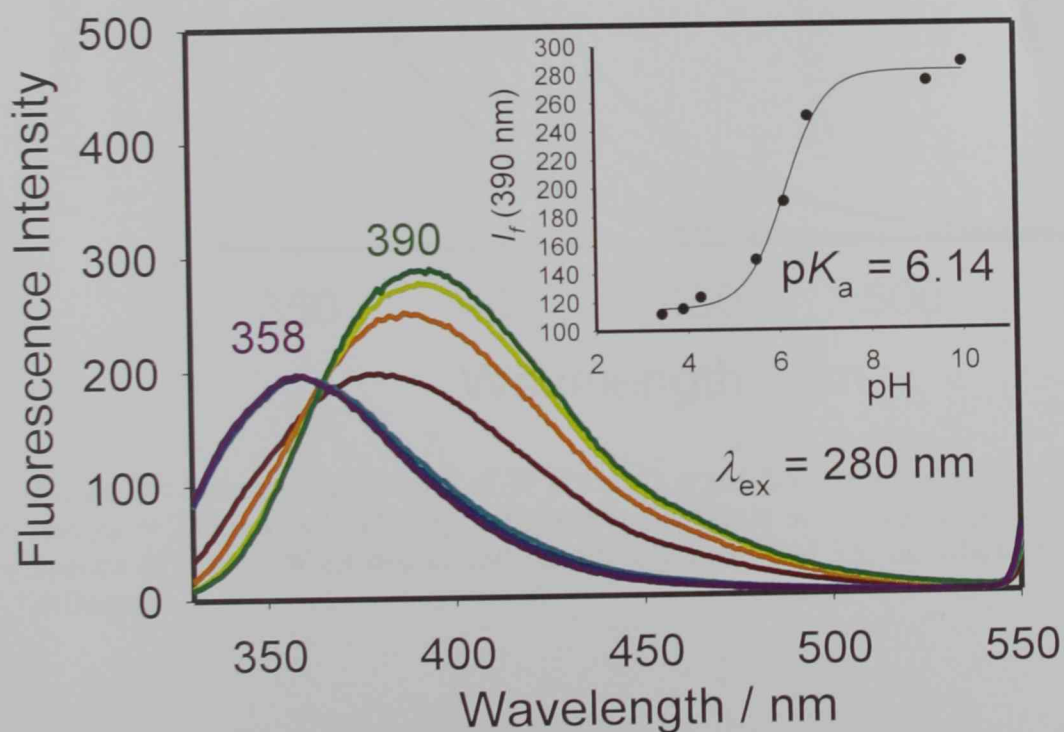


Figure 23: Fluorescence spectra of W (25 μM) at different pH values with excitation at 280 nm; the inset shows the experimental fit to a sigmoidal function (solid line), which gives $\text{p}K_a = 6.14 \pm 0.05$. Slit widths were 5 nm for excitation and 10 nm for emission

This could be ascribed to extended electron delocalization in the excited-state structure of the formed deprotonated form in parallel to the behaviors of isomers in the ground-state [53]. It must be noted that open and cyclic forms, despite having similar emission peaks, gave different emission profile when excited at 280 nm and 320 nm as illustrated in Figure 24, confirming the persistence of intramolecular proton transfer from the open form to cyclic tautomer (Figure 6) in the excited state.

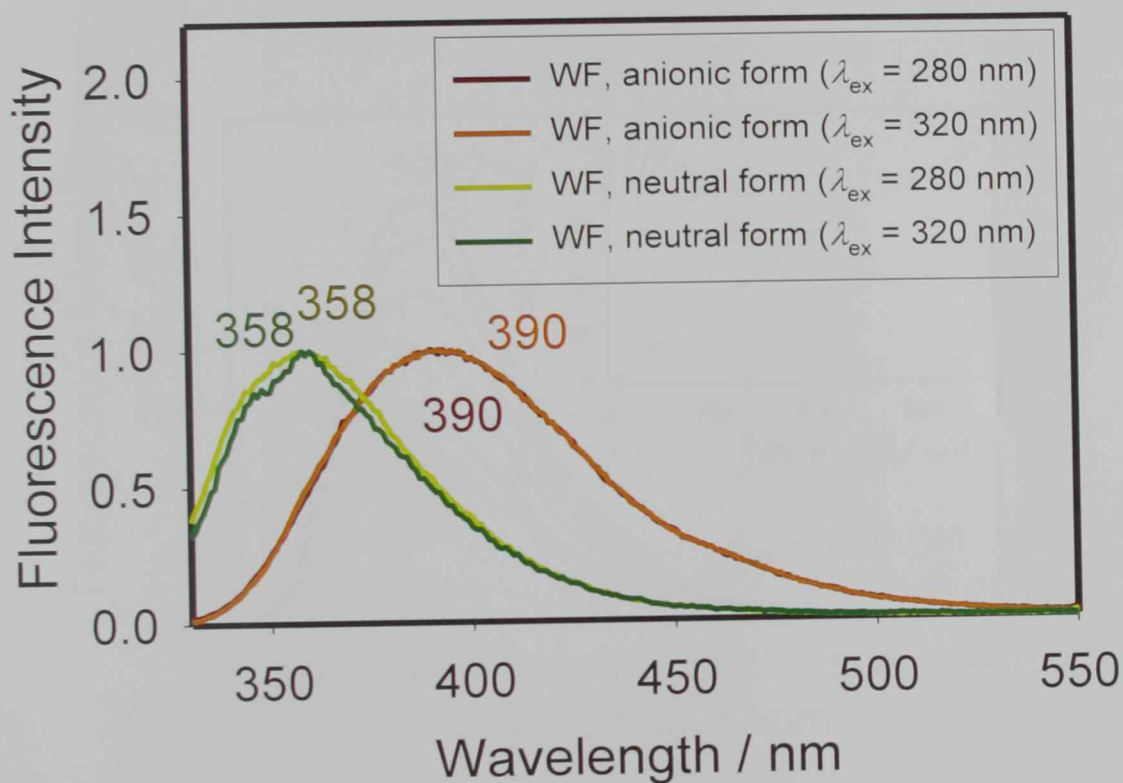


Figure 24: Fluorescence spectra of W (25 μM) at pH 2.5 and 10 with $\lambda_{\text{ex}} = 320 \text{ nm}$ and $\lambda_{\text{ex}} = 280 \text{ nm}$ as labeled by a distinct color. While no change in peak position, spectra of neutral W excited at 280 nm (dark yellow) and 320 nm (dark green) are different

As far as the effects of CDs on emission of W, contrarily to the observed three-fold fluorescence enhancement upon inclusion of W⁻ form at pH 9 (Figure 25; excitation at 320 nm) with binding affinity of 266 M^{-1} in agreement previous reports [38], [46], [50], the protonated (open or cyclic) forms at pH 3 showed very weak enhancement in fluorescence upon addition of 10 equivalents of host as illustrated in Figures 26-27. These contradictable results warrant further investigations using time-resolved fluorescence spectroscopy.

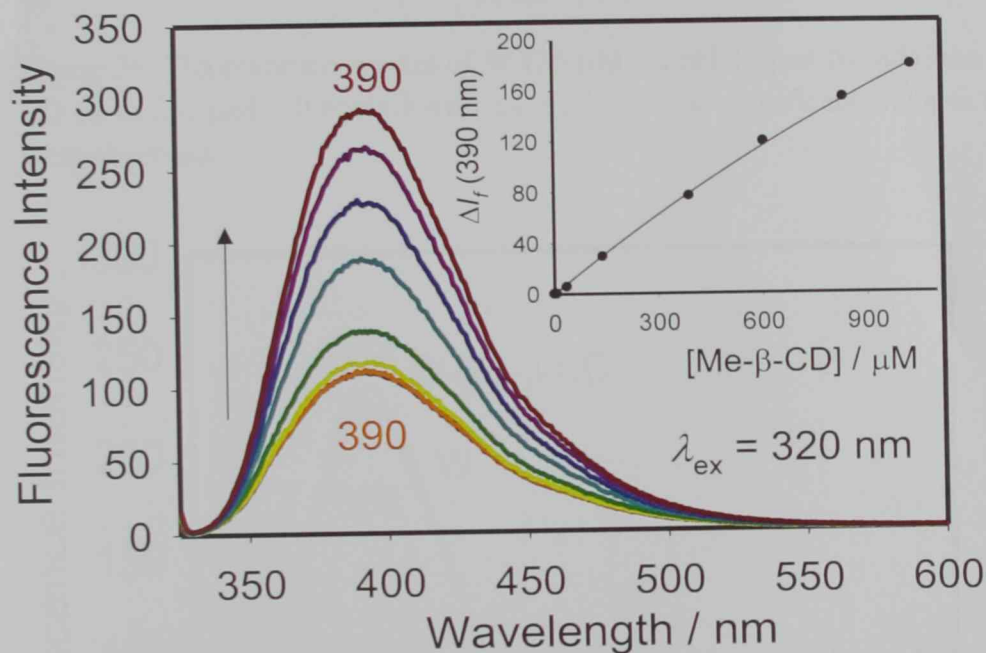


Figure 25: Fluorescence titration of W ($25 \mu\text{M}$) with Me- β -CD at pH 9 with excitation at 320 nm the inset shows the corresponding titration curve and the 1:1 binding fit (solid line) with $K = (2.66 \pm 0.06) \times 10^2 \text{ M}^{-1}$

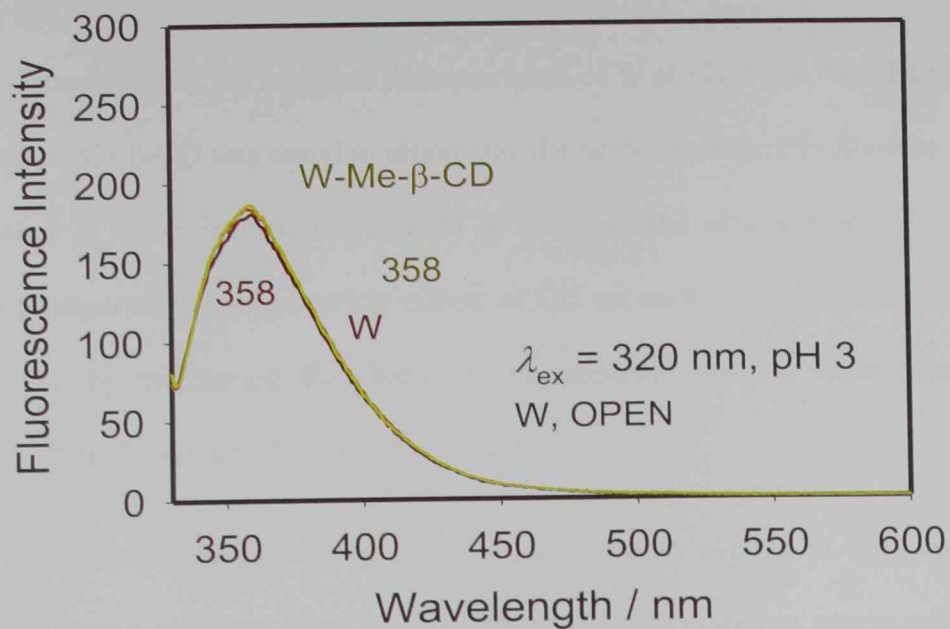


Figure 26: Fluorescence spectra of W (25 μM) at pH 3 upon the addition of Me- β -CD up to 250 μM (10 equiv.) with $\lambda_{\text{ex}} = 320 \text{ nm}$, no significant changes in spectra were observed

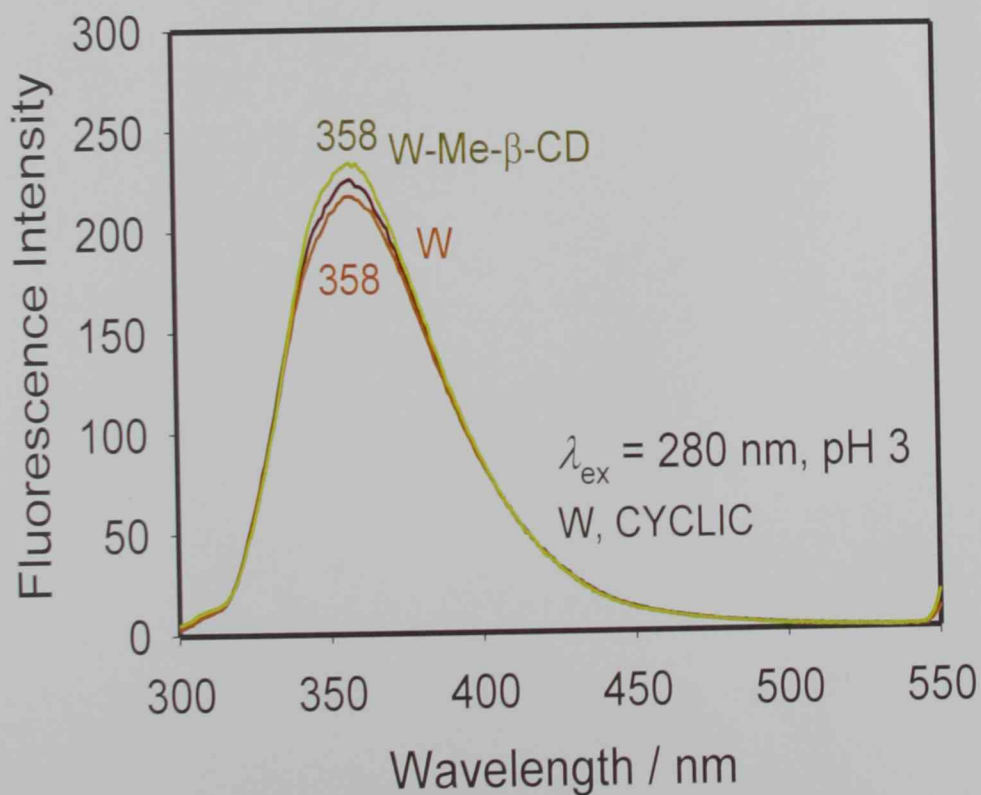


Figure 27: Fluorescence spectra of W (25 μM) at pH 3 upon the addition of Me- β -CD up to 250 μM (10 equiv.) with $\lambda_{\text{ex}} = 280 \text{ nm}$, no significant changes in spectra were observed

3.5 Fluorescence Lifetime Measurements/Decay-Associated Spectra (DAS)

Time-resolved fluorescence measurements of W at pH 3 and 9 in absence and presence of Me- β -CD was opted to rationalize the no enhancement in fluorescence of protonated W upon inclusion compared to those results observed at pH 9. Also sought to separate the sequestering effects of CD on each protonated isomer (open vs. cyclic), by monitoring the change in excited-state lifetime when selectively exciting W at 320 nm and 280 nm, respectively.

Previously, the two forms were simultaneously excited at 300 nm [45]. Emission decays collected every 10 nm across the entire emission spectra of free W at pH 3 upon excitation at 320 nm and 280 were globally fitted to monoexponential decays as shown in Figures 32-33 (Appendix) and tabulated in Table 2.

Table 2: Spectroscopic and photophysical data of W at 25 μ M in absence and in presence of Me- β -CD (1.0 mM). The DAS maximum for each lifetime is given in bracket

Drug Forms	$\lambda_{\text{abs}} / \text{nm}$	$\lambda_{\text{ex}} / \text{nm}$	$\lambda_{\text{em}} / \text{nm}$	τ_1 / ns ($\lambda_{\text{em}} / \text{nm}$)	τ_2 / ns ($\lambda_{\text{em}} / \text{nm}$)
W, OPEN (pH 3)	305	320	358	< 0.1	
W, CYCLIC (pH 3)	282	280	358	< 0.2	
W- (pH 9)	308	320	390	< 0.2	
W-Me- β -CD, OPEN (pH 3)	306	320 ^a	358	< 0.1 (358)	2.0 \pm 0.4 ^b (375)
W-Me- β -CD (pH 9)	309	320	390	< 0.2 (390)	1.22 \pm 0.02 ^b (390)

^a Upon excitation at 280 nm, no change in excited-state lifetime was observed upon inclusion at pH 3. ^b Values are presented as mean \pm standard deviation. The estimated experimental error was 20% and 1.6% for the complex lifetimes at pH 3 and 9, respectively.

The excited-state lifetimes appear within our instrument resolution of 90 ps in agreement with others [45]. However, when Me- β -CD complex were excited at pH 3 both mono-exponential and biexponential decays were observed as shown in Figures 34-35 (Appendix).

Although addition of CD at pH 3 did not affect the position of peaks (390 nm), excited-state lifetime increased but only when the complex was excited at 320 nm, and not 280 nm (Figure 28, 34 and 35), supporting that Me- β -CD host favors the open tautomer form. Even though emission decays of drugs were collected in Figures 28-29 upon addition of 40 equivalents of host (as limited by its solubility in water of about 15 mM), complete formation of complex has not been achieved due to relatively weak binding constant.

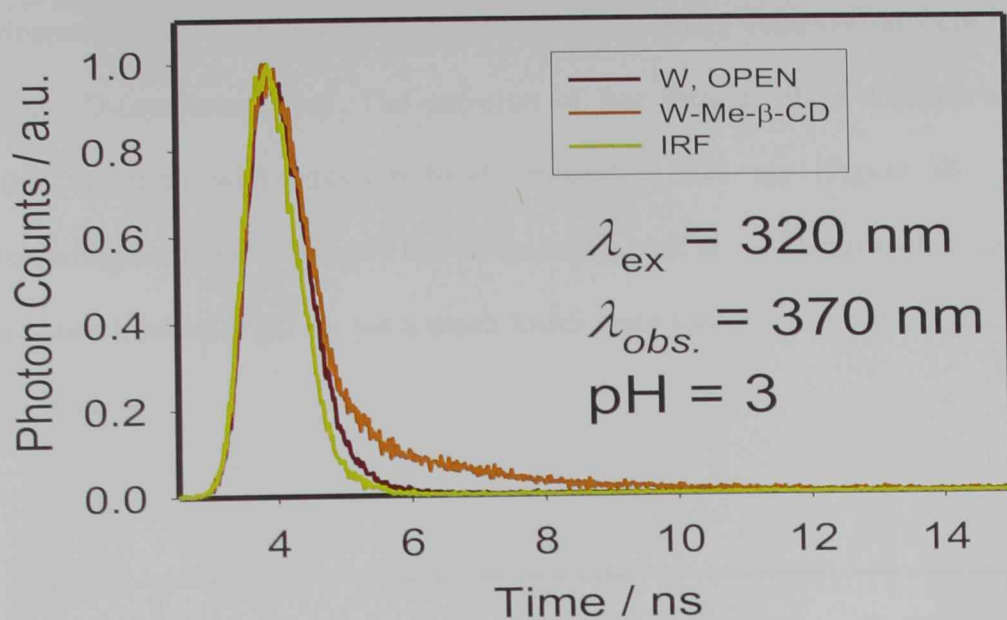


Figure 28: Emission decays collected at 370 nm for W at 25 μM in absence and in presence of Me- β -CD (1.0 mM) upon exciting at 320 nm. IRF is the instrument response function of ~ 90 ps

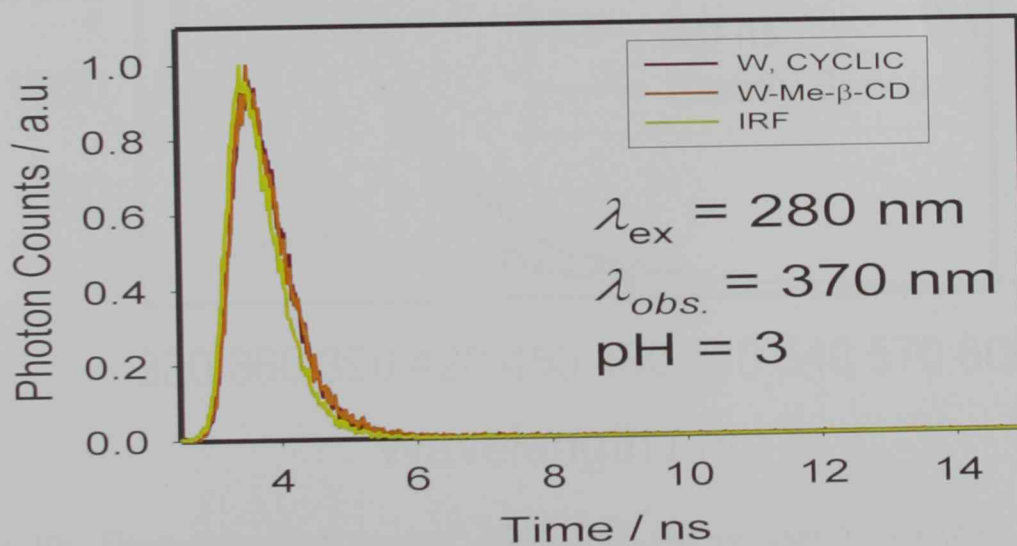


Figure 29: Emission decays collected at 370 nm for W at 25 μM in absence and in presence of Me- β -CD (1.0 mM) upon exciting at 280 nm. No change in fluorescence lifetime upon excitation at 280 nm. IRF is the instrument response function of ~ 90 ps

Accordingly, the corresponding DAS spectra in Figures 30-31 (see experimental section) are thus best interpreted by assuming contribution from both free and CD-complexed drug. The emission of free drug at pH 3 dominates the emission spectrum with emission band centered at 358 nm (Figure 30). The corresponding complex at this pH has an emission peak at ~ 375 nm with a longer excited-state lifetime ~ 2.0 ns, yet a much lower emission quantum yield (0.006 vs. 0.0005 in Table 3).

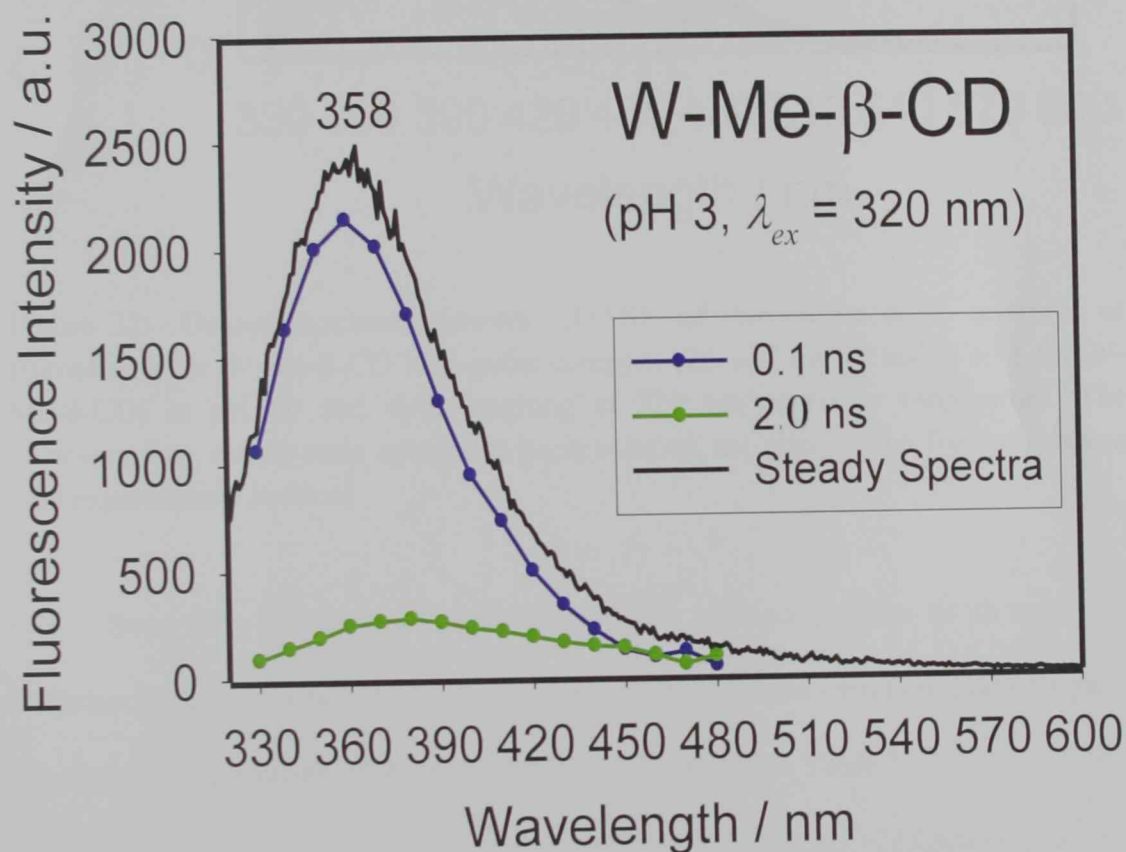


Figure 30: Decay-associated spectra (DAS) of two-component mixture of fluoroophores for W-Me- β -CD host-guest complex (25 μ M for W and 1 mM for W-Me- β -CD) at pH 3 upon exciting at 320 nm at room temperature. The corresponding steady-state spectra of each solution are also shown for comparison (see experimental section)

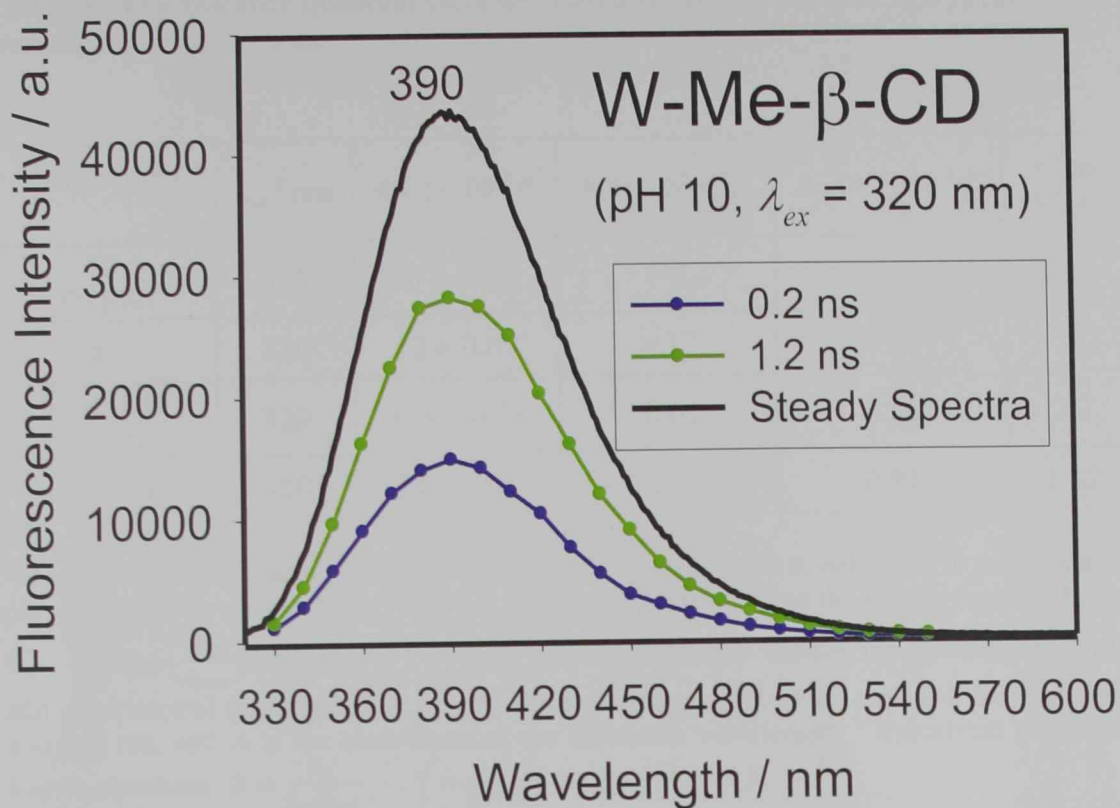


Figure 31: Decay-associated spectra (DAS) of two-component mixture of fluophores for W-Me-β-CD host-guest complex (25 μM for W and 1 mM for W-Me-β-CD) at pH 10 and upon exciting at 320 nm at room temperature. The corresponding steady-state spectra of each solution are also shown for comparison (see experimental section)

Separation of the two lifetimes by DAS method enables us to calculate radiative (k_r) and non-radiative rate constants (k_{nr}) alongside emission quantum yield (Φ_F) upon complexation of W to Me-β-CD, as illustrated in Table 3.

Table 3: Fluorescence quantum yield Φ_F , Radiative rate constant k_r , and Non-radiative rate constant k_{nr}

Drug Forms	λ_{ex}/nm	$\Phi_F (\times 10^{-3})^a$	$k_r (\times 10^7 \text{ s}^{-1})^b$	$k_{nr} (\times 10^9 \text{ s}^{-1})^b$	τ/ns (Mean)
W OPEN	320	6 ± 0.01	> 6	> 10	< 0.1
W ⁻	320	12 ± 0.01	> 12	> 10	< 0.1
Me- β -CD OPEN	320	0.5 ± 0.01	0.02	0.50	2.0
W ⁻ -Me- β -CD	320	24 ± 0.01	2.0	0.81	1.22

^aMeasured using warfarin (W) in phosphate-buffered saline (PBS, pH \sim 7.4, 10 mM sodium phosphate) as the standard ($\Phi_F = 0.012$) [46], and calculated using the known equation [55]:

$\Phi_{unk} = \Phi_{std} \left(\frac{I_{unk}}{A_{unk}} \right) \left(\frac{A_{std}}{I_{std}} \right) \left(\frac{n_{unk}}{n_{std}} \right)^2$, where n is the refractive indices for the standard (std) and experimental (unk) solvents, I is the fluorescence integral of the emission between 300 and 550 nm, and A is the absorbance at the excitation wavelength. ^b Calculated using the known equations: $\Phi = \frac{k_r}{k_r + k_{nr}}$, $\tau = \frac{1}{k_r + k_{nr}}$, $k_{nr} = \frac{1 - \Phi}{\tau}$, and $k_r = \frac{\Phi}{\tau}$.

The variations of k_r values in the absence and presence of CD are difficult to explain because of the unreliable measurement of lifetimes in water. However, the red shifts of emission peaks of W upon complexation to Me- β -CD hosts at pH 3 despite their non-polar hydrophobic and rigid cavity along with concomitant significant decrease of the k_r values by \sim 2 orders of magnitude, from $k_r = \sim 6 \times 10^7$ to $2 \times 10^5 \text{ s}^{-1}$, means that the enhancement in excited-state lifetimes is best described by a radiative-rate law [55].

This argument is supported by the boarder UV-visible spectrum of W when compared to that spectrum in water upon inclusion to CD, as shown in Figure 14 and in light of Strickler-Berg equation [55]. Karlsson et al. [45] pointed out the possibility of attributing the longer lifetime of W observed in ethanol (0.45 ns, $\lambda_{ex} = 295 \text{ nm}$) to the formation of solute-solvent hydrogen-bonded complexes.

Dondon et al. [56] also posited that for other 4-hydroxycoumarin derivatives similar hydrogen-bonded complexes could have formed between the coumarin lactone group and the CD secondary hydroxyl groups. It transpires that there is a plausible explanation to the fluorescence behaviors of protonated W-CD systems that similar hydrogen-bonded complexes have formed between CD secondary hydroxyl group and the carbonyl group of open form, which the cyclic form lacks.

It is worth to mention that the low binding affinity of our W-CD system in the present study limits its potential use for biomedical applications as a large fraction of free guest will always persist at any concentration of the added host.

However, understanding the factors that enhance the binding constants of W with CDs such as the formation of hydrogen bonded complexes [45], [56] and the presence of methyl groups [49] alongside the investigation of pH effects on the complex stability should preclude further studies in the future on W-CD systems for the establishment of pH-driven stimuli-responsive systems.

Emission decays collected at pH 9 Figure 36 and 37 (Appendix) gave DAS spectra (Figure 31) that demonstrated opposite effects induced by CD addition. In agreement with previous reports [46], [51], [57], the complex at pH 9 excited at 320 nm has a higher emission yields than that of free drug with excited-state lifetime of 1.22 ns and no change in peak position at ~ 390 nm (Table 3). Vasquez *et al.*, suggested encapsulation of deprotonated W forms considerably suppresses the vibronic modes that provide pathways for non-radiative transitions between the excited and ground states of W, causing a decrease in the rates of the non-radiative decay processes.

Indeed, calculation supports these findings and the operation of energy-gap law in which the excited state S_1 is more polar than the ground state S_0 . Consequently, S_1 state will be much more destabilized in a nonpolar medium than the S_0 state. Such an increase in energy gap results in a decrease in k_{nr} [58].

While non-radiate rate constants in Table 3 decreases ~ 12 times upon inclusion in the hydrophobic interior of Me- β -CD, radiative rate constants decreases only ~ 6 times. The increase in lifetimes and associated decrease in k_{nr} of other 4-hydroxycoumarin derivatives [56] from below 0.5 ns to 1.1–1.3 ns (depending on the derivative) was also observed upon sequestration of dyes into β -CD macromolecules.

Chapter 4: Conclusion

In this work, time-resolved fluorescence spectra measured using picoseconds laser diode at 280 nm and 320 nm with selective exciting of isomers of W were conducted utilizing both global and target analysis to give more specific information about nature and dynamics of excited-states of warfarin in water and inside cyclodextrins. An increase in excited-state lifetime of warfarin was observed upon its interaction with Me- β -CD with selective interactions towards the open form. The increase in lifetime of open form was explained by radiative rate law, while that of deprotonated form by energy-gap law. Lower acidity of the open form have been demonstrated.

Fluorescence properties of W inside cyclodextrins in aqueous solutions have attracted attentions in several occasions due to their implications in bioanalytical quantification of W in commercial pesticides [50], [59]–[61] or biological liquids [62] and in utilizing W as fluorescent probes or site marker to study drug-protein interactions [63], [64].

In addition to fluorescence properties better understanding of the interactions of W with cyclodextrin in ground-state should lead to better control on modulating its pK_a values in solution [38], [49], with implications in analytical separation of drug by electrophoresis [53] liquid chromatography [65], [66], as well as its formulations [67] or clinical studies [68].

In summary, these results give the structures present in cyclodextrin environment in ground-state and explain their kinetic behaviors in excited-state using quantitative time-resolved fluorescence tools, collectively leading to better

understanding on the role of cyclodextrin on manipulating the function of this anticoagulant drug in ground and excited states.

4.1 Recommendations

The following recommendations are suggested as possible ways to extend this study:

1- Study the host-guest interactions of W with different macromolecules such as cucurbit[n]uril and other different cyclodextrins.

2- Warfarin was selected because it is commercially used as drug that favors blood bleeding, while finding a stimuli (chemical competitor) that tune the form (open and cyclic) could control blood clotting/bleeding balance in the future.

3- Study the photochemistry of other drugs and the encapsulation of these drugs with different macromolecules.

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Appendix

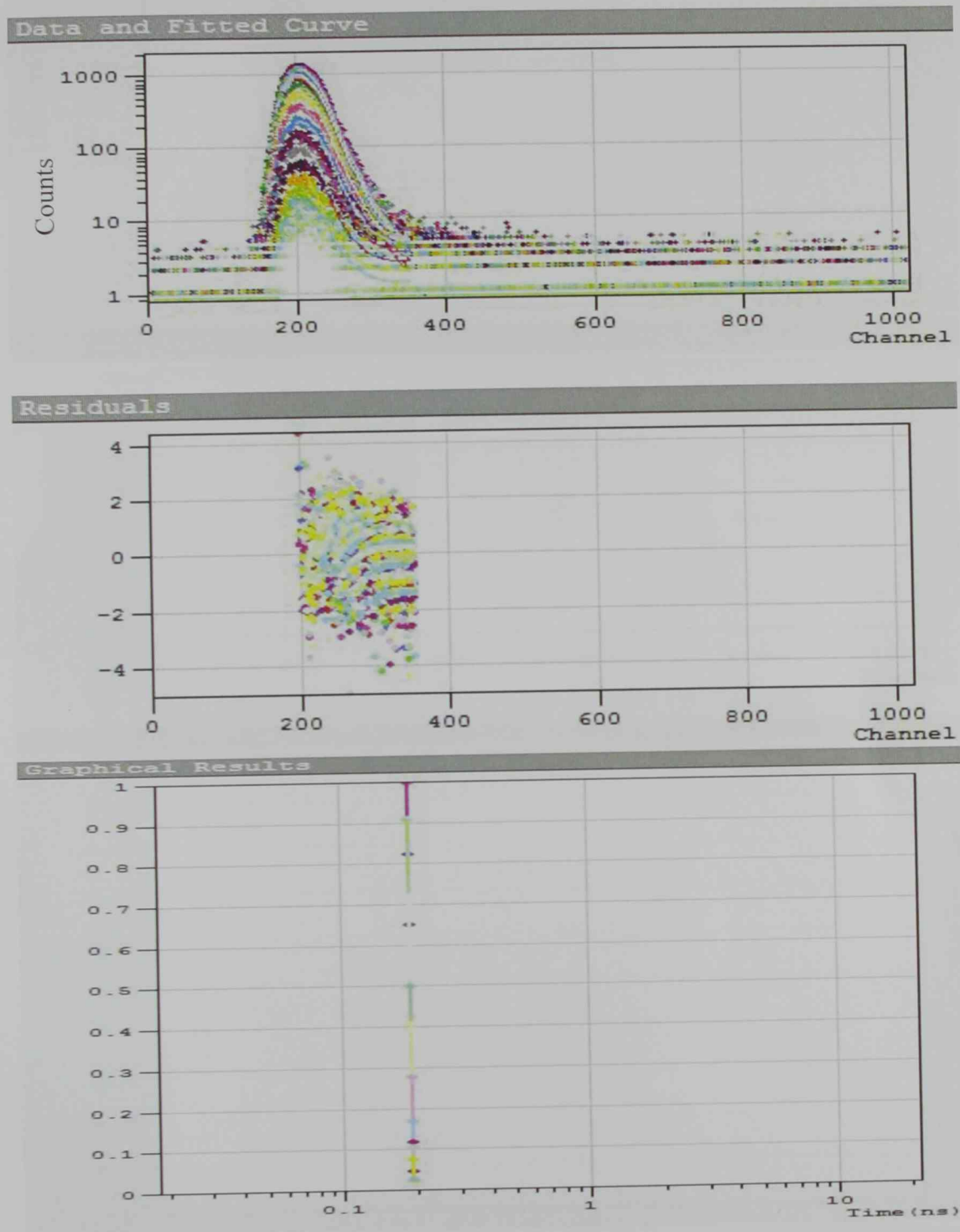


Figure 32: Collected emission decays measured over the emission spectrum of W at pH 3, excited at 320 nm from 330 to 490 nm every 10 nm with a dwell time of 50 s at each wavelength. Data at each wavelength were fitted to a mono-exponential model convoluted with IRF ~ 90 ps (as shown in the residuals)

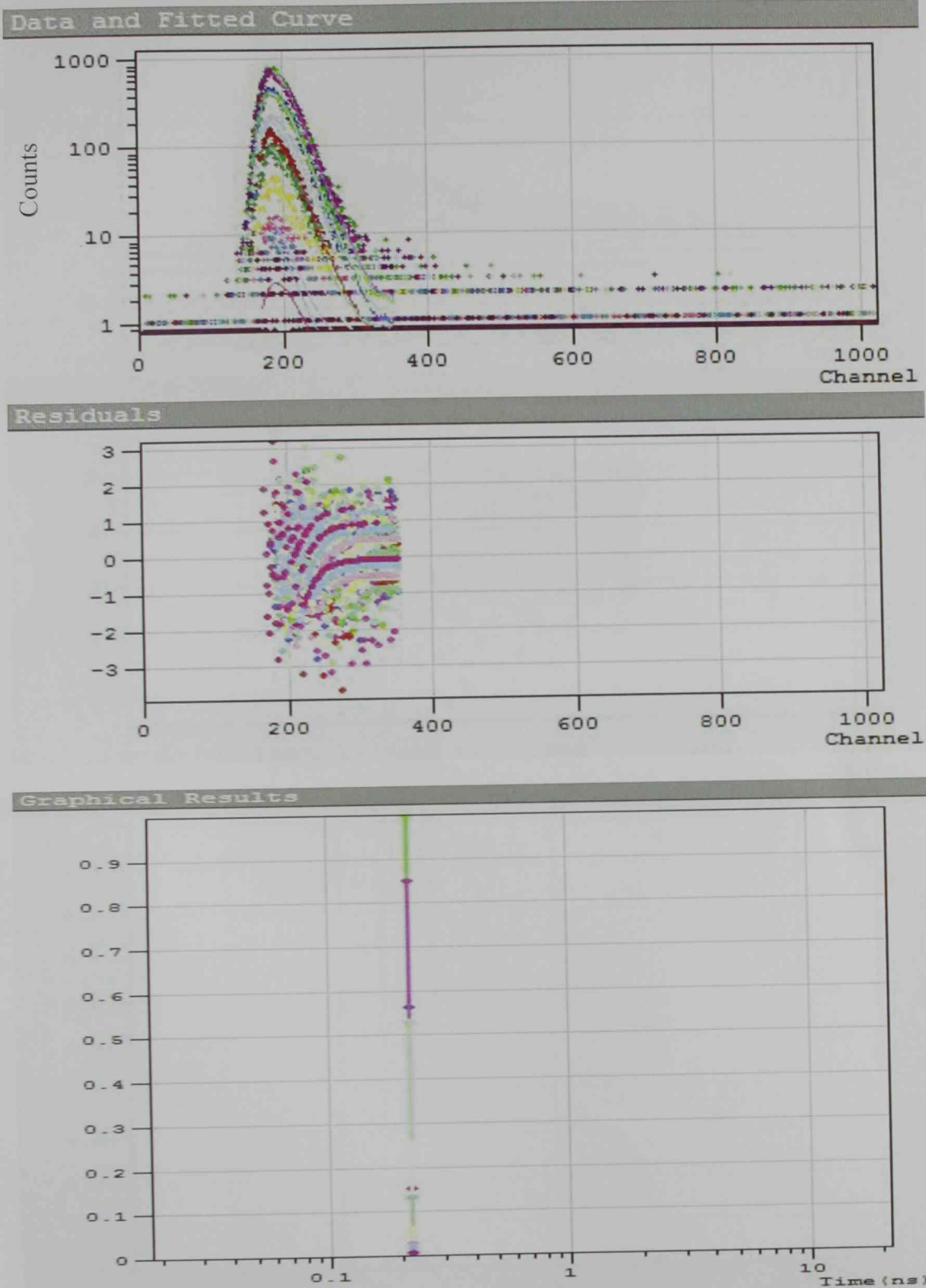


Figure 33: Collected emission decays measured over the emission spectrum of W at pH 3, excited at 280 nm from 310 to 510 nm every 20 nm with a dwell time of 50 s at each wavelength. Data at each wavelength were fitted to a mono-exponential model convoluted with IRF ~ 90 ps (as shown in the residuals)

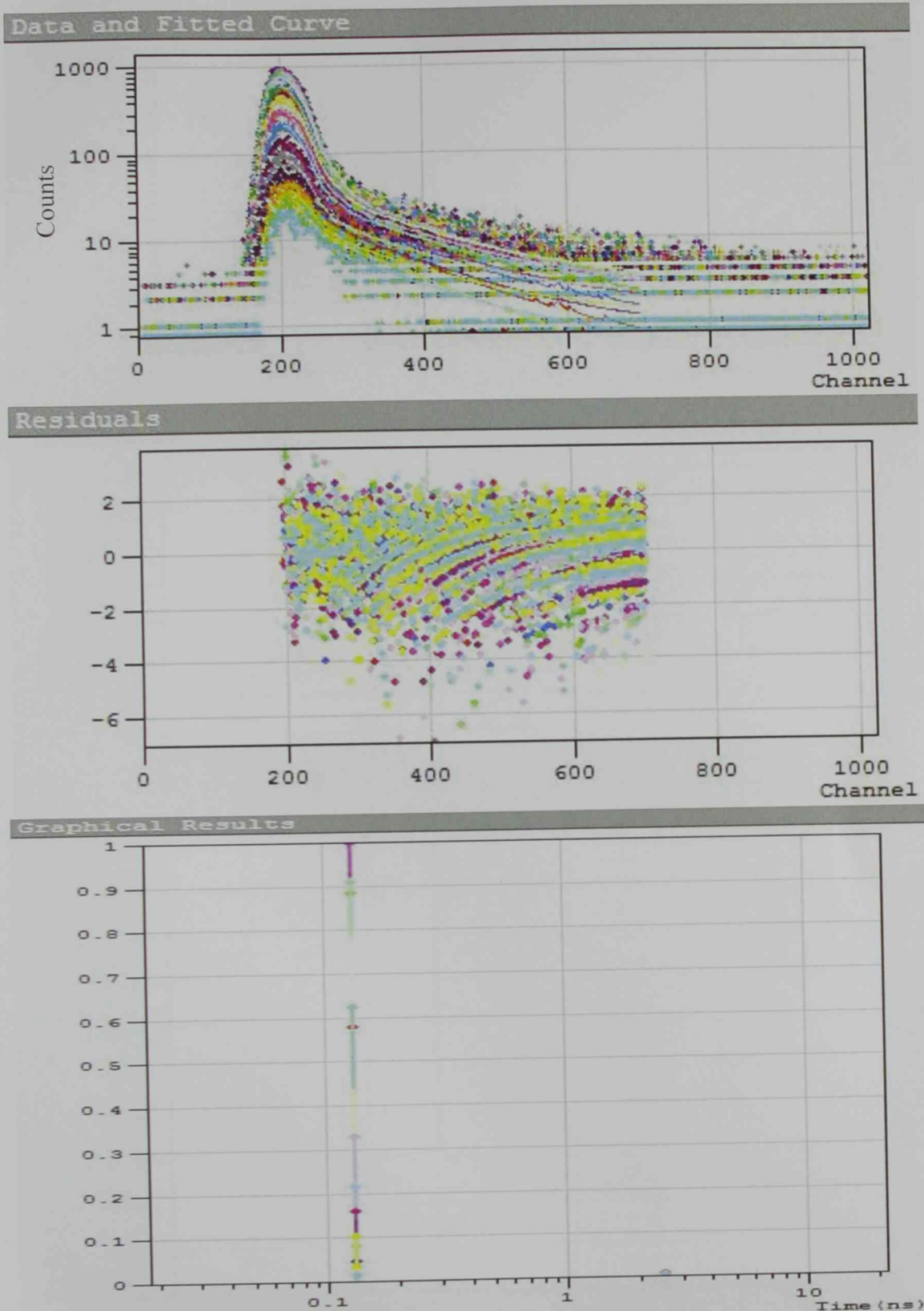


Figure 34: Collected emission decays measured over the emission spectrum of W-Me- β -CD at pH 3, excited at 320 nm (Figure 30) from 330 to 480 nm every 10 nm with a dwell time of 50 s at each wavelength. Data at each wavelength were fitted to a bi-exponential model convoluted with IRF \sim 90 ps (as shown in the residuals)

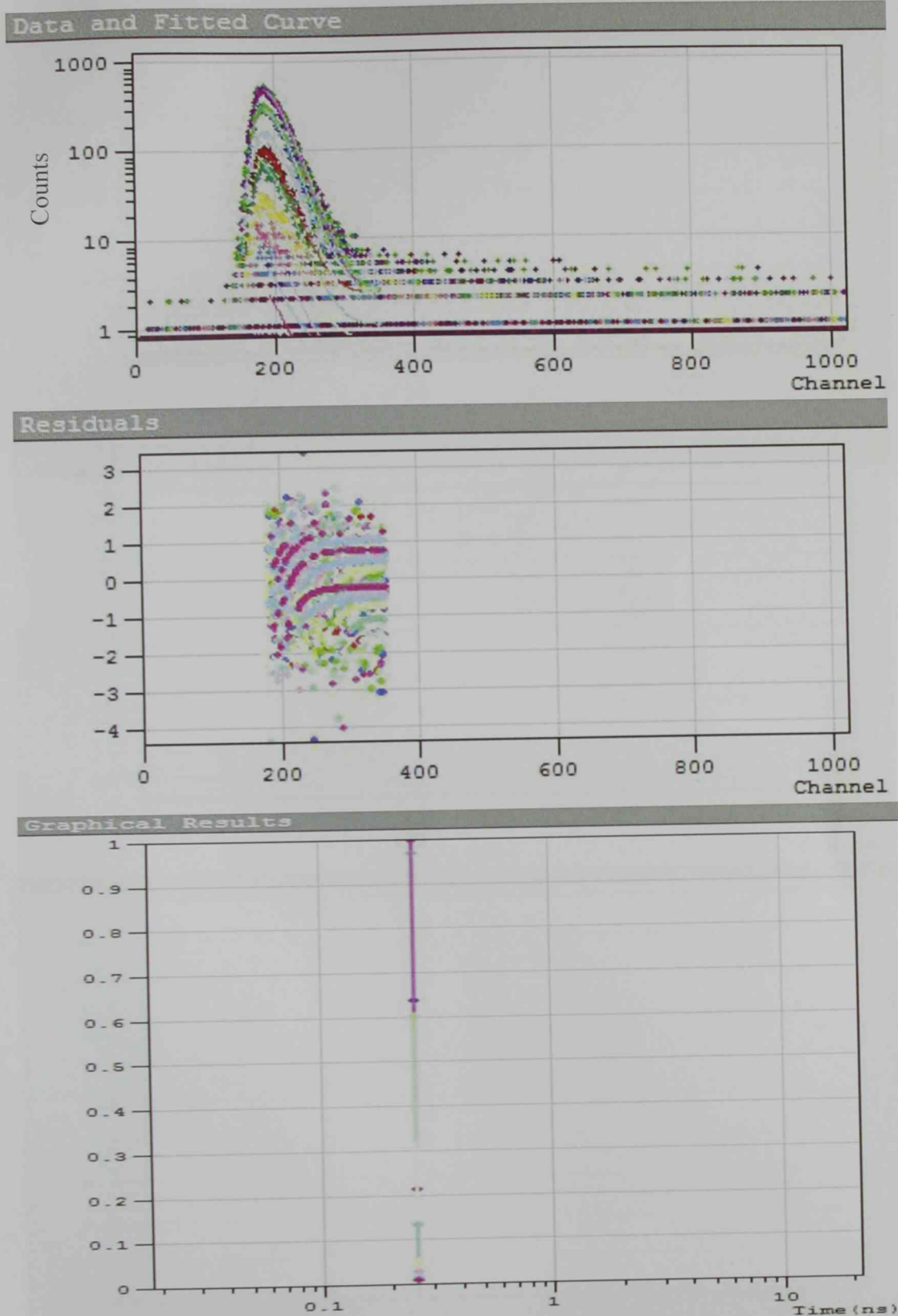


Figure 35: Collected emission decays measured over the emission spectrum of W-Me- β -CD at pH 3, excited at 280 nm from 310 to 510 nm every 20 nm with a dwell time of 50 s at each wavelength. Data at each wavelength were fitted to a mono-exponential model convoluted with IRF \sim 90 ps (as shown in the residuals)

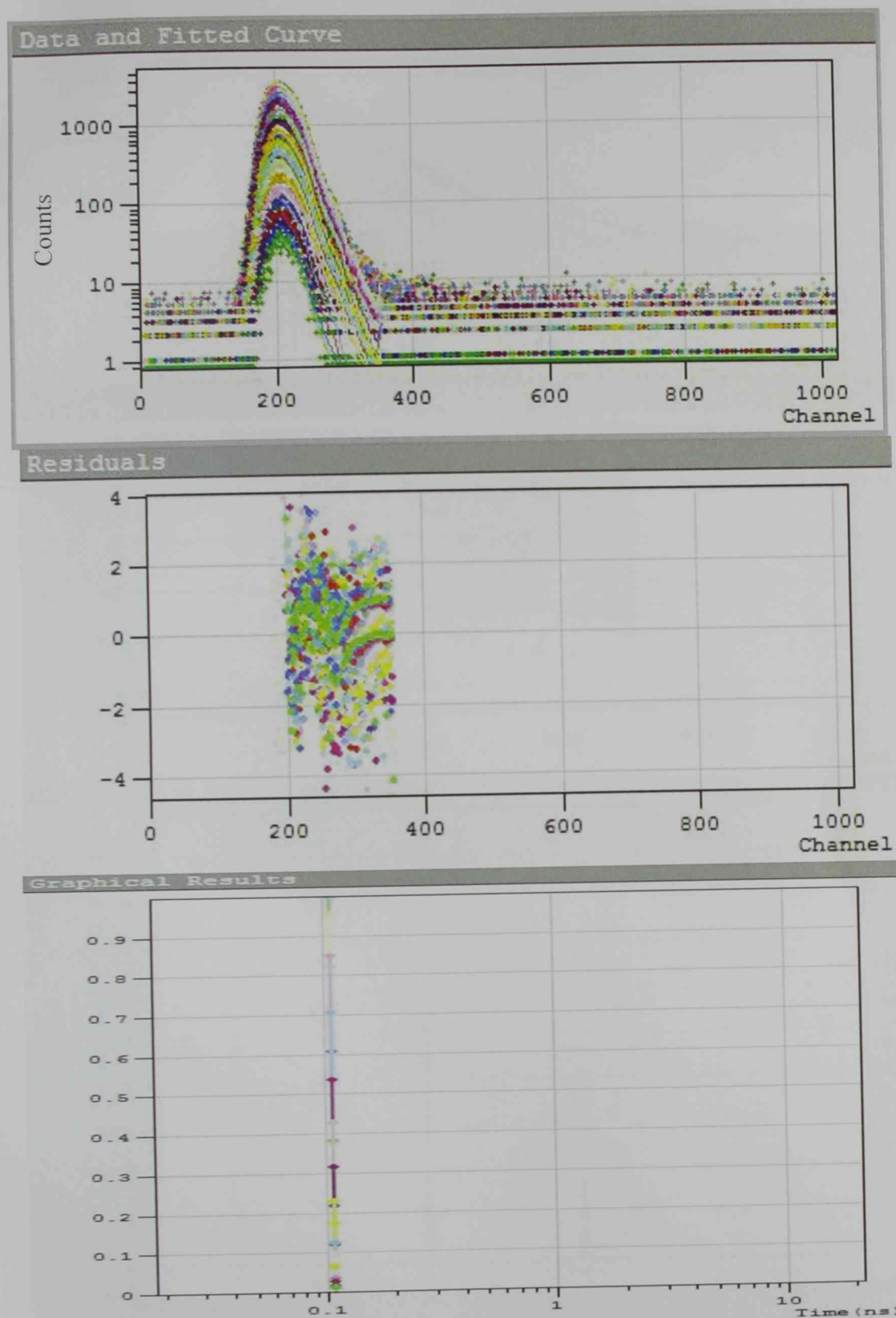


Figure 36: Collected emission decays measured over the emission spectrum of W at pH 9, excited at 320 nm from 330 to 550 nm every 10 nm with a dwell time of 50 s at each wavelength. Data at each wavelength were fitted to a mono-exponential model convoluted with IRF ~ 90 ps (as shown in the residuals)

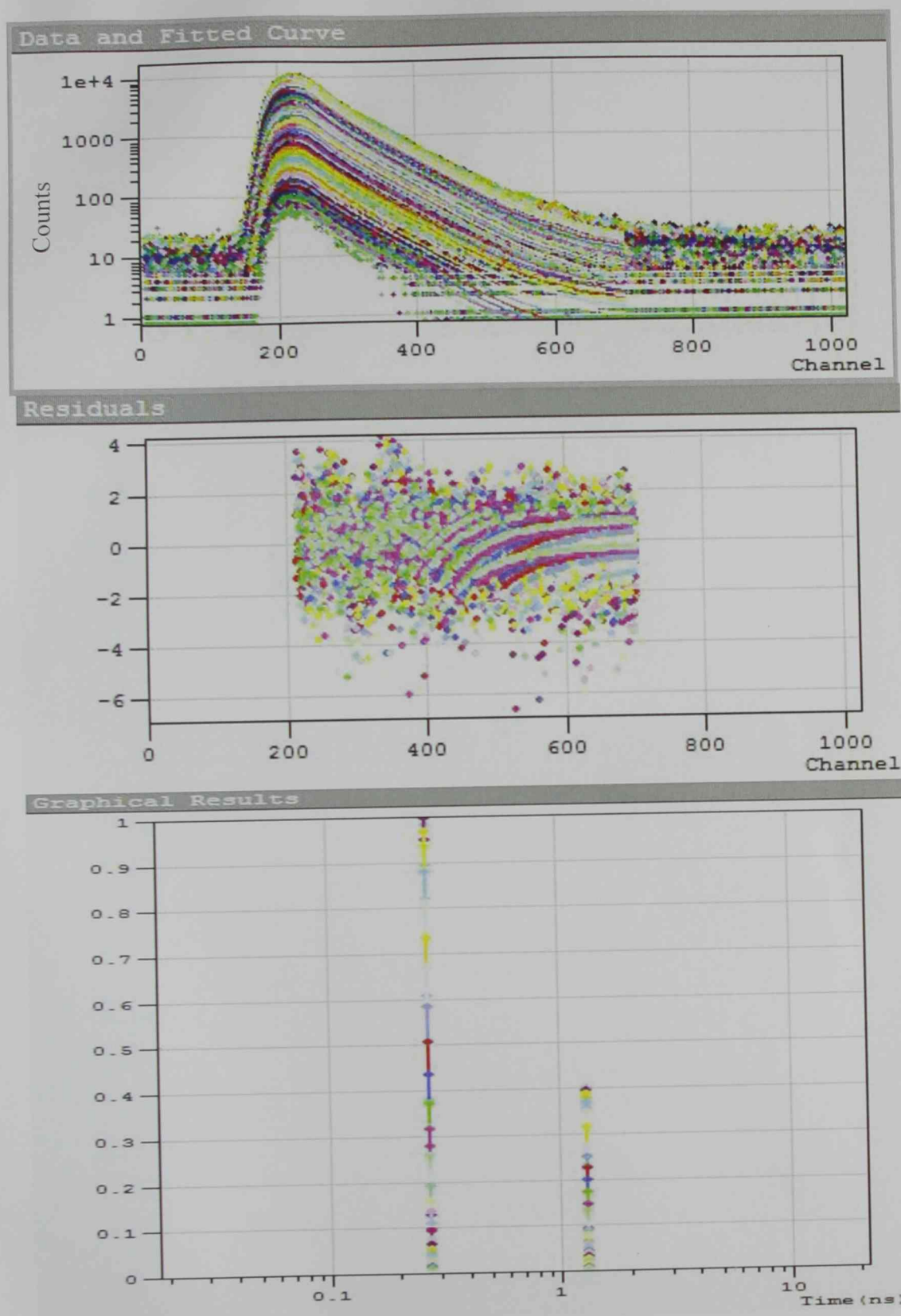


Figure 37: Collected emission decays measured over the emission spectrum of W-Me- β -CD at pH 9, excited at 320 nm (Figure 31) from 330 to 550 nm every 5 nm with a dwell time of 50 s at each wavelength. Data at each wavelength were fitted to a bi-exponential model convoluted with IRF \sim 90 ps (as shown in the residuals)