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**BADANIE ODDZIAŁYWAŃ POCHODNYCH
BENZOESANU METYLU I BIFENYLU
Z WYBRANYMI ZŁOŻONYMI
MAKROCZĄSTECZKAMI CHEMICZNYMI
I BIOLOGICZNYMI**

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Wstęp

W ostatnich kilkudziesięciu latach obserwuje się wyraźny wzrost zainteresowania badaniami oddziaływań wewnątrz- i międzycząsteczkowych w układach złożonych [1-4]. Nie ulega wątpliwości, że zrozumienie mechanizmów oddziaływań międzymolekularnych w różnych środowiskach ma duże znaczenie nie tylko z punktu widzenia chemików czy fizyków, ale również biochemików, medyków czy technologów materiałowych. Sumarycznym efektem interdyscyplinarnych działań naukowych było stworzenie nowej dziedziny naukowej, koncentrującej się na projektowaniu i syntezowaniu zupełnie nowych i niewystępujących w naturze struktur hierarchicznych oraz badaniu właściwości istniejących i nowo powstałych struktur złożonych. Tę dziedzinę wiedzy, której znaczenie gwałtownie wzrosło wraz z przyznaniem Nagrody Nobla *D. J. Cramowi*, *J. M. Lehmanowi* oraz *C. Pedersenowi* w 1987 roku za ich wkład w badania nad cząsteczkami, które oddziałują w wysoce selektywny sposób z innymi cząsteczkami, nazwano *chemią supramolekularną* [5].

Tak burzliwy rozwój fizykochemii supramolekularnej nie byłby możliwy, gdyby proponowane rozwiązania pozostały tylko w laboratoriach badawczych. Związki makrocykliczne okazały się również interesujące ze względu na ich liczne możliwości aplikacyjne w wielu dziedzinach życia. Część z nich znalazła zastosowania w farmacji, medycynie, rolnictwie, nanotechnologii, czy przemyśle kosmetycznym [6-11]. Pojawianie się nowych klas związków makrocyklicznych oferuje nowe możliwości m.in. w badaniach farmakokinetycznych. Nowe nośniki mogą być wykorzystywane jako transportery, czyli składniki tabletek, maści, syropów, kropli do oczu, czy aerozoli do nosa [12-13]. Istotną cechą tych związków jest to, że często zwiększają one pozorną rozpuszczalność leków hydrofobowych w osoczu krwi i stymulują ich dostarczenie do wnętrza komórek. W rolnictwie związki supramolekularne stymulują rozwój roślin. Wchodzą one w skład sztucznych nawozów, środków leczniczych i owadobójczych. W nanotechnologii kompleksy wykorzystywane są jako stabilizatory substancji czułych na światło, temperaturę, czy obecność tlenu (np. witaminy). Można zatem stwierdzić, że liczne układy supramolekularne znalazły wiele zastosowań praktycznych, choć nie zawsze ich struktura wewnętrzna i mechanizm powstawania są w pełni wyjaśnione i zrozumiałe [14].

Oprócz wspomnianych powyżej licznych możliwości aplikacyjnych omawianych układów, związki makrocykliczne są niezmiernie ciekawym obiektem z punktu widzenia możliwości systematycznych badań spektroskopowych. Dzięki wykorzystaniu technik stacjonarnej i rozdzielonej w czasie spektroskopii oraz spektroskopii magnetycznego rezonansu jądrowego możliwe jest określenie dynamiki procesów prowadzących do powstawania stabilnych kompleksów inkluzyjnych typu „gospodarz-gość”, jak również określenie ich stechiometrii oraz stabilności. Z czysto naukowego punktu widzenia, niezmiernie ciekawe jest badanie kompleksów supramolekularnych w przypadku których molekula „gościa” podlega procesowi fotoindukowanego wewnątrzcząsteczkowego przeniesienia elektronu lub protonu (*Intramolecular Electron Transfer* (IET), *Intramolecular Proton Transfer* (IPT)), oraz tworzeniu międzycząsteczkowych wiązań wodorowych. Można stwierdzić, że systematyczne badanie przebiegu różnych procesów fotofizycznych i fotochemicznych zachodzących w układzie molekula organiczna (w szczególności molekula typu ICT)–makrocykliczny związek organiczny (nośnik) wydaje się rzeczą niezwykle interesującą, nie tylko z punktu widzenia czysto teoretycznego (tj. rozumienia mechanizmów procesów prowadzących do powstania stabilnego kompleksu), ale również ze względu na możliwość zastosowania związków makrocyklicznych w wielu dziedzinach życia.

Równolegle do badań przebiegu różnych procesów fotofizycznych i fotochemicznych zachodzących w układzie molekula organiczna–związek makrocykliczny, wiele grup badawczych skupia swe zainteresowania nad badaniami oddziaływań substancji biologicznie czynnych z różnymi biomolekułami w organizmie. Zrozumienie, na poziomie molekularnym, oddziaływań lek–biomolekuła jest niezmiernie ważne, przede wszystkim, z punktu widzenia skuteczności i czasu działania substancji czynnej. Mając powyższe na uwadze, zrozumiałym staje się fakt wykorzystania różnych technik badawczych do badania procesów zachodzących w układzie molekula organiczna–albumina surowicy zwierzęcej. Poznanie procesu wiązania molekuly organicznej z białkiem pochodzenia zwierzęcego, które jest traktowane jako białko modelowe dla albuminy ludzkiej jest decydujące dla zrozumienia procesów zachodzących w układach biologicznych.

W świetle przedstawionych powyżej informacji celem nadrzędnym niniejszej rozprawy doktorskiej było dogłębne zrozumienie procesów prowadzących do powstania stabilnych kompleksów inkluzyjnych pomiędzy wybranymi molekułami organicznymi (pochodne

benzoesu metylu oraz pochodna bifenylu) a różnymi supramolekularnymi nośnikami makrocyklicznymi tj. α -, β -, γ -cyklodekstryny, kukurbit[7]uryle, 4-sulfonowe kaliks[6]areny oraz wyjaśnienie mechanizmów oddziaływań pomiędzy dwoma pochodnymi benzoesu metylu a białkami pochodzenia zwierzęcego (BSA). Przeprowadzone, z wykorzystaniem technik stacjonarnej oraz rozdzielonej w czasie spektroskopii oraz spektroskopii magnetycznego rezonansu jądrowego badania, przedstawione w poniższych publikacjach:

A1 K. Baranowska, M. Józefowicz, „*Spectroscopic studies of inclusion complexation between ortho derivatives of p-methylaminobenzoate and α - and γ -cyclodextrins*”, Journal of Molecular Liquids 265 (2018) 140-150, IF₂₀₁₈ = 4.56, MNISW = 100,

A2 K. Baranowska, M. Mońka, A. Kowalczyk, N. Szpakowska, Z. Kaczyński, P. Bojarski, M. Józefowicz, “*Spectroscopic studies of the excited-state intramolecular proton and electron transfer processes of methyl benzoate derivatives in cucurbit[7]uril nanocage*”, Journal of Molecular Liquids 318 (2020) 113921, IF₂₀₂₀ = 6.17, MNISW = 100,

A3 K. Baranowska, M. Mońka, A. Kowalczyk, Z. Kaczyński, P. Bojarski, M. Józefowicz, “*Spectroscopic studies on the supramolecular interactions of methyl benzoate derivatives with p-sulfocalix[6]arene macrocycles*” Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy 303 (2023) 123131, IF₂₀₂₃ = 4.3, MNISW = 140,

A4 K. Baranowska, A. Bajorek, M. Pietrzak, M. Józefowicz, „*Preferential encapsulation of different conformer of ethyl 5-(4-dimethylaminophenyl)-3-amino-2,4-dicyanobenzoate in γ -cyclodextrins*”, Journal of Molecular Liquids 302 (2020) 112430, IF₂₀₂₀ = 6.17, MNISW = 100,

A5 K. Baranowska, M. Mońka, P. Bojarski, M. Józefowicz, *Insight into Molecular Interactions of Two Methyl Benzoate Derivatives with Bovine Serum Albumin*, International Journal of Molecular Sciences 22 (2021) 11705, IF₂₀₂₁ = 6.208, MNISW = 140.

miały na celu:

- ustalenie możliwych ścieżek dezaktywacji elektronowo wzbudzonych molekuł, w przypadku których obserwuje się zjawisko ESPIT, ICT i TICT wewnątrz wybranych nośników supramolekularnych, jak również ocenę roli międzycząsteczkowych wiązań wodorowych w procesie kompleksowania,
- ustalenie stechiometrii, geometrii i trwałości badanych kompleksów inkluzyjnych,
- określenie wpływu obecności nośników makrocyklicznych na zjawisko fotoindukowanego wewnątrzcząsteczkowego przeniesienia protonu i elektronu,
- określenie wpływu budowy przestrzennej konformerów **EDMAADCy** na proces tworzenia kompleksów inkluzyjnych,
- wyjaśnienie natury i mechanizmów oddziaływań pomiędzy pochodnymi benzoesu metylu (I i II) a albuminą surowiczą wołową.

Rozdział 1

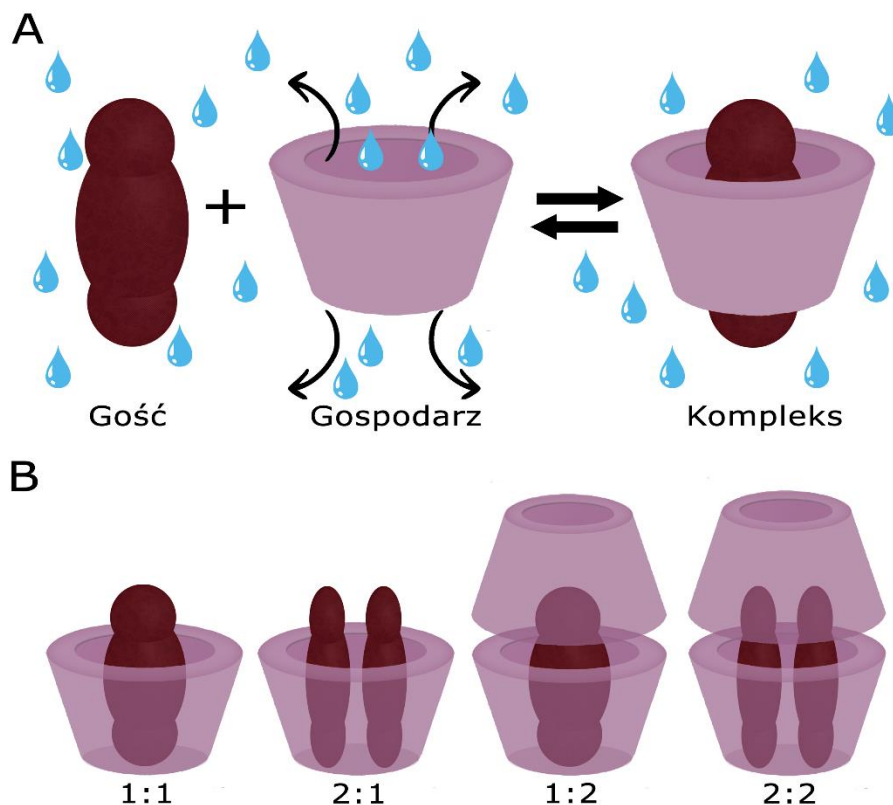
Kompleksy inkluzyjne typu „gospodarz-gość” - „gospodarze” w układach supramolekularnych

Na przestrzeni ostatnich kilkudziesięciu lat obserwuje się duże zapotrzebowanie na nowe innowacyjne leki wpisujące się w nurt terapii celowanej. Wiele substancji, mających ogromny potencjał leczniczy, zostaje odrzuconych podczas procesu badań przedklinicznych i klinicznych m. in. ze względu na ich słabą rozpuszczalność w wodzie. Jedną z metod, która może poprawić rozpuszczalność leku, a co się z tym wiąże jego biodostępność, jest utworzenie kompleksów inkluzyjnych między lekiem a nośnikami makrocyklicznymi. Korzyści wynikające z zastosowania leków w postaci kompleksów inkluzyjnych to również jego zwiększona bioaktywność i stabilność, szybkość biotransformacji oraz redukcja efektów niepożądanych. Ponadto farmaceutyki, które są zainkludowane do wnęki supramolekularnego nośnika są mniej narażone na wilgoć, co istotne jest przede wszystkim przy wysoce higroskopijnych substancjach czynnych. Co więcej, związki makrocykliczne chronią substancje lecznicze wrażliwe na światło, utlenianie, temperaturę czy degradację [15].

Z punktu widzenia licznych istniejących i potencjalnych zastosowań różnych związków makrocyklicznych to cyklodekstryny, kukurbituryly, kaliksareny, pillarareny, etery koranowe oraz cyklofany stanowią bazę układów supramolekularnych, których badania cieszą się niesłabnącym zainteresowaniem nie tylko wśród fizyków, chemików, biologów, ale także farmaceutów i medyków. Dzięki zdolnościom do tworzenia kompleksów inkluzyjnych z innymi mniejszymi związkami organicznymi lub nieorganicznymi nośniki te są szeroko badane w kierunku możliwości budowania różnych architektur supramolekularnych [16-18].

Tworzenie kompleksów inkluzyjnych polega na wnikanii rozmaitych cząsteczek, jonów a nawet rodników do wnętrza makrocyklicznego nośnika, czemu zwykle towarzyszy zmiana ich właściwości fizykochemicznych. Proces ten może odbywać się na zasadzie całkowitego lub częściowego wypełnienia hydrofobowej wnęki nośnika przez wybraną cząsteczkę [15]. Tego typu kompleksy nazywane są kompleksami inkluzyjnymi typu „gospodarz-gość”, gdzie funkcję „gospodarza” pełnią supramolekularne nośniki, a „gościa” kompleksowane związki. W

zależności od wzajemnej komplementarności cząsteczek (dopasowanie elektronowo–energetyczne oraz geometryczno–sferyczne) kompleksy inkluzyjne mogą być układami o różnej stechiometrii (Rys. 1).



Rys. 1 (A) Schematyczne przedstawienie tworzenia kompleksów inkluzyjnych z nośnikami makrocyclicznymi (B) Kompleksy inkluzyjne typu „gospodarz-gość” i ich wybrane stechiometrie 1:1, 2:1, 1:2, 2:2.

Jak już wspomniano, proces kompleksowania w roztworach polega na wnikaniu cząsteczki „gościa” do wnętrza hydrofobowej wnęki makrocyclicznego nośnika „gospodarza” (Rys. 1). Tworzenie kompleksów inkluzyjnych przebiega samorzutnie dzięki oddziaływaniom elektrostatycznym, typu van der Waalsa, hydrofobowym, a także na skutek słabych wiązań wodorowych, przy czym nie dochodzi do tworzenia wiązań kowalencyjnych [19]. W pierwszym etapie procesu kompleksowania badanych molekuł ze związkami makrocyclicznymi w roztworach wodnych wewnątrz torusa nośnika wytwarzają się słabe wiązania wodorowe z cząsteczkami polarnego rozpuszczalnika. Następnie cząsteczki wody zostają wyparte z torusa przez molekuly „gościa”, czego konsekwencją jest obniżenie energii konformacyjnej molekuly nośnika. Cząsteczka „gościa” zostaje zatrzymana we wnęcie dzięki wcześniej wspomnianym

oddziaływaniom. Należy podkreślić, że procesowi kompleksowania supramolekularnego nośnika towarzyszy zrywanie (osłabianie) międzycząsteczkowych wiązań wodorowych, które występują między inkludowaną cząsteczką a cząsteczkami wody [20-21].

Na zdolność nośników makrocyklicznych do tworzenia kompleksów inkluzyjnych z cząsteczkami „gościa” wpływają dwa główne czynniki. Pierwszy związany jest z dopasowaniem geometryczno-sferycznym. Oznacza to, że istotnym jest dopasowanie rozmiaru cząsteczki „gościa” do wielkości wnęki „gospodarza”. Jeżeli dopasowanie nie jest spełnione to umieszczenie molekuly wewnątrz nośnika staje się niemożliwe z dwóch powodów: (i) rozmiar molekuly „gościa” jest zbyt duży i nie mieści się wewnątrz makrocyklicznego „gospodarza”, (ii) mały rozmiar cząsteczki „gościa” uniemożliwia utrzymanie się molekuly we wnęce makrocyklicznego nośnika. Drugim znaczącym aspektem jest dopasowanie elektronowo-energetyczne pomiędzy różnymi składnikami systemu t.j. nośnik supramolekularny, cząsteczka „gościa” i rozpuszczalnik. W procesie tworzenia kompleksu „gospodarz-gość” niekorzystne energetycznie oddziaływania, które występują pomiędzy polarną cząsteczką wody, a niepolarną cząsteczką nośnika zostają zastąpione korzystnymi energetycznie oddziaływaniami pomiędzy cząsteczką „gościa” a hydrofobowym wnętrzem torusa makrocyklicznego nośnika [22-24].

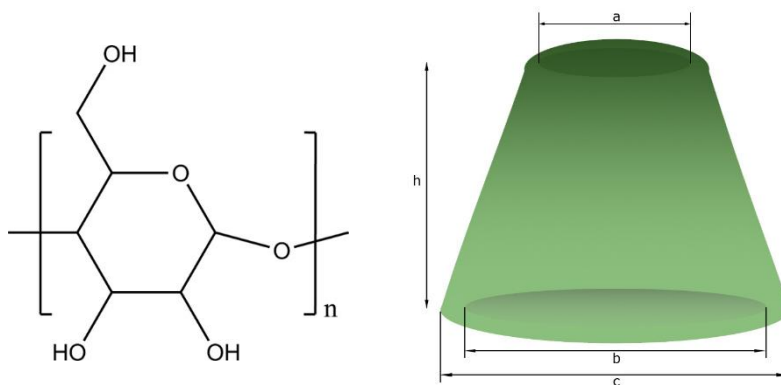
1.1 Wybrane związki makrocykliczne – cyklodekstryny (CD), kukurbit[7]uryle (CB[7]) oraz 4-sulfonowe kaliks[6]areny (SCA[6])

Spośród różnych znanych do tej pory klas związków makrocyklicznych różniących się budową, kształtem, wielkością hydrofobowych wnęk i sposobem wytwarzania na przestrzeni ostatnich kilku dziesięcioleci największym zainteresowaniem cieszą się cyklodekstryny. Są one wykorzystywane m.in. jako nośniki leków, fotouczulacze w terapii ukierunkowanej oraz w kompleksach z lekami mogą zwiększać ich działanie przeciwwirusowe i/lub przeciwbakteryjne.

Cyklodekstryny po raz pierwszy zostały otrzymane przez *Villiersa* w 1891 roku [25], natomiast ich główne właściwości fizykochemiczne i technika ich izolacji została opisana przez uważanego za twórcę podstaw chemii cyklodekstryn *Schardingera* w 1903 roku [26]. Związki te należą do grupy cyklicznych oligosacharydów złożonych z kilku podjednostek α -

glukopirany. Cyklodekstryny, określane mianem *cyklodekstryn pierwszej generacji*, obejmują trzech przedstawicieli składających się z 6 (α -CD), 7 (β -CD), i 8 (γ -CD) jednostek cukrowych [27]. Cyklodekstryny w roztworze przyjmują kształt ściętego stożka (toroidu) o otwartych obu końcach, gdzie wewnątrz kształtowane jest przez atomy wodoru i odpowiednie glikozydowe mostki tlenowe [28].

Dzięki swojej unikatowej budowie (drugorzędowe grupy hydroksylowe na szerszej a pierwszorzędowe grupy hydroksylowe na węższej krawędzi torusa) cyklodekstryny posiadają właściwości hydrofilowe, a zatem dobrze rozpuszczają się w wodzie. Wnętrze tych cukrów, pozbawione grup hydroksylowych, jest hydrofobowe. Charakterystyczny kształt cyklodekstryn pozwala wnikać



Właściwości	α -CD	β -CD	γ -CD
Ilość jednostek glukowy	6	7	8
Wzór empiryczny	$C_{36}H_{60}O_{30}$	$C_{42}H_{70}O_{35}$	$C_{48}H_{80}O_{40}$
Minimalna średnica wnętrza (Å)(a)	4.4	5.8	7.4
Maksymalna średnica wnętrza (Å)(b)	5.7	7.8	9.5
Średnica zewnętrzna (Å)(c)	13.7	15.3	16.9
Wysokość torusa (Å)(h)	7.8	7.8	7.8
Objętość wnęki (Å ³)	174	262	427
Masa molowa (g/mol)	972	1135	1297

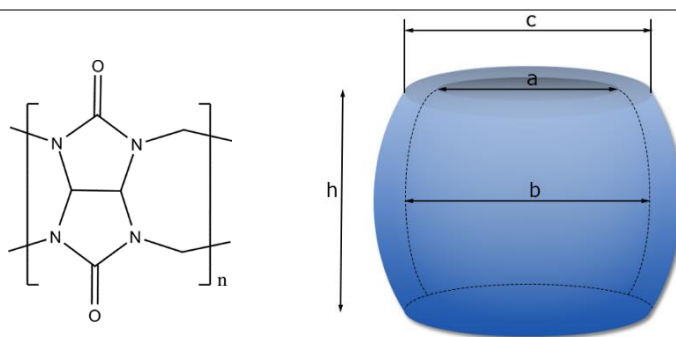
Tab. 1 Wybrane parametry strukturalne cyklodekstryn [27-28]

różnym cząsteczkom do wnętrza torusa w całości lub częściowo [28].

Inną grupą makrocyklicznych związków organicznych, które dzięki swej budowie mogą tworzyć kompleksy inkluzyjne z różnymi związkami są kukurbit[n]uryle (CB[n]). Związki te zostały zsyntezowane przez *Behrenda* w 1905 roku, zaś ich struktura krystaliczna została poznana dopiero w 1981 roku dzięki pracom *Mocka* i jego współpracowników [29-30]. Kukurbit[n]uryle składają się z jednostek *n-glikourylowych* (5-10) połączonych parami

mostków metylowych [31]. Podobnie jak w przypadku cyklodekstryn, kukurbit[n]uryle charakteryzują się sztywną strukturą, wewnątrz której znajdują się wnęki hydrofobowe o różnej objętości. Nośniki makrocykliczne: CB[6], CB[7] i CB[8] mają porównywalne objętości i rozmiary wnęk z odpowiednio α -CD, β -CD i γ -CD. Należy jednak pamiętać, że podobieństwa w rozmiarach omawianych wielkości związków supramolekularnych (CD i CB) nie przekładają się na podobieństwa ich struktur i właściwości wiążących. Różnica w budowie między dwoma omawianymi nośnikami, wynika ściśle ze sposobu ich wytwarzania. Cyklodekstryny są to cykliczne węglowodany otrzymywane podczas enzymatycznej degradacji skrobi, natomiast synteza kukurbit[n]urili polega na polimeryzacji katalizowanej kwasem glikolurylu i formaldehydu w efekcie czego powstaje sztywna, cykliczna cząsteczka „gospodarza” [32].

Należy wyraźnie podkreślić, że cyklodekstryny mające kształt ściętego stożka (toroidu), z drugorzędowymi grupami hydroksylowymi znajdującymi się przy szerszej krawędzi torusa oraz pierwszorzędowymi grupami hydroksylowymi przy węższej krawędzi nie są idealnie cykliczne w przeciwieństwie do CB, które charakteryzują się zachowaną symetrią z obu możliwych stron



Właściwości	CB[6]	CB[7]	CB[8]
Ilość jednostek	6	7	8
glikourylowych			
Wzór empiryczny	C ₃₆ H ₃₆ N ₂₄ O ₁₂	C ₄₂ H ₄₂ N ₂₈ O ₁₄	C ₄₂ H ₄₈ N ₃₂ O ₁₆
Średnica wnętrza (Å)(a)	3.9	5.4	6.9
Średnica portalu (Å)(b)	5.8	7.3	8.8
Średnica zewnętrzna(Å)(c)	14.4	16.0	17.5
Wysokość torusa (Å)(h)	9.1	9.1	9.1
Objętość wnęki (Å ³)	164	279	479
Masa molowa (g/mol)	997	1163	1329

Tab. 2 Wybrane parametry strukturalne kukurbit[n]urili (n=6-8) [29-30]

kompleksowania. Różnice w strukturze molekularnej nośnika przekładają się niewątpliwie na mechanizm oddziaływań międzycząsteczkowych molekuł „gościa” z nośnikiem makrocyklicznym. Badania eksperymentalne oraz teoretyczne obliczenia kwantowo-chemiczne wykazują, że stałe równowagi tworzenia kompleksu inkluzyjnego z nośnikiem CB

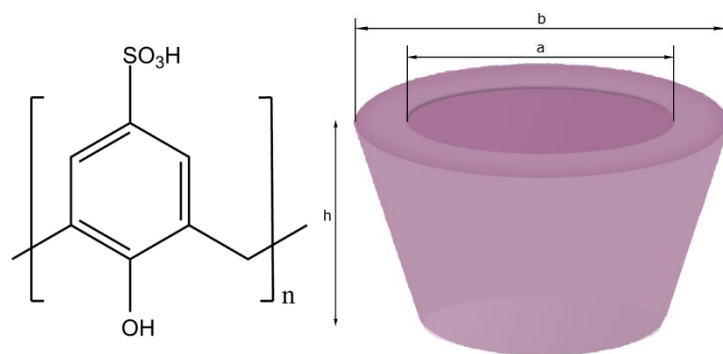
są nawet o kilka rzędów wielkości wyższe niż w przypadku układu z cyklodekstrynami [33-34]. Liczne dane literaturowe potwierdzają przedłużenie stabilności i aktywności leku zainkludowanego do wnętrza kukurbit[n]uryli w porównaniu z układem lek-CD [35]. Z całej rodziny kukurbit[n]urili tylko CB[7] i CB[8] są rozpuszczalne w wodzie, co czyni te makrocykliczne nośniki najbardziej przydatnymi w badaniach układów transportujących substancje farmakokinetyczne.

Kaliks[n]areny [CA] stanowią kolejną grupę związków makrocyklicznych mogącą tworzyć stabilne kompleksy typu „gospodarz-gość” z różnymi cząsteczkami gościa. Te znacząco różniące się kształtem od cyklodekstryn, jak i kukurbit[n]uryli związki organiczne zbudowane są z cyklicznie ułożonych jednostek fenylowych, których ilość ($n=4-20$) określa rozmiar pierścienia makrocyklicznego [36-38]. Związki te zostały odkryte przez *Baeyera* ponad 100 lat temu podczas badań reakcji fenoli z aldehydami [39]. W 1942 roku *Zinke* i *Ziegler* w wyniku uproszczenia procesu syntezy otrzymali krystaliczny produkt oraz scharakteryzowali jego strukturę cykliczną [40].

W przypadku posiadających kształt podobny do kielicha kaliksarenów (stąd ich nazwa od greckiego *kalyx* – kielich) można wyróżnić dwa główne obszary. Węższa, posiadająca charakter hydrofilowy krawędź greckiej wazy (obrzeże dolne) zakończona jest fenyłowymi grupami hydroksylowymi (-OH) podczas gdy szerszą hydrofobową krawędź (obrzeże górne) stanowią podstawniki w pozycji „para” pierścieni aromatycznych. Warto w tym miejscu podkreślić, że średnica wnętrza CA[6] jest porównywalna z β -CD [41]. Podobnie jak w przypadku wcześniej omówionych związków makrocyklicznych, kaliksareny posiadają wnękę hydrofobową, do której mogą przyłączać się mniejsze cząsteczki bądź jony tworząc kompleksy typu „gospodarz-gość”. Pomimo tego, iż na pierwszy rzut oka, kaliksareny mają podobny kształt do cyklodekstryn istnieją istotne różnice w budowie tych dwóch omawianych rodzin związków makrocyklicznych. Mianowicie, CD mają dość sztywną strukturę cząsteczki, podczas gdy CA są cząsteczkami elastycznymi zdolnymi do niewielkiego zginania, jak również posiadają zdolność do całkowitego odwrócenia pierścienia [41]. Niestety, w przeciwieństwie do cyklodekstryn słaba rozpuszczalność w wodzie kaliks[n]arenów ogranicza ich zastosowanie biomedyczne.

W ciągu ostatnich kilkunastu lat nastąpił istotny wzrost zainteresowania wielu grup badawczych sulfonowymi pochodnymi kaliksarenu (SCA) głównie ze względu na ich wysoką rozpuszczalność w wodzie, selektywną zdolność do tworzenia wiązań z mniejszymi cząsteczkami, dimerami czy jonami. Para-sulfonowe kaliks[n]areny (n=4-8) zostały po raz pierwszy opisane przez *Shinkaia* w 1984 roku [42].

SCA w przeciwieństwie do cyklodekstryn przyjmują różne konformacje w zależności od ilości jednostek fenylowych. SCA[4], jako najmniejszy stabilny nośnik przyjmuje kształt ściętego „stożka”, a niewielki rozmiar wnęki makrocyklicznej (por. Tab. 3) umożliwia tworzenie kompleksów inkluzyjnych z niewielkimi cząsteczkami, czy też jonami. Kolejny w grupie związków SCA[6] ma możliwość tworzenia bardziej skomplikowanych struktur



Właściwości	SCA[4]	SCA[6]	SCA[8]
Ilość jednostek fenylowych	4	6	8
Wzór empiryczny	C ₂₈ H ₂₄ O ₁₆ S ₄	C ₄₂ H ₃₆ O ₂₄ S ₆	C ₅₆ H ₄₈ O ₃₂ S ₈
Średnica wewnętrzna (Å)(a)	3.0	7.6	11.7
Średnica zewnętrzna (Å)(b)	5.9	4.96	9.23
Wysokość torusa (Å)(h)	11.75	16.24	22.4
Masa molowa (g/mol)	745	1117	1489

Tab. 3 Wybrane parametry strukturalne p-sulfonowych kaliks[n]arenów (n=4, 6, 8) [34-35]

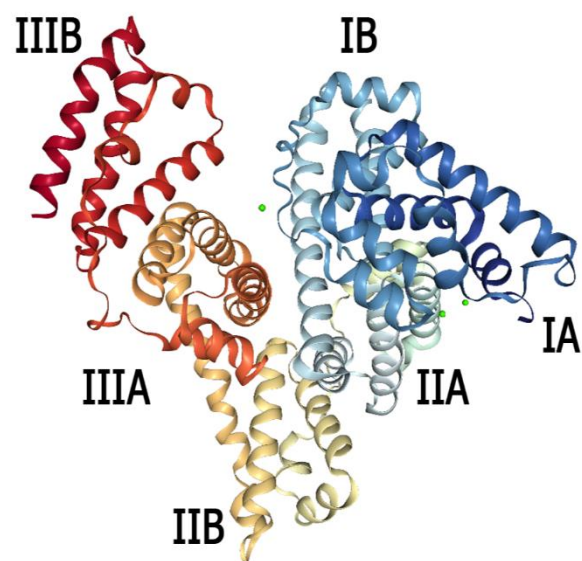
supramolekularnych niż SCA[4] ponieważ jego wnęki jest większa i charakteryzuje się giętką strukturą molekuly [39,43]. SCA[6] może przyjmować więcej konformacji w roztworze wodnym, jednak najczęściej występuje w konformacji częściowego stożka [39,43]. Ostatni nośnik z rodziny para-sulfonowych kaliks[n]arenów to SCA[8], który charakteryzuje się znacznie większą i jeszcze bardziej elastyczną strukturą niż SCA[4] i SCA[6]. W związku z tym nośnik ten może tworzyć skomplikowane kompleksy inkluzyjne z cząsteczkami o budowie sferycznej.

1.2 Wybrane złożone makrocząsteczki biologiczne BSA i HSA

Jak już wspomniano, lek zanim dotrze do docelowego receptora podlega oddziaływaniu z różnymi biomolekułami. Jednym z takich procesów jest wiązanie leku z albuminą surowicy ludzkiej (HSA z ang. „*Human Serum Albumin*”). Zjawisko to ma istotny wpływ m.in. na skuteczność oraz czas działania farmaceutyku.

Albuminy to białka, które najobficiej (ponad 60% wszystkich białek) występują w osoczu krwi. Odgrywają one znaczącą rolę w transporcie różnych substancji w organizmie (hormony, kwasy tłuszczowe, witaminy, leki czy jony metali) przenosząc je do docelowych tkanek. Biorąc pod uwagę fakt, iż albumina jest uważana za najistotniejszą z białek osocza krwi w ostatnich latach grupy naukowców zajmujących się badaniami z zakresu farmakokinetyki leków skierowały swoją uwagę na aminokwasy pochodzenia zwierzęcego np. albuminę wołową („*bovine serum albumin*” (BSA)),

która jest traktowana jako białko modelowe dla albuminy ludzkiej. Co więcej, te naturalne proteiny wykazujące zdolność do tworzenia wiązań z różnorodnymi molekułami charakteryzują się dobrą rozpuszczalnością w wielu środowiskach (są hydrofilowe) oraz łatwą krystalizacją. Albumina surowicza wołowa, podobnie jak albumina ludzka składa się z około 580 kwasów aminowych w pojedynczych łańcuchach polipeptydowych i 17 mostków dwusiarczkowych. Białko pochodzenia



Rys. 2 Struktura albuminy surowicy bydlęcej (BSA) [A4]

zwierzęcego ma w 80% takie same sekwencje jak HSA. Zgodnie z nomenklaturą *Sudlowa* BSA posiada trzy główne domeny (I, II i III), z których każda dzieli się na dwie subdomeny (IA i IB, IIA i IIB, IIIA i IIIB) (Rys. 2) [44-46]. Na podstawie danych literaturowych [47-49] sugeruje się dwa możliwe obszary wiążące, które umiejscowione są w subdomenach IIA i IIIA.

Główne składniki hydrofobowych wnęk to reszty aminokwasowe takie jak tryptofan (Trp), tyrozyna (Tyr) oraz fenyloalanina (Phe). Chociaż HSA i BSA wykazują podobne właściwości konformacyjne, ich właściwości spektroskopowe są różne. Jako, że za absorpcję i fluorescencję białek odpowiedzialne są wcześniej wspomniane aminokwasy aromatyczne różnica głównych charakterystyk spektroskopowych związana jest z faktem, iż BSA ma dwie różne cząsteczki tryptofanu (Trp 134 i Trp 212), które charakteryzują się własną fluorescencją podczas gdy HSA ma tylko jedną resztę Trp 214 [46]. Cząsteczki tryptofanu Trp 134 są obecne na powierzchni pierwszej domeny (**IB**), podczas gdy tryptofan Trp 212 znajduje się wewnątrz hydrofobowej kieszeni znajdującej się w drugiej domenie (**IIA**).

Proces wiązania cząsteczki leku z albuminą w organizmie ludzkim, jak i zwierzęcym, jest bardzo złożony i często jest analizowany z wykorzystaniem technik stacjonarnej i rozdzielonej w czasie spektroskopii UV-Vis. Jak już wzmiankowano, analiza podstawowych charakterystyk luminescencyjnych (widma absorpcji i emisji, czasy życia fluorescencji, wydajności kwantowe) podstawowych aminokwasów aromatycznych (tryptofan, tyrozyna i fenyloalanina) może dostarczyć cennych informacji na temat międzycząsteczkowych oddziaływań lek–albumina. Pomimo tego, że wydajności kwantowe tryptofanu i tyrozyny w roztworze są zbliżone w białkach natywnych fluorescencja pochodząca od tyrozyny jest silnie wygaszana w wyniku transferu elektronowej energii wzbudzenia między tyrozyną a tryptofanem. Pamiętając o niskiej wydajności kwantowej fluorescencji fenyloalaniny (w białkach dodatkowo obniżone z powodu bezpromienistego transferu elektronowej energii wzbudzenia między fenyloalaniną a tyrozyną i fenyloalaniną a tryptofanem) można stwierdzić, że właściwości fluorescencyjne zarówno albuminy surowicy wołowej, jak i ludzkiej wynikają głównie z promienistej dezaktywacji bardzo wrażliwego na otoczenie tryptofanu.

Rozdział 2

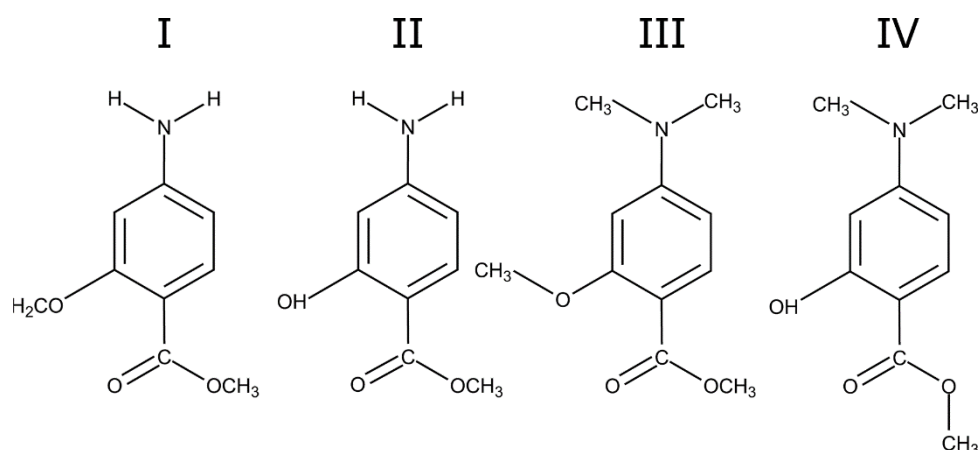
Związki typu ES IPT i TICT, jako „goście” w układach supramolekularnych

Fotoindukowane wewnątrzcząsteczkowe i międzycząsteczkowe przeniesienie ładunku (protonu lub elektronu) to procesy, które odgrywają kluczową rolę w wielu podstawowych procesach biologicznych i chemicznych [50-54]. Mechanizm tych zjawisk jest silnie zależny od struktury chemicznej danej molekuly oraz środowiska, w którym się znajduje. W okresie ostatnich kilkudziesięciu lat wielu naukowców próbowało dogłębnie badać procesy fotoindukowanego wewnątrzcząsteczkowego przeniesienia elektronu (IET – *Intramolecular Electron Transfer*) i protonu (IPT – *Intramolecular Proton Transfer*). Szczególnie ciekawe okazały się badania procesu fotoindukowanego wewnątrzcząsteczkowego przeniesienia elektronu związanego z zmianą geometrii układu (TICT – *Twisted Intramolecular Charge Transfer*) oraz zjawiska tworzenia międzycząsteczkowych wiązań wodorowych. Badania te były interesujące zarówno z punktu widzenia czysto teoretycznego (tj. poznanie mechanizmów procesów zachodzących w badanym układzie), jak również ze względu na szerokie możliwości wykorzystania takich związków w różnych obszarach życia (czujniki fluorescencyjne, barwniki laserowe, fotostabilizatory UV, sondy fluorescencyjne, molekularne elementy elektroniczne, obiekty na bazie których produkowane są leki i wiele więcej) [55-59]. Warto w tym miejscu nadmienić, że równolegle do badań związków typu ICT znajdujących się w jednorodnych środowiskach o różnej polarności, w ostatnich latach poświęcono znaczną uwagę badaniom interakcji między cząsteczkami organicznymi typu ICT, a supramolekularnymi nośnikami, zwracając szczególną uwagę na oddziaływania niespecyficzne i specyficzne [50-54].

2.1 Pochodne benzoesanu metylu

W niniejszej rozprawie doktorskiej jako obiekt badawczy wybrano m. in. grupę molekuł, które są estrami metylowymi kwasów: *o*-metoksy *p*-metyloaminobenzoesowego (I), *o*-hydrokso *p*-metyloaminobenzoesowego (II), *o*-metoksy *p*-dimetyloaminobenzoesowego (III),

o-hydroksy *p*-dimetyloaminobenzoesowego (IV) (Rys. 3). Prace Kashy i współpracowników [60-63] oraz Heldta i współpracowników [64-68] jednoznacznie wskazują, że właściwości fotofizyczne i fotochemiczne badanych molekuł istotnie zależą zarówno od rodzaju podstawników, jak i środowiska w którym się znajdują. Badania przy wykorzystaniu technik stacjonarnej i rozdzielonej w czasie spektroskopii oraz teoretycznych obliczeń kwantowo-chemicznych pozwoliły na zaproponowanie schematycznego diagramu poziomów energetycznych tłumaczącego możliwe ścieżki dezaktywacji elektronowo wzbudzonych badanych molekuł (Schemat 1) [62].

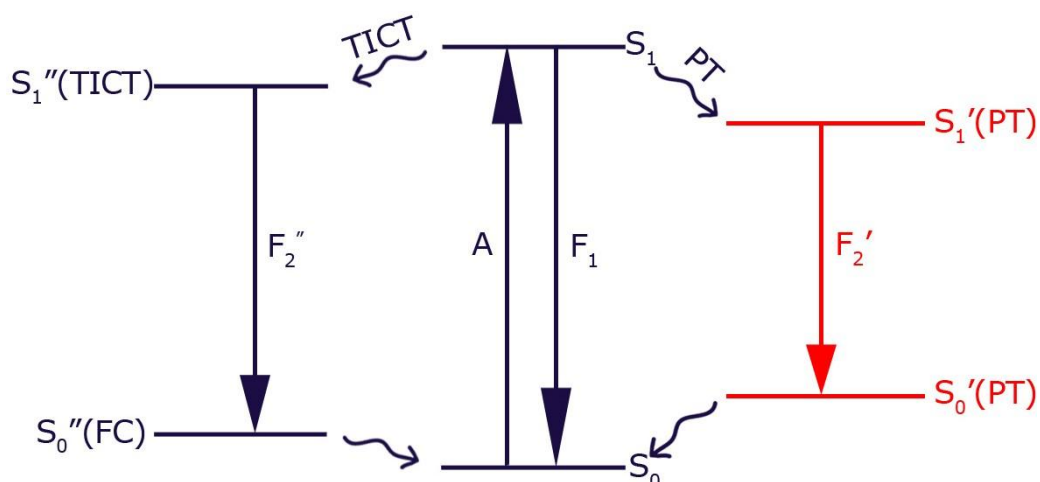


Rys. 3 Wzory strukturalne estrów metylowych kwasów: *o*-metoksy *p*-metyloaminobenzoesowego (I), *o*-hydroksy *p*-metyloaminobenzoesowego (II), *o*-metoksy *p*-dimetyloaminobenzoesowego (III) i *o*-hydroksy *p*-dimetyloaminobenzoesowego (IV).

W przypadku molekuly I znajdującej się w rozpuszczalniku aprotycznym zaobserwowano wyłącznie emisję krótkofalową ze stanu lokalnie wzbudzonego $S_1(LE) \rightarrow S_0$ (LE–*locally excited*), podczas, gdy w tym samym środowisku molekula II oprócz fluorescencji normalnej wykazuje fluorescencję pochodzącą od tautomeru powstałego w stanie wzbudzonym w wyniku wewnątrzcząsteczkowego transferu protonu $S'_1(PT) \rightarrow S'_0(PT)$ (Schemat 1).

Dalsze badania eksperymentalne i teoretyczne prowadzone przez wcześniej wspomniane grupy naukowców pozwoliły stwierdzić, że w aprotycznych rozpuszczalnikach molekula III posiada dwa pasma emisyjne: fluorescencję normalną $S_1(LE) \rightarrow S_0$ oraz fluorescencję związaną z fotoindukowanym wewnątrzcząsteczkowym przeniesieniem elektronu $S_1''(TICT) \rightarrow S''_0(FC)$ (po wzbudzeniu molekuly następuje skręcenie grupy dimetyloaminowej, które prowadzi do przyjęcia przez wzbudzoną molekułę układu

przestrzennie różniącego się od układu atomów w stanie podstawowym). Ostatnia molekula z grupy badanych pochodnych benzoesanu metylu (**IV**) wykazuje aż trzy mody emisyjne: fluorescencję normalną $S_1(LE) \rightarrow S_0$, fluorescencję pochodzącą od tautomeru powstałego w stanie wzbudzonym $S'_1(PT) \rightarrow S'_0(PT)$ oraz fluorescencję powstałą w wyniku wewnątrzcząsteczkowego przeniesienia elektronu $S_1''(TICT) \rightarrow S''_0(FC)$. Obecność trzech pasm emisyjnych w widmach fluorescencji została potwierdzona na drodze ich selektywnego wykluczania poprzez dobór odpowiedniego środowiska [60-62].



Schemat 1 Schematyczny diagram poziomów energetycznych przedstawiający możliwe ścieżki dezaktywacji elektronowo wzbudzonych badanych pochodnych benzoesanu metylu. Przejścia promieniste zaznaczone linią ciągłą a bezpromieniste „falą”.

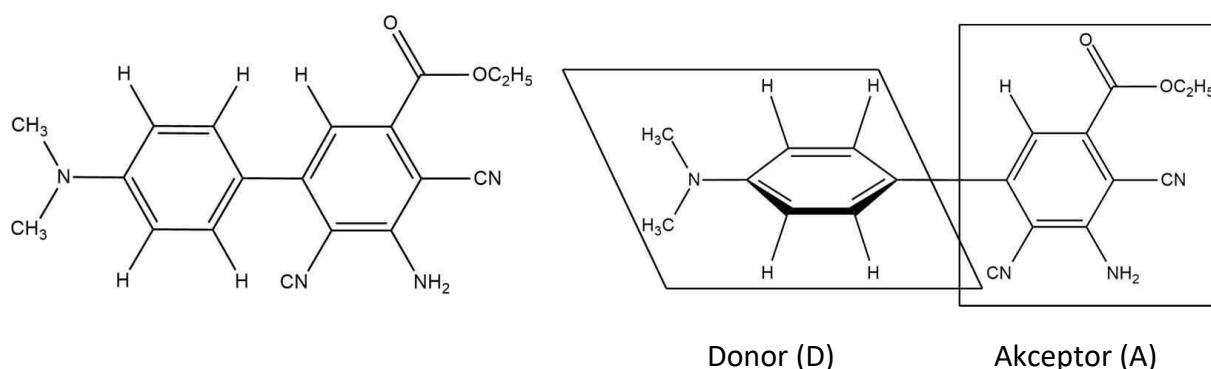
Warto w tym miejscu nadmienić, że wybór obiektów badawczych był podyktowany nie tylko faktem, iż pochodne benzoesanu metylu posiadają bardzo interesujące właściwości fotofizyczne i fotochemiczne ale również mają liczne możliwości aplikacyjne. Pochodne benzoesanu metylu **I** i **II** są składnikami olejków, maści przeciwzapalnych i przeciwbólowych. Mają one właściwości przeciwgrzybicze i antibakteryjne [69]. Duża część estrów m. in. **I** i **II** posiada charakterystyczny zapach (owocowy lub kwiatowy) dzięki czemu znalazły one liczne zastosowania w przemyśle spożywczym i perfumeryjnym, gdzie stanowią skład kompozycji zapachowych [70]. Pochodne benzoesanu metylu są wykorzystywane jako substancje wyjściowe do produkcji leków [71]. Molekuły **III** i **IV**, które wykazują procesy typu CT znalazły zastosowania jako sondy luminescencyjne [72], w elektronice kwantowej [73], jak również są wykorzystywane w produkcji materiałów tryboaktywnych do tonerów kserograficznych [74].

2.2 Pochodna bifenyłu

Jak już wspomniano, jednym z głównych celów poznawczych niniejszej dysertacji było zrozumienie mechanizmów oddziaływań pomiędzy cząsteczkami wykazującymi fotoindukowane wewnątrzcząsteczkowe przeniesienie ładunku a wybranymi nośnikami makrocyklicznymi. Chcąc poszerzyć wiedzę na temat tych oddziaływań, a w szczególności określić wpływ geometrii cząsteczek „gościa” na trwałość powstałych kompleksów, jako drugi obiekt badawczy wybrano dwuchromoforowy, donorowo-akceptorowy (D-A) związek aromatyczny–5-(4-aminofenylo)-3-dimetyloamino-2,4-dicyjanobenzoesan etylu (**EDMAADCy**) (Rys. 4). W przypadku tej molekuly, zbudowanej z dwóch pierścieni aromatycznych połączonych pojedynczym wiązaniem, fotowzbudzenie inicjuje proces wewnątrzcząsteczkowego przeniesienia elektronu. Mechanizm tego zjawiska był szczegółowo analizowany przez fotofizyków z grupy prof. Heldta [75-76]. Wykazali oni, że w przypadku badanej pochodnej bifenyłu znajdującej się w środowisku niepolarnym bądź silnie polarnym i aprotycznym, widmo fluorescencji posiada tylko jedno, szerokie, krótkofalowe pasmo (emisja ze stanu lokalnie wzbudzonego), natomiast w środowisku średniopolarnym zaobserwowano dwumodową fluorescencję tj. emisję ze stanu lokalnie wzbudzonego oraz emisję konformeru powstałego w stanie wzbudzonym w wyniku wewnątrzcząsteczkowego przeniesienia elektronu [75-76]. Co więcej, zauważono, że widma fluorescencji (natężenie, kształt i położenie) silnie zależą od długości fali światła wzbudzającego. Zapostulowano, że zmiany te, w połączeniu z niejednorodnym poszerzeniem elektronowych pasm absorpcyjnych i emisyjnych, stanowią dowód, na to że już w stanie podstawowym związek **EDMAADCy** tworzy układ spektroskopowo niejednorodny (przestrzennie różne konformery, które powstają w wyniku stosunkowo dużej swobody rotacji wokół pojedynczego wiązania C-C, łączącego dwa pierścienie aromatyczne) [75-76].

Istnienie przestrzennie różnych konformerów badanej donorowo-akceptorowej molekuly w stanie podstawowym potwierdziła analiza teoretyczna (obliczenia kwantowo-chemiczne). W wyniku obliczeń ustalono, iż najbardziej korzystna energetycznie konformacja w stanie S_0 odpowiada sytuacji, w której oba chromofory są ustawione do siebie niemal prostopadle („*perpendicular form*”) [76]. Z drugiej strony niewielkie zmiany w wartościach energii stanu podstawowego, wywołane obrotem płaszczyzn obu chromoforów w przedziale

między 60° a 120° potwierdzają, że badany związek w stanie podstawowym należy traktować jako zbiór konformerów o różnym kącie pomiędzy grupami donorową i akceptorową (układ spektroskopowo niejednorodny). W pracach [75-76] na podstawie analizy wyników eksperymentalnych wspartych obliczeniami kwantowo-chemicznymi ustalono, że w środowisku średniopolarnym krótkofalowe pasmo emisji związane jest z promienistą dezaktywacją lokalnie wzbudzonego konformeru, gdzie oba chromofory są ustawione w stosunku do siebie prostopadle „*perpendicular form*” podczas gdy długofalowe pasmo to emisja znajdującego się w elektronowo wzbudzonym stanie z wewnątrzcząsteczkowym przeniesieniem elektronu konformeru, dla którego kąt pomiędzy chromoforami wynosi 45° („*flattened form*”). Ustalono, również, że za obserwowaną jednomodową emisję w rozpuszczalnikach niepolarnych głównie odpowiada akceptorowa część badanego związku D-A.



Rys. 4 Wzór strukturalny badanej molekuly 5-(4-aminofenilo)-3-dimetyloamino-2,4-dicyjanobenzoesan etylu (EDMAADCy).

Podobnie jak to miało miejsce w przypadku pochodnych benzoesu metylu, wybór dwuchromoforowej, donorowo-akceptorowej cząsteczki do badań procesu kompleksowania z wybranymi nośnikami makrocyclicznymi podyktowany był również dwoma zasadniczymi powodami. Pierwszy z nich wynika z potrzeby dogłębnego zrozumienia mechanizmów reakcji oddziaływań cząsteczka wykazująca fotoindukowane wewnątrzcząsteczkowe przeniesienie elektronu–związek makrocycliczny. Drugi powód związany jest z badaniem związku, który posiada bardzo interesujące możliwości aplikacyjne. Jak pokazano w pracach [77-79] związek ten może być wykorzystany jako sonda fluorescencyjna do monitorowania postępu polimeryzacji oraz w elektronice molekularnej, biorąc pod uwagę, jej nieliniowe właściwości optyczne.

Rozdział 3

Oddziaływania pochodnych benzoesu metylu z wybranymi nośnikami makrocyklicznymi

Jednym z celów współczesnej spektroskopii molekularnej jest badanie oddziaływań między różnymi molekułami organicznymi i/lub nieorganicznymi, a związkami supramolekularnymi. W świetle wzmiankowanych w rozdziale 1 informacji spośród wielu znanych związków makrocyklicznych szczególna uwaga naukowców skupiła się na trójce głównych przedstawicieli: α -, β - i γ -cyklodekstryny, kukurbit[n]uryly i sulfonowe kaliks[n]areny ze względu na swoją przestrzenną budowę, stabilną strukturę i przede wszystkim unikalne zdolności do tworzenia kompleksów inkluzyjnych typu „gospodarz-gość”. W niniejszym rozdziale zbadano, z wykorzystaniem technik stacjonarnej i czasowo-rozdzielczej spektroskopii oraz spektroskopii magnetycznego rezonansu jądrowego (^1H NMR), oddziaływania czterech pochodnych benzoesu metylu z cyklodekstrynami, kukurbit[n]urydami i sulfonyowymi kaliks[n]arenami, które dzięki swej budowie są zdolne do tworzenia kompleksów inkluzyjnych. Analiza uzyskanych wyników eksperymentalnych pozwoliła uzyskać informację na temat możliwych ścieżek dezaktywacji elektronowo wzbudzonych molekuł wewnątrz wybranych nośników supramolekularnych, jak również ocenić, jaką rolę w procesie kompleksowania pełnią międzycząsteczkowe oddziaływania uniwersalne (dipol-dipolowe) i specyficzne (międzycząsteczkowe wiązania wodorowe). Ustalono również stechiometrię badanych kompleksów inkluzyjnych oraz określono orientację molekuły wewnątrz makrocyklicznego nośnika. Wyniki prac zostały zaprezentowane w trzech publikacjach naukowych:

A1 K. Baranowska, M. Józefowicz, „*Spectroscopic studies of inclusion complexation between ortho derivatives of p-methylaminobenzoate and α - and γ -cyclodextrins*”, *Journal of Molecular Liquids* 265 (2018) 140-150, IF₂₀₁₈ = 4.56, MNISW = 100.

A2 K. Baranowska, M. Mońka, A. Kowalczyk, N. Szpakowska, Z. Kaczyński, P. Bojarski, M. Józefowicz, „*Spectroscopic studies of the excited-state intramolecular proton and electron transfer processes of methyl benzoate derivatives in cucurbit[7]uril nanocage*”, *Journal of Molecular Liquids* 318 (2020) 113921, IF₂₀₂₀ = 6.17, MNISW = 100.

A3 K. Baranowska, M. Mońka, A. Kowalczyk, Z. Kaczyński, P. Bojarski, M. Józefowicz, „*Spectroscopic studies on the supramolecular interactions of methyl benzoate derivatives with*

3.1 Widma absorpcji i emisji pochodnych benzoesanu metylu w wodnych roztworach wybranych nośników supramolekularnych

Ze względu na fakt, iż badania tworzenia kompleksów inkluzyjnych między wybranymi pochodnymi benzoesanu metylu a nośnikami makrocyklicznymi były prowadzone w środowisku wodnym ważnym ich elementem było wyjaśnienie mechanizmu zjawiska tworzenia się międzycząsteczkowych kompleksów z wiązaniem wodorowym oraz wpływu wiązań wodorowych na zjawisko fotoindukowanego wewnątrzcząsteczkowego przeniesienia ładunku. Aby zrozumieć naturę oddziaływań między badanymi molekułami a cząsteczkami rozpuszczalnika (uniwersalne (dipol-dipolowe) *versus* specyficzne (międzycząsteczkowe wiązania wodorowe)) przeprowadzono szereg badań eksperymentalnych oraz obliczeń kwantowo-mechanicznych [A1, 80-81].

Jak pokazano w pracy [A1], w przypadku molekuł I i II znajdujących się w mieszaninie THF-H₂O długofalowe pasmo absorpcji ulega coraz większemu przesunięciu „ku czerwieni” wraz ze wzrostem ułamka molowego wody. To batochromowe, nieliniowe przesunięcie, w połączeniu z wyraźnymi zmianami kształtu, szerokości połówkowej oraz wartości molowego współczynnika absorpcji długofalowego pasma absorpcyjnego są zrozumiałe w kontekście dwóch konkurencyjnych procesów: zjawiska solwatacji preferencyjnej oraz tworzenia różnych kompleksów z cząsteczkami wody. Analiza wyników eksperymentalnych wsparta obliczeniami kwantowo-chemicznymi pokazała, że w sytuacji gdy ułamek molowy wody był mniejszy niż 0.9 ($0 < x_{H_2O} < 0.9$) obserwuje się kompleksy z międzycząsteczkowym wiązaniem wodorowym, z tym że tylko pojedyncza cząsteczka wody została przyłączona do badanych związków (wiązanie wodorowe między azotem grupy -NH₂, a wodorem cząsteczki wody – „*monohydrated complex*”). Dla dużego ułamka molowego wody ($x_{H_2O} > 0.9$) możliwe jest powstanie kompleksów, w których dwie cząsteczki wody są połączone z badanymi molekułami (elementem łączącym są grupy -NH₂ i -COOCH₃ – „*dihydrated complex*”). Analiza widm fluorescencji molekuł I i II w czystym polarnym tetrahydrofuranie i polarnej, protycznej wodzie oraz w ich mieszaninach o różnym ułamku molowym wody jednoznacznie wskazuje, że ułamki molowe poszczególnych składników mieszaniny w otocze solwatacyjnej fotowzbudzonej

badanej molekuly różnią się od wartości ułamków molowych określających roztwór jako całość co świadczy o występowaniu zjawiska solwatacji preferencyjnej w stanie wzbudzonym.

Podsumowując, można stwierdzić, że otrzymane wyniki dla związków będących w stanie wzbudzonym są w pełni zrozumiałe biorąc pod uwagę występowanie dwóch konkurencyjnych procesów tj. solwatacja preferencyjna i specyficzne oddziaływania, którego efektem jest tworzenie różnego rodzaju międzycząsteczkowych wiązań wodorowych [A1].

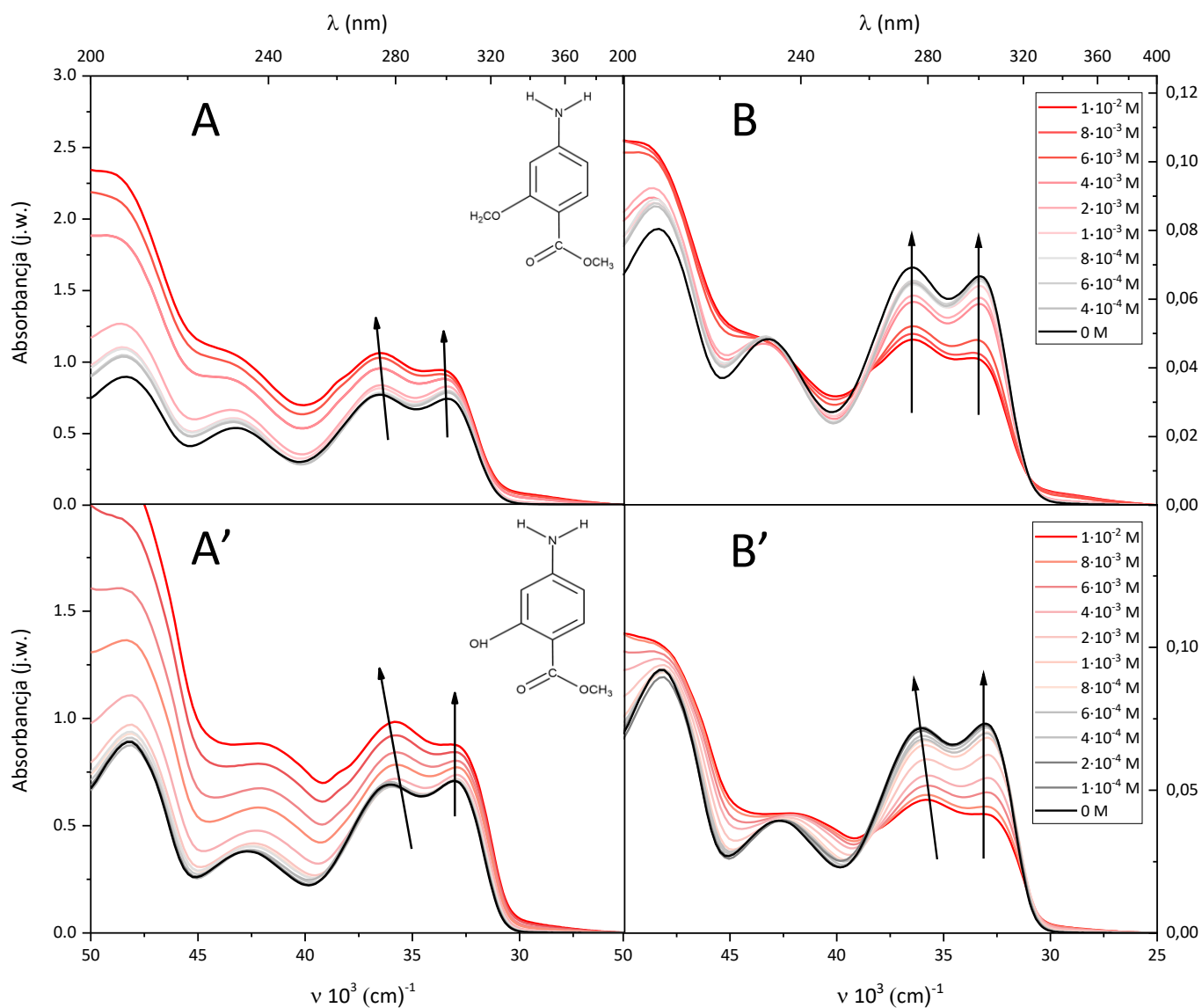
W świetle powyższych informacji można stwierdzić, że metody spektroskopowe należą do bardzo użytecznych technik stosowanych do oceny natury oddziaływań substancja rozpuszczona–rozpuszczalnik (zarówno czysty, jednorodny rozpuszczalnik, jak i dwuskładnikowa mieszanina rozpuszczalników). Jak pokazano w pracach [A1-A3], cenne informacje na temat mechanizmów procesu tworzenia się kompleksów inkluzyjnych pochodna benzoesanu metylu–związek makrocykliczny w stanie podstawowym uzyskano na podstawie szczegółowej analizy stacjonarnych widm absorpcji pochodnych benzoesanu metylu znajdujących się w roztworach wodnych o różnym stężeniu związku makrocyklicznego (α -, β - i γ -cyklodekstryny, kukurbit[7]uryle i sulfonowe kaliks[6]areny). Poniżej zestawiono najważniejsze wyniki i spostrzeżenia przedstawione w publikacji [A1] oraz otrzymane na podstawie dodatkowych, niepublikowanych wyników dla związków I i II znajdujących się w wodnych roztworach zawierających różne stężenia α -, β -, γ -cyklodekstryn:

- wzrost stężenia α -CD powoduje bardzo niewielkie zmiany w widmach absorpcji (kształt, położenie, szerokość połówkowa), co sugeruje, że w stanie podstawowym nie powstają stabilne kompleksy inkluzyjne między związkami I i II a α -cyklodekstylinami,
- obecność β -CD powoduje bardzo istotne zmiany w widmach absorpcji (por. niepublikowane dotychczas widma absorpcji molekuł I i II w obecności β -CD przedstawione na Rys. 5). Zmiany wartości molowego współczynnika absorpcji pasma długofalowego (przejście elektronowe ($S_0 \rightarrow S_1$)), przesunięcie pasm absorpcji w stronę fal krótkich oraz wyraźna obecność punktu izobestycznego znamionuje istnienie równowag między badaną molekułą (I lub II) a kompleksem inkluzyjnym (I- β -CD i II- β -CD). Utworzenie kompleksu jest zrozumiałe biorąc pod uwagę fakt, że średnica wnęki β -CD jest większa niż α -CD. Wzajemna komplementarność badanych związków

tj. dopasowanie geometryczno–sferyczne przekłada się na powstanie stabilnego układu,

- zmiany w widmach absorpcji, które zostały zaobserwowane dla nośnika, który charakteryzuje się największym rozmiarem wnęki spośród badanych cyklodekstryn jakim jest γ -cyklodekstryna, są bardzo znaczące. Podobnie jak to miało miejsce w przypadku β -CD wyraźne zmiany wartości molowego współczynnika absorpcji dwóch długofalowych pasm absorpcyjnych, wyraźne przesunięcie hipsochromowe, obecność punktu izobestycznego to przesłanki świadczące o powstaniu stabilnego kompleksu z γ -CD,
- warto w tym miejscu wspomnieć, że Józefowicz wraz z współautorami [82-83] pokazali, że w przypadku molekuł **III** i **IV** stabilne kompleksy w stanie podstawowym powstają w obecności β - i γ -CD, podczas gdy dla układu z α -CD nie znaleziono przesłanek potwierdzających powstanie trwałego kompleksu.

W celu określenia wpływu struktury przestrzennej badanych związków makrocyklicznych na proces tworzenia stabilnych kompleksów inkluzyjnych w stanie podstawowym, w pracach [A2] i [A3], przeanalizowano wpływ obecności kukurbit[7]uryli i sulfonowych kaliksarenów na widma absorpcji czterech pochodnych benzoesanu metylu znajdujących się w wodnym roztworze. Dla wszystkich badanych układów zaobserwowano, że wraz ze wzrostem stężenia CB[7] długofalowe pasmo absorpcji ulega stopniowym zmianom, czego przejawem jest m.in niewielkie przesunięcie pasm w kierunku fal krótkich oraz wyraźna obecność punktu izobestycznego. Zaobserwowane zmiany w widmach absorpcji molekuł, które posiadają grupę hydroksylową (**II** i **IV**) są zdecydowanie większe niż te obserwowane dla pochodnych benzoesanu metylu pozbawionych grupy –OH tj. **I** i **III**. Zaobserwowane rezultaty wskazują, że kompleksy inkluzyjne między badanymi molekułami organicznymi a CB[7] powstają w stanie podstawowym, z tym że występuje zasadnicza różnica w geometrii i trwałości utworzonych kompleksów (**I** i **III** *versus* **II** i **IV**). Analiza widm absorpcyjnych w obecności SCA[6] potwierdza, że również w tym układzie badane cząsteczki tworzą kompleksy inkluzyjne w stanie podstawowym.



Rys. 5 Widma absorpcji (A, A') i znormalizowane widma absorpcji (B i B') ($c=5 \cdot 10^{-5}$ M) w roztworach wodnych zawierających różne stężenia β -CD.

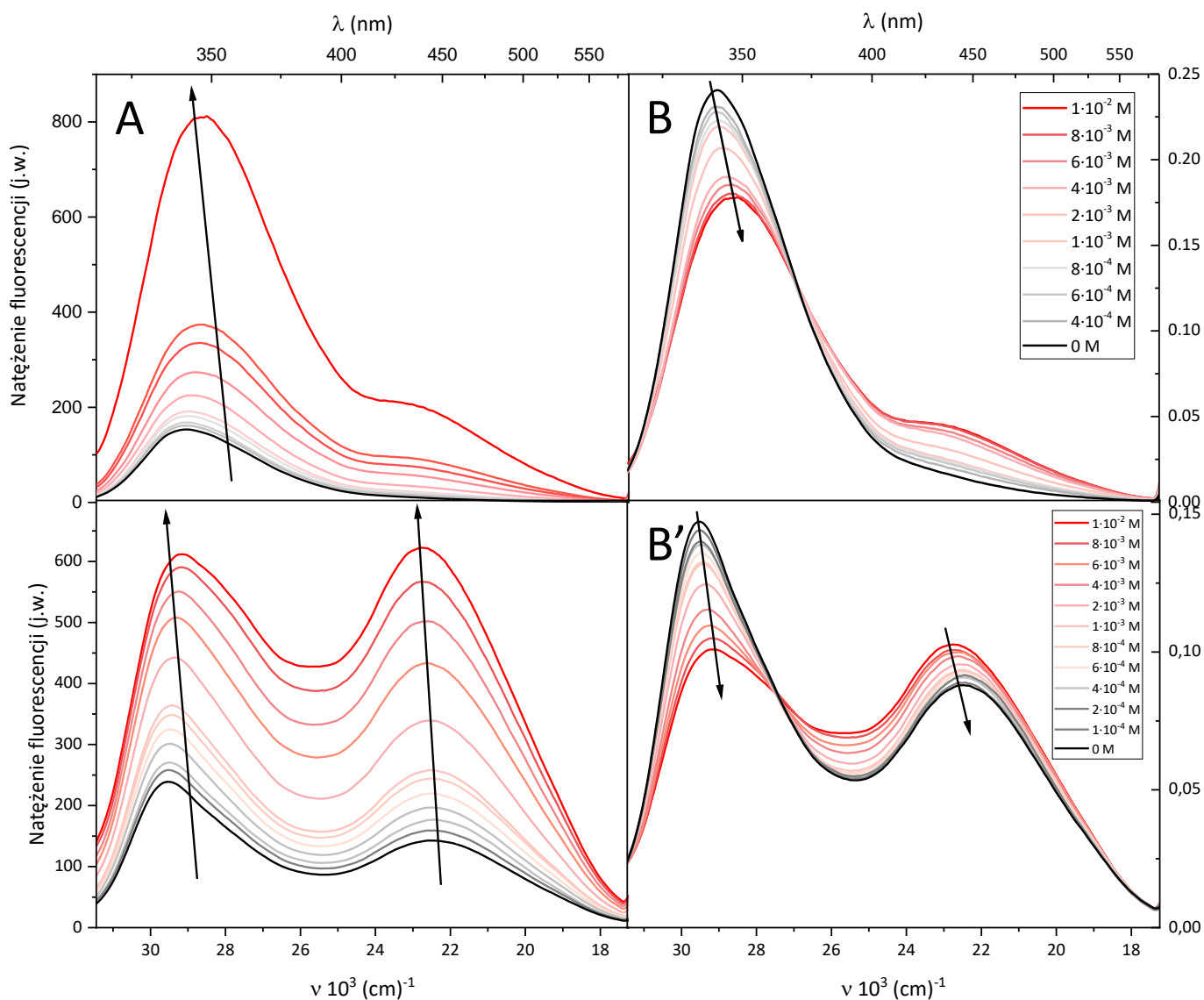
Aby zrozumieć mechanizm molekularny tworzenia kompleksów inkluzyjnych pomiędzy pochodnymi benzoianu metylu a wybranymi nośnikami makrocyclicznymi w stanie wzbudzonym, szczegółową analizę otrzymanych wyników eksperymentalnych w układzie molekula organiczna–związek makrocycliczny należy odnieść do omówionych wyżej fotoindukowanych procesów wewnątrzcząsteczkowych tzn. uwzględnić fakt, iż każda z badanych molekuł w środowisku wodnym oprócz fluorescencji ze stanu LE (I); LE i ESIPT (II); LE, TICT (III); LE, ESIPT i TICT (IV) charakteryzuje się również promienistą dezaktywacją kompleksów z międzycząsteczkowym wiązaniem wodorowym.

Aby uzyskać pełniejsze zrozumienie wewnątrz- i międzycząsteczkowych procesów molekularnych w stanie wzbudzonym występujących w układzie pochodna benzoianu

metylu–wybrany nośnik makrocykliczny, badania rozpoczęto od analizy widm emisji w wodnych roztworach CD, CB[7] i SCA[6]. Analiza danych eksperymentalnych zaprezentowanych w pracach [A1-A3], które zostały uzupełnione niepublikowanymi dotąd wynikami przedstawionymi m.in. na Rys. 6, wskazuje, że dla wszystkich badanych układów, dodanie α -, β - i γ -cyklodekstryn, kukurbit[7]uryli i sulfonowych kaliks[6]arenów powoduje istotny wzrost intensywności pasm fluorescencji z jednoczesną obecnością punktów izobestycznych w znormalizowanych do pola widmach emisji i niewielkie przesunięcie pasm w kierunku fal krótkich. Takie zmiany w zachowaniu widmowym w obecności nośników wskazują, że nastąpiła wyraźna zmiana środowiska w najbliższym otoczeniu (sferze solwatacyjnej) badanych molekuł. Wzrost intensywności pasm fluorescencji wywołany obecnością α -, β -, γ -CD, CB[7] i SCA[6] jest w pełni rozumiały, biorąc pod uwagę fakt, iż zainkludowanie wybranych molekuł do wnętrza makrocyklicznego nośnika powoduje zerwanie wiązań wodorowych, które w istocie wygaszają fluorescencję. Ponadto, hipsochromowe przesunięcie pasma krótkofalowego wskazuje również, że mikrośrodowisko badanych cząsteczek organicznych zmieniło się na mniej polarne (wnęka makrocykliczna). Zaobserwowane zmiany w widmach emisji molekuł I-IV miareczkowanych roztworem związku makrocyklicznego pozwoliły na wyciągnięcie następujących wniosków na temat zjawiska fotoindukowanego wewnątrzcząsteczkowego przeniesienia protonu i elektronu (ESIPT i TICT) oraz międzycząsteczkowych wiązań wodorowych zachodzących w obecności związku makrocyklicznego:

- dodanie do wodnego roztworu molekuły I α -, β -, γ -CD, CB[7] i SCA[6] skutkuje istotnym wzrostem intensywności fluorescencji pasma LE oraz jego przesunięciem w kierunku fal krótkich. Jak już wspomniano, to przesunięcie hipsochromowe jest efektem zmiany mikrośrodowiska na mniej polarne występujące we wnętrzu nośnika,
- w przypadku molekuły II znajdującej się w wodnych roztworach związków makrocyklicznych, dla której widmo emisji jest wynikiem promienistej dezaktywacji co najmniej 3 centrów luminescencyjnych (emisja ze stanu LE, ESIPT oraz kompleksu z międzycząsteczkowym wiązaniem wodorowym) obserwuje się wzrost wydajności kwantowej fluorescencji wraz ze wzrostem stężenia nośnika, przy czym najsilniejszy wzrost intensywności pasma ESIPT obserwuje się w obecności CB[7]. Zauważone prawidłowości sugerują, że będący w stanie wzbudzonego układu II-CB[7] cechuje

największa, spośród badanych układów, wzajemna komplementarność (najlepsze dopasowanie elektronowo-energetyczne oraz geometryczno-sferyczne). Wyraźny wzrost prawdopodobieństwa zajścia procesu ESIPT jest dowodem na zainkludowanie cząsteczki do wnętrza nośnika (ESIPT jest możliwy jedynie we wnętrzu),



Rys. 6 Widma emisji (A, A') i znormalizowane widma emisji (B, B') I i II ($c=5 \cdot 10^{-5}$ M) w roztworach wodnych zawierających różne stężenia β -CD.

- w przypadku molekuly III obserwuje się różny wpływ badanego nośnika supramolekularnego na intensywność fluorescencji różnych pasm emisyjnych. Wzrost stężenia cyklodekstryn i sulfonowych kaliks[6]arenów w sąsiedztwie charakteryzującej się 3-modową fluorescencją (LE, TICT, kompleks z wiązaniem wodorowym) molekuly III powoduje znaczący wzrost intensywności fluorescencji pasma LE, przy niewielkim wzroście intensywności pasma TICT. Dopiero dla dużego stężenia molowego CD i

- SCA[6] ($c_{CD} > 5 \cdot 10^{-3} \text{ M}$, $c_{SCA[6]} > 10^{-2} \text{ M}$) emisja ze stanu TICT staje się bardziej prawdopodobna. Powyższe zmiany należy tłumaczyć możliwością pełnej izolacji badanej molekuly **III** od środowiska wodnego poprzez całkowite „uwięzienie” cząsteczki gościa w wnęce makrocyklicznej co przekłada się na możliwość rotacji grupy $-N(\text{CH}_3)_2$ i w konsekwencji uaktywnienie procesu TICT. Z drugiej strony niewielki wzrost intensywności pasma fluorescencji po stronie długofalowej (TICT) w obecności „gospodarza” CB[7] sugeruje, że mogło nastąpić zainkludowanie molekuly **III** do wnętrza makrocyklicznego nośnika CB[7], z tym, że grupa dimetyloaminowa ($-N\text{Me}_2$) pozostała na zewnątrz nośnika (możliwość oddziaływania z rozpuszczalnikiem (wodą)),
- dodanie do wodnego roztworu molekuly **IV** cyklodekstryn oraz sulfonowych kaliks[6]arenów powoduje znaczący wzrost intensywności fluorescencji pasma LE i ESIPT przy jednoczesnym, nieznacznym wzroście intensywności fluorescencji ze stanu TICT. Zmiany te sugerują możliwość utworzenia kompleksu typu „gospodarz-gość”, przy czym wydaje się, że mało efektywnemu fotoindukowanemu procesowi przeniesienia elektronu nie towarzyszy rotacja grupy dimetyloaminowej. Podobnie jak w przypadku molekuly **III** dopiero bardzo duże stężenie nośników CD i SCA[6] ($c_{CD} > 5 \cdot 10^{-3} \text{ M}$, $c_{SC[6]} > 10^{-2} \text{ M}$) powoduje zauważalny wzrost intensywności pasma TICT, który wynika z faktu zainkludowania cząsteczki „gościa” do wnęki „gospodarza”, dzięki czemu istnieje możliwość rotacji grupy $-N(\text{CH}_3)_2$. W przypadku układu **IV**-CB[7] dla całego użytego zakresu stężeń CB[7] nie zaobserwowano obecności pasma fluorescencji TICT. Powyższe zachowanie sugeruje, że geometria powstałego w stanie wzbudzonym kompleksu inkluzyjnego umożliwia zajście procesu ESIPT, przy jednoczesnym braku możliwości rotacji grupy dimetyloaminowej ze względu na wiązanie wodorowe pomiędzy $-N(\text{CH}_3)_2$ a wodą.

3.2 Pomiary czasowo-rozdzielcze pochodnych benzoesanu metylu w wodnych roztworach wybranych nośników supramolekularnych

Aby uzyskać informacje na temat dynamiki procesów wewnątrz i międzycząsteczkowych dla czterech pochodnych benzoesanu metylu w obecności związków makrocyklicznych zastosowano technikę rozdzielonej w czasie spektroskopii. W niniejszej rozprawie doktorskiej dokonano analizy czasowo-rozdzielczych widm emisyjnych oraz krzywych gaśnięcia fluorescencji badanych pochodnych benzoesanu metylu w roztworach

wodnych cyklodekstryn, kukurbit[7]uryli oraz sulfonowych kaliks[6]arenów. Czasy życia fluorescencji (τ_i) oraz współczynniki A_i opisujące udział procentowy składowej zaniku fluorescencji o czasie zaniku τ_i zestawiono w Tab. 4-8.

Krzywą gaśnięcia fluorescencji molekuł **I** i **II** w wodzie oraz roztworach wodnych α - i γ -cyklodekstryn można dość dobrze dopasować stosując funkcje dwuwykładnicze z krótszym składnikiem τ_1 około 50-80 ps i dłuższym wynoszącym $\tau_2 \sim 150$ -300 ps (Tab. 4 i 6). Dokładne wyjaśnienie pochodzenia wszystkich składowych wymagało odniesienia tych wyników do otrzymanych wcześniej przez Kashę i współpracowników [60-63] oraz Heldta i współpracowników [64-68] czasów życia fluorescencji omawianych związków znajdujących się w jednorodnych aprotycznych i protycznych środowiskach. Na podstawie wspomnianych prac można stwierdzić, że w środowisku wodnym występują dwa centra emitujące: wzbudzony kompleks z międzycząsteczkowym wiązaniem wodorowym z czasem zaniku τ_1 (wiązanie wodorowe między pochodnymi benzoesanu metylu a cząsteczkami wody (**I**-H₂O, **II**-H₂O) powoduje skrócenie czasu gaśnięcia fluorescencji) oraz wzbudzona cząsteczka niezwiązana wiązaniem wodorowym z molekułami wody (molekuła **I** lub **II**).

Z danych zestawionych w Tab. 4 i 6 jednoznacznie wynika, że wraz ze wzrostem stężenia związku makrocyklicznego (α - i γ -CD) obserwuje się systematyczny spadek udziału procentowego składowej zaniku τ_1 z jednoczesnym wzrostem udziału procentowego składowej zaniku τ_2 . Taka prawidłowość sugeruje, że badane molekuly dostały się do wnętrza makrocyklicznego nośnika, które chroni pochodne benzoesanu metylu przed bezpośrednim kontaktem z cząsteczkami wody. Ograniczenie interakcji międzycząsteczkowych (**I**-H₂O, **II**-H₂O), zmniejsza liczbę kompleksów z wiązaniem wodorowym, czego przejawem jest zmniejszenie udziału procentowego A_1 . Należy zauważyć, że w sytuacji, kiedy stężenie γ -CD jest większe niż $c=10^{-2}$ M w gaśnięciu fluorescencji **II** pojawia się dodatkowo składowa τ_3 , której udział procentowy rośnie wraz ze wzrostem stężenia. Czas gaśnięcia τ_3 należy przypisać promienistej dezaktywacji molekuly **II**, która utworzyła kompleks inkluzyjny o stechiometrii 1:2 z γ -CD. Obecność kompleksów o stechiometrii 1:1 i 1:2 została potwierdzona na podstawie dalszych analiz (patrz rozdział 3.3).

		α-CD								
	Stężenie (10 ⁻⁴) [M]	τ ₁ [ps]	A ₁ [%]	τ ₂ [ps]	A ₂ [%]	τ ₃ [ps]	A ₃ [%]	τ ₄ [ps]	A ₄ [%]	χ ²
I	0	60	92	240	8					1.17
	5	70	84	185	16					1.24
	10	94	82	280	18					1.14
	50	66	74	300	26					1.22
	100	80	58	260	42					1.18
	200	70	40	305	60					1.14
	400	64	21	287	79					1.04
	500	82	12	284	88					1.17
II	0	34	88	120	12					1.11
	5	48	74	205	26					1.08
	10	32	68	148	32					1.21
	50	24	64	160	36					1.14
	100	62	44	180	52	420	4			1.16
	200	38	36	212	48	512	16			1.21
	400	72	24	148	51	447	25			1.18
	500	54	18	160	47	506	35			1.24
	Stężenie (10 ⁻⁴) [M]	<τ> [ps]		<A> [%]		τ ₃ [ps]	A ₃ [%]	τ ₄ [ps]	A ₄ [%]	χ ²
III*	10	110		43		450	47	3570	10	1.0
	25	140		51		470	36	3940	12	1.12
	50	170		33		500	38	4080	29	0.99
	100	430		25		400	65	4350	10	1.04
	500	270		25		630	16	4090	59	1.11
IV*	0	50		78		320	22			1.17
	5	70		66		410	34			1.24
	50	70		62		440	38			1.11
	500	70		33		400	32	3210	35	1.07

Tab. 4 Czasy życia fluorescencji pochodnych benzoesu metylu I, II, III i IV ($c = 5 \cdot 10^{-5}$ M) w roztworach wodnych zawierających różne stężenia α-CD.

- III* dane uzyskane z publikacji [82]
- IV* dane uzyskane z publikacji [83]

Niestety, ze względu na różny czas, w którym dokonywane były pomiary czasowo-rozdzielonych widm emisyjnych, rejestracja czasów zaniku fluorescencji z rozdzielczością czasową kilkunastu ps była tylko możliwa dla molekuly I i II w wodnych roztworach α- i γ-CD. Dla wszystkich późniejszych pomiarów (pochodne benzoesu metylu w wodzie oraz wodnych roztworach β-CD, CB[7] i SCA[6]) krzywą zaniku fluorescencji można było w zadowalający sposób dopasować stosując funkcje dwu- i trzykrotności, przy czym czasowa zdolność rozdzielcza wynosiła wówczas około 30-50 ps. W tej sytuacji występującą w gaśnięciu fluorescencji najkrótszą składową (około 100 ps) należy interpretować jako średni czas życia

następujących dwóch centrów luminescencyjnych: cząsteczki z międzycząsteczkowym wiązaniem wodorowym oraz molekuly swobodnej, niezwiązanej wiązaniem wodorowym.

Podsumowując wyniki badań z wykorzystaniem metod rozdzielonej w czasie spektroskopii określono wpływ obecności nośników makrocyklicznych na zjawisko fotoindukowanego wewnątrzcząsteczkowego przeniesienia protonu i elektronu. Najważniejsze wnioski i spostrzeżenia przedstawiono poniżej:

	β-CD							χ ²
	Stężenie (10 ⁻⁴) [M]	<τ> [ps]	<A> [%]	τ ₃ [ps]	A ₃ [%]	τ ₄ [ps]	A ₄ [%]	
I	0	100	100					1.11
	6	100	100					1.20
	40	120	100					1.05
	60	140	100					1.07
	80	180	100					1.12
	100	210	100					1.12
II	0	90	100					1.10
	6	130	100					1.09
	40	140	100					1.04
	60	130	84	730	16			1.15
	80	210	42	520	58			1.17
	100	210	30	550	70			1.13
IV*	0	50	78	320	22			1.17
	5	40	70	350	21			1.31
	50	40	75	320	25			1.15
	500	10	53	380	30	2920	17	1.05

Tab. 5 Czasy życia fluorescencji pochodnych benzoesu metylu I, II i IV ($c = 5 \cdot 10^{-5}$ M) w roztworach wodnych zawierających różne stężenia β-CD.

IV* dane uzyskane z publikacji [83]

- w przypadku molekuly II, będącej cząsteczką typu ESPIT, wzrost stężenia nośnika makrocyklicznego powoduje, że w zaniku fluorescencji pojawia się dodatkowa składowa, której udział procentowy rośnie wraz ze wzrostem stężenia nośnika. Co więcej, wartość tego czasu jest porównywalna z czasem życia stanu ESIPT molekuly II znajdującej się w jednorodnym, aprotycznym środowisku [60-68]. Wyniki te potwierdzają, że molekula II wnika do wnętrza nośnika (α-, β-, γ-CD, CB[7] i SCA[6]), a proces izolacji II od środowiska wodnego prowadzi do uaktywnienia procesu fotoindukowanego wewnątrzcząsteczkowego przeniesienia protonu w stanie wzbudzonym,

- dynamika fotoindukowanych procesów dla molekuly III, będącej związkiem typu TICT, różni się w zależności od rodzaju nośnika makrocyklicznego użytego do badań. Podobnie, jak to miało miejsce w przypadku molekuly II, wzrost stężenia nośnika powoduje pojawienie się nowej składowej τ_4 , której obecność w układach z α -CD i SCA[6] należy przypisać emisji ze stanu TICT. Takie przyporządkowanie wynika z faktu, iż czas życia stanu TICT molekuly III znajdującej się w rozpuszczalnikach aprotycznych jest porównywalny z czasem τ_4 [por. prace 60-68]. Jak już wzmiankowano zjawisko fotoindukowanego wewnątrzcząsteczkowego przeniesienia elektronu, któremu towarzyszy rotacja grupy $-N(CH_3)_2$ jest możliwe jedynie w sytuacji, gdy cząsteczka zainkludowała do wnętrza nośnika. Co więcej, dopasowanie geometryczno-sferyczne w takim układzie musi pozwalać na swobodny obrót grupy dimetyloaminowej. Jest to możliwe zarówno w sytuacji, gdy układ posiada stechiometrię 1:1 z grupą $-N(CH_3)_2$ znajdującą się całkowicie we wnętrzu nośnika lub kompleks inkluzyjny ma stechiometrię 1:2, gdzie cząsteczka jest w pełni uwięziona we wnętrzu dwóch nośników, co skutkuje pełną izolacją od środowiska wodnego,

		γ-CD								
	Stężenie (10 ⁻⁴) [M]	τ_1 [ps]	A ₁ [%]	τ_2 [ps]	A ₂ [%]	τ_3 [ps]	A ₃ [%]	τ_4 [ps]	A ₄ [%]	χ^2
I	0	54	88	180	12					1.27
	5	44	83	146	17					1.12
	10	80	74	220	26					1.21
	50	84	78	256	22					1.14
	100	62	66	184	30	120	4			1.16
	200	80	58	180	26	212	16			1.08
	400	58	32	200	43	147	25			1.21
	500	72	22	164	43	206	35			1.16
II	0	92	64	180	8					1.27
	5	84	58	225	16					1.12
	10	72	54	320	28					1.14
	50	71	46	268	29					1.21
	100	63	33	200	35	460	2			1.16
	200	48	28	358	40	520	12			1.13
	400	35	20	404	33	600	32			1.08
	500	24	34	380	28	580	38			1.19

Tab. 6 Czasy życia fluorescencji wybranych pochodnych benzoesanu metylu ($c = 5 \cdot 10^{-5}$ M) w roztworach wodnych zawierających różne stężenia γ -CD.

- w przypadku molekuly III nowo pojawiająca się w obecności CB[7] składowa τ_4 stanowi potwierdzenie utworzenia kompleksu w stanie wzbudzonym, przy czym wartość czasu

τ_4 (wyraźnie różna od τ_{TICT} w rozpuszczalnikach aprotycznych) sugeruje, iż w tym przypadku nie występuje całkowite wewnętrzcząsteczkowe przeniesienie ładunku, któremu towarzyszy skręcenie grupy $-N(CH_3)_2$ a jedynie ma miejsce częściowe przeniesienie ładunku. Taki proces możliwy jest w sytuacji gdy w nowo powstałym kompleksie grupa dimetyloaminowa pozostaje na zewnątrz nośnika i może specyficznie oddziaływać z cząsteczkami wody,

		CB[7]						
	Stężenie (10^{-4}) [M]	$\langle\tau\rangle$ [ps]	$\langle A \rangle$ [%]	τ_3 [ps]	A_3 [%]	τ_4 [ps]	A_4 [%]	χ^2
I	0	290	100					1.02
	1	260	100					1.18
	2	350	100					1.10
	4	330	100					1.12
	0	370	100					1.20
II	1	380	90	990	10			1.05
	2	360	86	630	14			1.10
	4	320	71	800	29			1.12
III	0	290	100					1.18
	1	270	100					1.21
	2	360	87			660	23	1.13
	4	660	71			860	29	1.19
IV	0	250	100					1.16
	1	300	96	1520	4			1.06
	2	230	36	1640	24	3850	42	1.19
	4	360	8	1480	26	3730	66	1.17

Tab. 7 Czasy życia fluorescencji pochodnych benzoesu metylu I, II, III, IV ($c=5 \cdot 10^{-5}$ M) w roztworach wodnych zawierających różne stężenia CB[7].

- kinetyka zaniku fluorescencji molekuly **IV** w obecności nośników makrocyclicznych CD, CB[7] i SCA[6] ma najbardziej złożony charakter. W obecności CD i SCA[6] o niewielkim stężeniu molekula **IV** emituje ze stanu lokalnie wzbudzonego oraz ESPIT. Dla najwyższych spośród użytych stężeń CD i SCA[6] (Tab. 4, 5 i 8) pojawia się dodatkowa ścieżka promienistej dezaktywacji związana z emisją ze stanu TICT. Rotacja grupy dimetyloaminowej jest możliwa dzięki utworzeniu kompleksów o stechiometrii 1:2 (**IV**-(CD)₂) (**IV**-(SCA[6])₂). W sytuacji, gdy molekula **IV** znajduje się w wodnych roztworach zawierających CB[7], krzywa zaniku fluorescencji posiada składową τ_3 , która charakteryzuje fluorescencję ze stanu ES IPT, oraz składową zaniku τ_4 przypisaną emisji ze stanu z częściowym przeniesieniem elektronu ICT.

		SCA[6]						
	Stężenie (10^{-4}) [M]	$\langle \tau \rangle$ [ps]	$\langle A \rangle$ [%]	τ_3 [ps]	A_3 [%]	τ_4 [ps]	A_4 [%]	χ^2
I	0	110	100					1.14
	1	120	100					1.07
	5	160	100					1.28
	10	170	100					1.07
	50	210	100					1.15
II	0	170	100					1.09
	1	180	37	850	63			1.03
	5	200	24	710	76			1.13
	10	220	5	550	95			1.25
	50	240	21	540	79			1.20
III	0	170	100					1.05
	1	180	100					1.09
	5	210	100					1.14
	10	230	5			2610	95	1.13
	50	250	8			2910	92	1.01
IV	0	170	100					1.12
	1	170	76	450	34			1.20
	5	220	44	670	56			1.05
	10	240	20	650	42	2350	38	1.10
	50	270	16	420	51	2310	33	1.21

Tab. 8 Czasy życia fluorescencji pochodnych benzoesu metylu I, II, III, IV ($c=5 \cdot 10^{-5}$ M) w roztworach wodnych zawierających różne stężenia SCA[6].

Analiza zależnych od czasu widm fluorescencji zarejestrowanych z wykorzystaniem kamery smugowej dostarczyła również bardzo cennych informacji na temat relaksacji cząsteczek ośrodka wokół badanego związku będącego w stanie wzbudzonym. Czasowa ewolucja położenia maksimum pasma fluorescencji została poddana analizie z wykorzystaniem funkcji korelacji rozpuszczalnika ($c(t)$) zaproponowanej przez *Maroncelliego* i *Fleminga* [84]. Ze względu na ograniczoną rozdzielczość czasową aparatury, wynoszącą około 50 ps, analizę przeprowadzono dla długofalowego pasma: ESIPT molekula II oraz TICT molekula IV. Dla cząsteczki II znajdującej się w obecności CB[7] i SCA[6] czasowa ewolucja funkcji $c(t)$ ma charakter jednowykładniczy z czasem relaksacji związanym z procesem solwatacji wynoszącym odpowiednio: $\tau_{\text{solv.}}(\text{CB}[7]) = 254$ ps oraz $\tau_{\text{solv.}}(\text{SCA}[6]) = 67$ ps. W przypadku układów IV-CB[7] oraz IV-SCA[6] czasowa ewolucja $c(t)$ ma odpowiednio charakter dwuwykładniczy ($\tau_{\text{solv.1}}(\text{CB}[7]) = 50$ ps oraz $\tau_{\text{solv.2}}(\text{CB}[7]) = 337$ ps) oraz jednowykładniczy $\tau_{\text{solv.}}(\text{SCA}[6]) = 70$ ps.

Otrzymane wyniki jednoznacznie wskazują na wyraźne wydłużenie czasu relaksacji związanego z procesem solwatacji w obecności badanych nośników w porównaniu z sytuacją,

gdy cząsteczka znajduje się w jednorodnym rozpuszczalniku o różnej polarności. Odnosząc otrzymane wyniki do wartości $\tau_{\text{solv}} < 1$ ps, którą otrzymał *Maroncelli* badając molekułę kumaryny 153 w wodzie, oraz innych jednorodnych rozpuszczalnikach o różnej polarności: etanolu, propanolu, butanolu, 2-propanolu [84] można stwierdzić, że w wodnych roztworach zawierających SCA[6] i CB[7] proces relaksacji solwatacyjnej jest co najmniej o dwa (SCA[6]) lub trzy (CB[7]) rzędy wolniejszy od sytuacji, gdy molekula luminezująca znajduje się w ośrodku jednorodnym. Powyższe prawidłowości można wytłumaczyć faktem zwiększenia liczby cząsteczek wody związanych z nośnikiem (utrata zdolności translacyjnych) w stosunku do liczby swobodnych molekuł H₂O [84].

3.3 Stechiometria kompleksów oraz stałe kompleksowania wyznaczone w oparciu o model Benesi-Hildebranda, Nigama i Durochera oraz Joba

Analiza danych doświadczalnych uzyskanych przy użyciu metod stacjonarnej i rozdzielonej w czasie spektroskopii pozwoliła na określenie stechiometrii i stałych tworzenia kompleksów inkluzyjnych, które powstały pomiędzy badanymi pochodnymi benzoesanu metylu a wybranymi nośnikami supramolekularnymi w stanie podstawowym i wzbudzonym. Do wyznaczenia stałych równowagowych i określenia stechiometrii badanych układów skorzystano z metod: *Benesi-Hildebranda* (B-H), regresji nieliniowej zaproponowanej przez *Nigama* i *Durochera* (N-D) oraz zależności *Joba* (J). Metody te pozwalają na wyznaczenie wartości stałych kompleksowania zarówno w przypadku, gdy utworzony został kompleks o stechiometrii 1:1 (K_1), jak i 1:2 (K_2) na podstawie zmian podstawowych charakterystyk luminescencyjnych (widma absorpcji i emisji, czasy życia fluorescencji) zależnych od stężenia nośnika. Należy zauważyć, że metoda B-H pozwala na wyznaczenie wartości stałych równowagowych, które są obarczone dużą niepewnością i dostarcza raczej szacunkowe niż dokładne wartości. Doskonałym narzędziem do właściwego określenia wartości K_1 i K_2 okazała się analiza danych pomiarowych w oparciu o metodę regresji nieliniowej (N-D) wykorzystującą wstępnie oszacowane parametry uzyskane przy wykorzystaniu metody *Benesi-Hildebranda*.

Zaobserwowane zmiany w charakterystykach luminescencyjnych czterech pochodnych benzoesanu metylu w obecności nośników makrocyclicznych (CDs, CB[7] i SCA[6]) pozwoliły

wyciągnąć ilościowe wnioski na temat stechiometrii oraz trwałości powstałych kompleksów. Na podstawie danych zestawionych w Tab. 9 można stwierdzić, że:

- wszystkie powstałe w stanie podstawowym kompleksy inkluzyjne (wyjątek stanowią układy III-SCA[6] oraz IV-SCA[6]) charakteryzują się stechiometrią 1:1. Należy przypomnieć, że oddziaływanie molekuł I i II z α -CD nie powoduje tworzenie stabilnego kompleksu w stanie podstawowym,
- spośród wszystkich badanych układów w stanie podstawowym największą komplementarnością, a co za tym idzie największą trwałością charakteryzują się układy z kukurbit[7]urydami, zaś najmniej stabilne są kompleksy z cyklodekstrynami. Tę samą prawidłowość obserwuje się dla kompleksów w stanie wzbudzonym,
- wszystkie badane układy mają znacznie większe powinowactwo wiązania cząsteczek „gościa” w stanie wzbudzonym niż w stanie podstawowym. Zachowanie to jest zrozumiałe, biorąc pod uwagę, że wartość elektrycznego momentu dipolowego badanych molekuł organicznych wzrasta w wyniku fotowzbudzenia ($\mu_e > \mu_g$),
- powstałe w stanie wzbudzonym kompleksy inkluzyjne I- α -CD, (I-IV)-CB[7] oraz I-II-SCA[6] posiadają stechiometrię 1:1, podczas gdy stechiometria 1:2 charakteryzuje układy: II- α -CD, (I-II)- β -CD, (I-II)- γ -CD oraz (III-IV)-SCA[6]. Stałe równowagowe we wzbudzonych układach z CB[7] są o 1-2 rzędy wielkości wyższe niż w dla kompleksów z α - i γ -CD. Jako, że wymiar wnęki hydrofobowej odgrywa istotną rolę w procesie tworzenia kompleksów, niewielkie wartości stałych K_1 i K_2 w układach z α - i γ -CD są zrozumiałe biorąc pod uwagę fakt, że z jednej strony zbyt mała wielkość wnęki α -CD uniemożliwia powstanie stabilnych kompleksów z pochodnymi benzoenu metylu, z drugiej strony, hydrofobowe wnętrze γ -CD jest zbyt duże w stosunku do rozmiaru „gościa”, co również skutkuje niską wzajemną komplementarnością cząsteczek.

Molekuły	Medium	$K_1^g[\text{M}^{-1}] / K_2^g[\text{M}^{-1}]$	$K_1^e[\text{M}^{-1}] / K_2^e[\text{M}^{-1}]$	
I	SCX[6]	640 ^a / -	660 ^a / -	
		530 ^b / -	550 ^b / -	
		300 ^d / -	680 ^c / -	
		490 ^e / -		
	CB[7]	1301 (± 3%) ^a	1642 (± 9%) ^a	
		1531 (± 7%) ^b	1626 (± 3%) ^b	
	α-CD	NSK ^a	15 (± 2) ^a	
		NSK ^b	14 (± 2) ^b	
	β-CD	329 ^a	687 ^a / 41 ^a	
		166 ^b	400 ^b / 53 ^b	
	γ-CD	32 (± 3) ^a	3 (± 0.8) ^a / 16 (± 2) ^a	
		38 (± 2) ^b	4.5 (± 0.5) ^b / 12 (± 1) ^b	
Trp	(2.1±0.1)×10 ⁴ ^a	(2.6±0.1)×10 ⁴ ^a		
BSA	(1.9±0.1)×10 ⁴ ^a	(2.1±0.1)×10 ⁴ ^a		
II	SCX[6]	610 ^a / -	930 ^a / -	
		630 ^b / -	1200 ^b / -	
		350 ^d / -	1440 ^c / -	
		480 ^e / -		
	CB[7]	1176 (± 17%) ^a	2153 (± 12%) ^a	
		840 (± 17%) ^b	4572 (± 21%) ^b	
	α-CD	NSK ^a	587 (± 63) ^a / 35 (± 5) ^a	
		NSK ^b	210 (± 20) ^b / 5 (± 2) ^b	
	β-CD	153 ^a	418 ^a / 25 ^a	
		89 ^b	564 ^b / 119 ^b	
	γ-CD	66 (± 7) ^a	187 (± 80) ^a / 47 (± 20) ^a	
		68 (± 6) ^b	379 (± 50) ^b / 15 (± 2) ^b	
Trp	(1.9±0.1)×10 ⁴ ^a	(2.6±0.1)×10 ⁴ ^a		
BSA	(2.3±0.1)×10 ⁴ ^a	(2.0±0.1)×10 ⁴ ^a		
III	SCX[6]	1250 ^a / 550 ^a	1040 ^a / 700 ^a	
		910 ^b / 450 ^b	1630 ^b / 1230 ^b	
		1500 ^d / 370 ^d	1150 ^c / 550 ^c	
		1090 ^e / 390 ^e		
	CB[7]	1053 (± 3%) ^a	1677 (± 15%) ^a	
		1647 (± 3%) ^b	1731 (± 9%) ^b	
	β-CD	-	106 ^f	
	IV	SCX[6]	1490 ^a / 550 ^a	1950 ^a / 1010 ^a
			1520 ^b / 500 ^b	2080 ^b / 1470 ^b
			1390 ^d / 620 ^d	1750 ^c / 310 ^c
			1720 ^e / 410 ^e	
		CB[7]	2250 (± 12%) ^a	3096 (± 9%) ^a
1925 (± 6%) ^b			4231 (± 16%) ^b	
α-CD		-	38 ^f / 52 ^f	
β-CD		-	326 ^f / 1520 ^f	

NSK – kompleks niestabilny

^a na podstawie danych otrzymanych z wykorzystanych metod spektroskopii stacjonarnej, stosując metodę Benesięgo-Hildebranda.

^b na podstawie danych z spektroskopii stacjonarnej przy użyciu metody regresji nieliniowej.

^c na podstawie danych z spektroskopii rozdzielonej w czasie, stosując metodę Benesiego-Hildebranda.

^d na podstawie danych z spektroskopii magnetycznego rezonansu jądrowego (¹H NMR), stosując metodę Benesiego-Hildebranda.

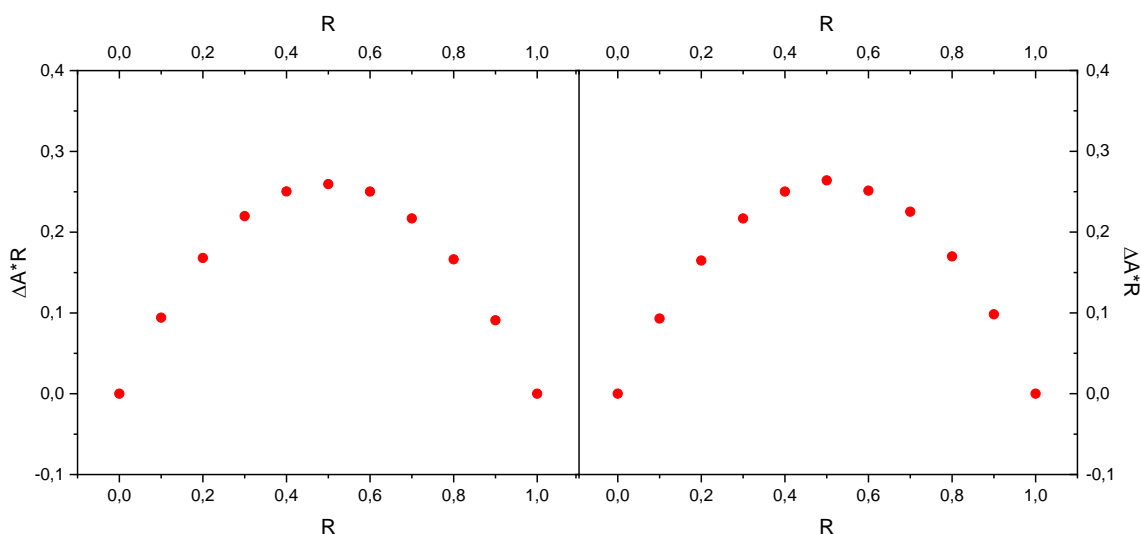
^e na podstawie danych spektroskopowych ¹H NMR przy użyciu regresji nieliniowej.

^f Z [82-83].

Tab. 9 Stałe równowagowe wybranych pochodnych benzoesanu metylu (I-IV) w roztworach wodnych w obecności cyklodekstryn (α , β i γ), kukurbit[7]uryli, sulfonowych kaliks[6]arenów (SCA[6]) oraz białka pochodzenia zwierzęcego (BSA) wyznaczone za pomocą metody Benesiego-Hildebranda i regresji nieliniowej.

- spośród trójki użytych do badań przedstawicieli najwyższe wartości stałych kompleksowania uzyskano dla CB[7]. Wynika z faktu najlepszego dopasowania geometryczno-sferycznego CB[7] z badanymi pochodnymi benzoesanu metylu $K_{CB[7]} > K_{SCA} > K_{CD}$. Co więcej, zbliżony rozmiar wnęk CB[7] i SCA[6] do β -CD potwierdza, że również dla tych układów powstają stabilne kompleksy,
- molekuly II oraz IV tworzą bardziej trwałe kompleksy inkluzyjne z CB[7] w stanie wzbudzonym niż I i III, co może sugerować, że pochodne hydroksylowe są głębiej osadzone we wnęce CB[7].

W celu potwierdzenia stechiometrii omawianych kompleksów inkluzyjnych zarówno w stanie podstawowym, jak i wzbudzonym, zastosowano metodę *Joba* zwaną również metodą zmian ciągłych. Metoda ta bazuje na analizie zmian wybranych charakterystyk luminescencyjnych (widma absorpcji, emisji, czasy życia fluorescencji) wywołanych miareczkowaniem nośnikiem makrocyclicznym, przy jednoczesnym zachowaniu stałej sumy stężeń molekuł „gościa” i „gospodarza”. Analiza przebiegu krzywej opisującej zmiany wartości absorbancji oraz intensywności fluorescencji w funkcji ułamka molowego nośnika (maksimum zlokalizowane przy ułamku molowym 0.5) potwierdza, że układy I-CB[7], I-SCA[6], II-CB[7], II-SCA[6], III-CB[7] oraz IV-CB[7] tworzą w stanie podstawowym kompleksy inkluzyjne o stechiometrii 1:1, podczas gdy dla układów III-SCA[6] i IV-SCA[6] maksimum w zależności *Joba* obserwowane jest przy 0.6 co potwierdza powstanie kompleksów inkluzyjnych o stechiometrii 1:2. Metodę *Joba* wykorzystano również do potwierdzenia stechiometrii kompleksów I- β -CD oraz II- β -CD. Na podstawie niepublikowanych dotąd badań eksperymentalnych (Rys.7) potwierdzono, że w stanie podstawowym powstają kompleksy inkluzyjne I- β -CD i II- β -CD o stechiometrii 1:1.

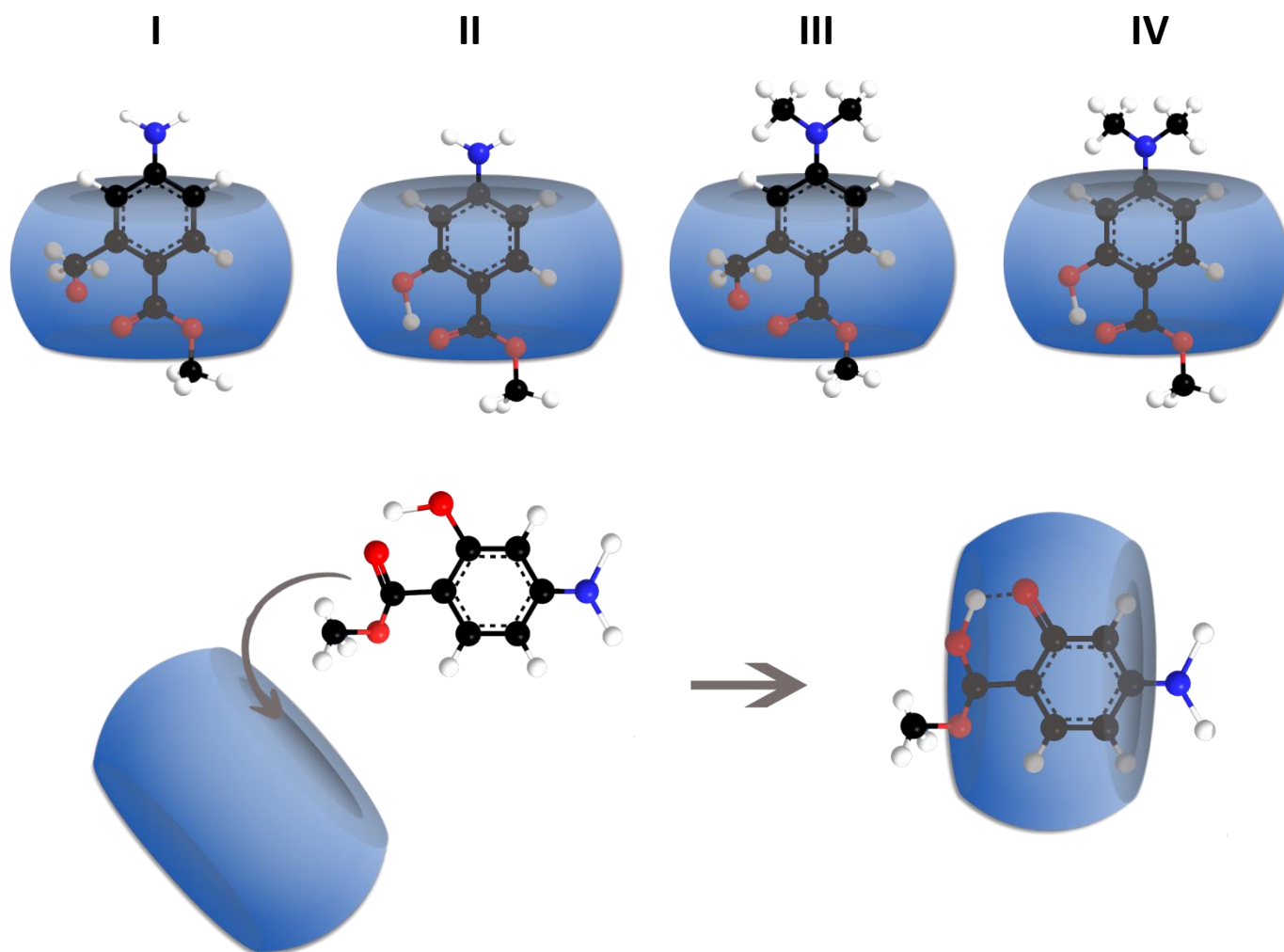


Rys. 7 Wykres Joba prezentujący zmiany absorbancji w funkcji ułamka molowego [β-CD] dla układów I-β-CD i II-β-CD.

3.4 Badania oddziaływań pochodnych benzoesanu metylu z kukurbit[7]urylami i sulfonowymi kaliks[6]arenami z wykorzystaniem techniki spektroskopii magnetycznego rezonansu jądrowego (^1H NMR)

Dotychczasowe badania jednoznacznie wykazały, że właściwości spektroskopowe molekuł, które utworzyły kompleksy z wybranymi związkami makrocyklicznymi istotnie różnią się o tych, jakie posiadają nieskompleksowane molekuły. W celu uzyskania szerszego spojrzenia na proces tworzenia kompleksów inkluzyjnych, w szczególności w celu potwierdzenia stechiometrii układów oraz ustalenia struktury geometryczno-przestrzennej powstałych kompleksów wykorzystano technikę magnetycznego rezonansu jądrowego (^1H NMR). Strukturę utworzonego kompleksu inkluzyjnego typu „gospodarz-gość” można określić na podstawie zmian przesunięć chemicznych protonów występujących w cząsteczkach badanych związków organicznych. W pierwszym etapie prac zbadano oddziaływanie pochodnych benzoesanu metylu z kukurbit[7]urylami. Zaobserwowane zmiany przesunięć chemicznych miały bardzo złożony charakter. Szczegółowa analiza tych przesunięć pozwoliła na zaprezentowanie schematu poglądowego powstałych struktur geometrycznych (Schemat 2). W przypadku molekuł posiadających grupę metoksyłową w położeniu orto (I i III) zauważono, że tylko część protonów szkieletu benzenowego jest osadzona wewnątrz nośnika makrocyklicznego (Schemat 2), podczas gdy fragment grupy metoksykarbonylowej ($-\text{COOCH}_3$) znajduje się na zewnątrz nośnika makrocyklicznego. W przypadku układów I-CB[7], III-CB[7],

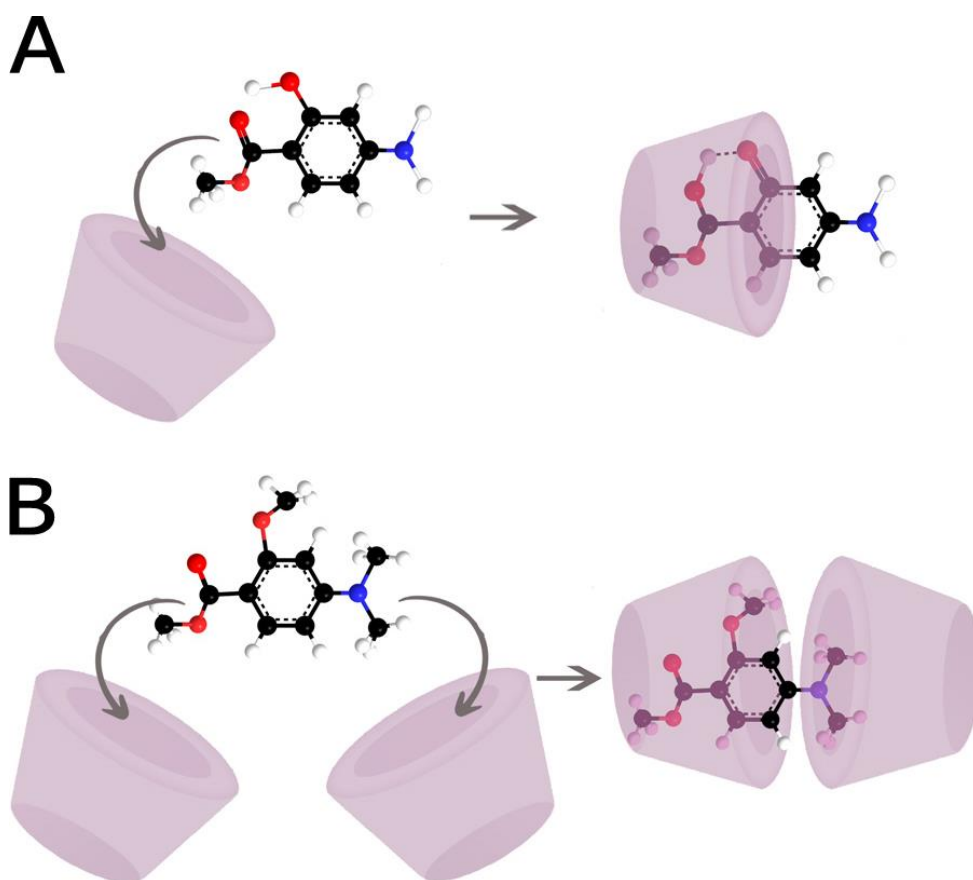
(molekuły posiadające grupę hydroksylową w pozycji orto) wszystkie protony pierścienia benzenowego znajdują się we wnętrzu CB[7] z fragmentem grupy $-\text{COOCH}_3$ usytuowanym poza wnęką nośnika. Podsumowując, można stwierdzić, iż badania z wykorzystaniem metod spektroskopii magnetycznego rezonansu jądrowego jednoznacznie potwierdziły, że badane pochodne benzoesu metylu zainkludowały do wnętrza kukurbit[7]uryli tworząc stabilne kompleksy inkluzyjne o stechiometrii 1:1.



Schemat 2 Schematycznie przedstawienie struktury geometryczno-przestrzennej kompleksów inkluzyjnych utworzonych między badanymi pochodnymi benzoesu metylu, a CB[7].

W celu określenia struktury geometryczno-przestrzennej kompleksów inkluzyjnych, które powstały między pochodnymi benzoesu metylu a sulfonowymi kaliks[6]arenami zastosowano również technikę spektroskopii ^1H NMR. Analiza danych doświadczalnych zaowocowała odnotowaniem bardzo interesujących prawidłowości:

- ze względu na strukturę przestrzenną powstałe kompleksy inkluzyjne można podzielić na dwie grupy: pochodne z podstawnikiem *metylaminowym* (I i II) oraz pochodne z podstawnikiem *dimetyloaminowym* (III i IV),
- pochodne I i II tworzą z sulfonowymi kaliks[6]arenami kompleksy inkluzyjne o stechiometrii 1:1 z częściowo zainkludowanym do wnętrza nośnika pierścieniem benzenowym (por. Schemat 3), podczas gdy w przypadku III-SCA[6] i IV-SCA[6] stechiometria układu wynosi 1:2, potwierdzając tym samym wcześniejsze obserwacje.



Schemat 3 Schematyczne przedstawienie struktury geometryczno-przestrzennej kompleksów inkluzyjnych utworzonych między pochodnymi benzoesanu metylu z podstawnikiem metylaminowym (I,II) (A) oraz dimetyloaminowym (III, IV) (B), a SCA[6].

Rozdział 4

Oddziaływanie pochodnej bifenyłu (EDMAADCy) z γ -cyklodestrydami

Jak już wzmiankowano jednym z celów pracy było określenie wpływu geometrii związku organicznego na proces tworzenia kompleksów inkluzyjnych z nośnikami makrocyklicznymi w stanie podstawowym i wzbudzonym. Do realizacji tego celu wykorzystano molekułę donorowo-akceptorową (5-(4-aminofenylo)-3-dimetyloamino-2,4-dicyjanobenzoetan etylu ((EDMAADCy)), która dzięki możliwości rotacji wokół pojedynczego wiązania między donorem i akceptorem tworzy już w stanie podstawowym układ spektralnie niejednorodny. Ze względu na rozmiary geometryczne molekuły luminezującej jako nośnik makrocykliczny wybrano γ -cyklodekstryny. Należy w tym miejscu wspomnieć, że badania z wykorzystaniem technik stacjonarnej i rozdzielonej w czasie spektroskopii były prowadzone w DMSO oraz mieszaninie dwóch rozpuszczalników DMSO–woda (4:1). Wybór takich środowisk był podyktowany słabą rozpuszczalnością EDMAADCy w czystej wodzie oraz faktem, iż jeden z rozpuszczalników dwuskładnikowej mieszaniny (H₂O) jest związkiem, który może tworzyć kompleksy z międzycząsteczkowym wiązaniem wodorowym z cząsteczką EDMAADCy, a co za tym idzie możliwe było zbadanie wpływu specyficznych oddziaływań molekuła luminezująca–protyczny rozpuszczalnik na proces tworzenia kompleksów. Wyżej wzmiankowanym zagadnieniom poświęcona jest praca:

A4 K. Baranowska, A. Bajorek, M. Pietrzak, M. Józefowicz, „Preferential encapsulation of different conformer of ethyl 5-(4-dimethylaminophenyl)-3-amino-2,4-dicyanobenzoate in γ -cyclodextrins”, Journal of Molecular Liquids 302 (2020) 112430, IF₂₀₂₀ = 6.17, MNISW = 100.

Jako, że badania oddziaływań pochodnej bifenyłu (EDMAADCy) z γ -cyklodestrydami były prowadzone w silnie polarnym DMSO oraz mieszaninie DMSO–woda zostały one poprzedzone określeniem właściwości spektroskopowych badanej molekuły D-A w tych środowiskach bez obecności nośnika makrocyklicznego. Badania spektroskopowe cząsteczki EDMAADCy w DMSO potwierdziły wcześniejsze ustalenia:

- w środowisku silnie polarnym obserwuje się pojedyncze, szerokie pasmo fluorescencyjne, którego kształt i położenie maksimum wyraźnie zależy od stężenia badanej molekuly oraz długości fali światła wzbudzającego,
- przy wzbudzeniu molekuly światłem o długości fali odpowiadającej maksimum widma absorpcji, obserwuje się pasmo emisyjne związane z fluorescencją konformeru gdzie donor i akceptor są ustawione prostopadle do siebie („*perpendicular form*” Schemat 4), podczas gdy wzbudzenie światłem o długościach fal z części antystokesowskiej widma absorpcji skutkuje selektywnym wzbudzeniem tych konformerów, dla których kąt pomiędzy D-A wynosi między 60° a 90° („*flattened form*”),
- położenie maksimum pasma emisji wykazuje istotną zależność od długości fali światła wzbudzającego tylko przy niskim stężeniu pochodnej bifenylu ($c < 5 \cdot 10^{-5}$ M). Wzrost stężenia **EDMAADCy** powoduje że układ staje się bardziej jednorodny spektroskopowo,
- zanik fluorescencji **EDMAADCy** w DMSO zarejestrowany dla różnych stężeń molekuly D-A ($5 \cdot 10^{-6}$ M $< c_{\text{EDMAADCy}} < 5 \cdot 10^{-4}$ M) jest dwuwykładniczy z pierwszą krótką składową τ_1 (1.92–2.92 ns) oraz drugą dłuższą τ_2 (5.92–7.14 ns). Udział procentowy każdej składowej zaniku silnie zależy od stężenia badanej molekuly i długości fali detekcji (470 nm i 510 nm). Ustalono, że czas gaśnięcia τ_1 należy przypisać emisji konformeru gdzie oba chromofory są ustawione w stosunku do siebie prostopadle, natomiast czas τ_2 odpowiada promienistej dezaktywacji formy „wypłaszczonej”. Wzrost stężenia **EDMAADCy** z $5 \cdot 10^{-6}$ M, do $5 \cdot 10^{-4}$ M powoduje, że udział procentowy składowej fluorescencji o czasie zaniku τ_1 maleje z 60% do 30%, przy jednoczesnym wzroście udziału drugiej składowej z 40% do 70% (Schemat 4).

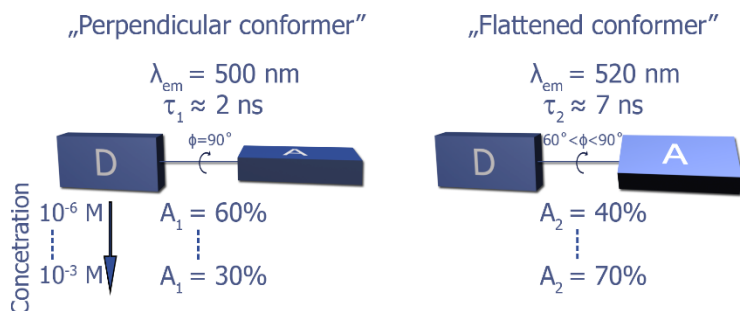
Uzupełnieniem powyższych badań było określenie właściwości fotofizycznych i fotochemicznych molekuly **EDMAADCy** w mieszaninie rozpuszczalników DMSO–woda (4:1), kładąc szczególny nacisk na zrozumienie wpływu międzycząsteczkowych wiązań wodorowych na podstawowe parametry spektroskopowe molekuly. Na podstawie analizy danych doświadczalnych (pomiar widm absorpcji, emisji, czasów gaśnięcia fluorescencji) oraz obliczeń kwantowo-chemicznych stwierdzono, że:

- promienista dezaktywacja elektronowo wzbudzonej molekuly **EDMAADCy** w dwuskładniowej mieszaninie rozpuszczalników DMSO–woda związana jest z emisją

lokalnie wzbudzonej cząsteczki, której konformacja charakteryzuje się wzajemną prostopadłością grupy donorowej i akceptorowej, emisją konformeru powstałego w stanie wzbudzonym w wyniku wewnątrzcząsteczkowego przeniesienia elektronu, któremu towarzyszy zmiana geometrii badanej molekuly oraz kompleksów z międzycząsteczkowym wiązaniem wodorowym.

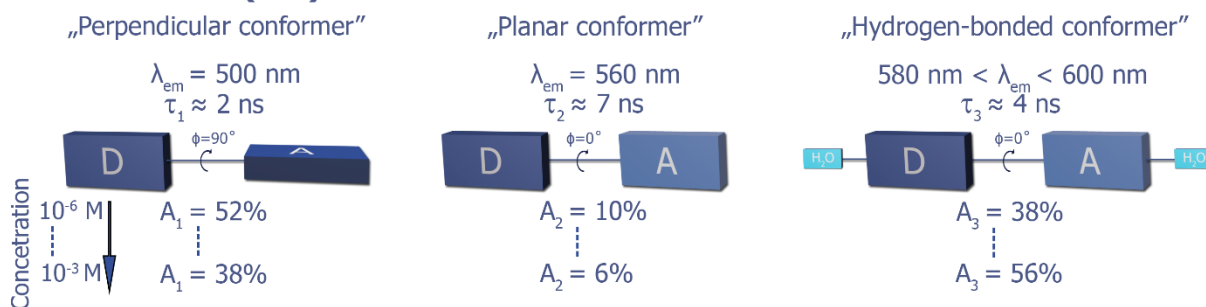
A

DMSO



B

DMSO-WATER (4:1)



Schemat 4 Schematyczne przedstawienie wpływu stężenia EDMAADCy na strukturę przestrzenną konformerów w roztworach DMSO (A) i DMSO–woda (B) [A4].

4.1 Podstawowe charakterystyki luminescencyjne molekuly EDMAADCy w roztworach DMSO w obecności γ -CD

Miareczkowanie znajdującej się w DMSO molekuly EDMAADCy nośnikiem makrocyclicznym skutkuje niewielkimi zmianami kształtu i położenia widm absorpcyjnych i emisyjnych. Zauważono subtelne zmiany zarówno intensywności pasma emisji, jak i niewielkie przesunięcie położenia maksimum w kierunku fal krótkich wywołane obecnością nośnika. Przesunięcie hipsochromowe wraz z obecnością punktów izobestycznych sugeruje, że

nastąpiła zmiana polarności środowiska, w którym znajduje się badana molekula w efekcie utworzenia kompleksu inkluzyjnego **EDMAADCy**- γ -CD.

$C_{[\gamma\text{-CD}]} [\text{M}]$	$\tau_1 [\text{ns}]$	$A_1 [\%]$	$\tau_2 [\text{ns}]$	$A_2 [\%]$	χ^2
0	2.76	64	7.34	36	1.31
$2.5 \cdot 10^{-4}$	2.63	63	7.61	37	1.06
$5 \cdot 10^{-4}$	2.54	61	7.01	39	1.11
10^{-3}	2.88	60	7.44	40	1.14
$2.5 \cdot 10^{-3}$	3.02	59	6.84	41	1.23
$5 \cdot 10^{-3}$	2.66	57	7.32	43	1.17

Tab. 10 Czasy życia fluorescencji **EDMAADCy** ($c=10^{-4}$ M) w roztworach DMSO zawierających różne stężenia γ -CD.

W celu określenia wpływu budowy przestrzennej konformerów na proces tworzenia kompleksów inkluzyjnych pomocne okazały się wyniki analiz krzywych zaniku fluorescencji otrzymane dla różnych stężeń γ -CD. Zanik fluorescencji badanej cząsteczki D-A w roztworze DMSO zawierającym różne stężenia γ -CD można w zadawalający sposób dopasować stosując funkcje dwuwykładnicze (Tab. 10) z krótszą składową zaniku τ_1 (~ 3 ns), której obecność należy przypisać fluorescencji „prostopadłej” formy **EDMAADCy** ($\phi=90^\circ$), oraz dłuższą składową τ_2 (~ 7 ns) związaną z promienistą dezaktywacją „wypłaszczonej formy” **EDMAADCy** ($60^\circ < \phi < 90^\circ$). Wyraźne różnice w udziałach procentowych poszczególnych składowych wywołane wzrostem stężenia γ -CD (udział A_1 maleje a A_2 wzrasta) świadczą o tym, że to konformery, gdzie chromofory nie są ustawione do siebie prostopadłe inkludują do wnęki cyklodekstryny tworząc kompleksy inkluzyjne.

4.2 Podstawowe charakterystyki luminescencyjne molekuly **EDMAADCy** w mieszaninie DMSO–woda w obecności γ -CD

Jak już wspomniano w celu określenia wpływu międzycząsteczkowych wiązań wodorowych na proces tworzenia kompleksów inkluzyjnych, przeprowadzono badania spektroskopowe molekuly **EDMAADCy** w roztworach DMSO–woda o różnym stężeniu γ -cyklodekstryn. Już pobieżna analiza wykazuje, że stacjonarne widma emisji ulegają bardziej wyraźnym zmianom po dodaniu γ -CD do roztworu DMSO–woda niż w przypadku czystego DMSO. Największą, ze spektroskopowego punktu widzenia, różnicą jest ta związana z ilością obserwowanych pasm fluorescencyjnych. W mieszaninie rozpuszczalników DMSO–woda cząsteczka **EDMAADCy** posiada dwa, wyraźne pasma emisyjne. Pierwsze, krótkofalowe pasmo przypisano fluorescencji ze stanu lokalnie wzbudzonego (LE) ($\phi=90^\circ$), natomiast pasmo po

stronie długofalowej związane jest z emisją konformeru, który powstał w stanie wzbudzonym w wyniku wewnątrzcząsteczkowego przeniesienia elektronu, dla którego kąt między chromoforami jest bliski 0. Zauważono, że wraz ze wzrostem stężenia nośnika makrocyklicznego intensywność długofalowego pasma emisji zwiększa się, podczas gdy intensywność pasma krótkofalowego pozostaje praktycznie niezmienną.

$C_{[\gamma\text{-CD}]} [\text{M}]$	$\lambda_{\text{obs}} [\text{nm}]$	$\tau_1 [\text{ns}]$	$A_1 [\%]$	$\tau_2 [\text{ns}]$	$A_2 [\%]$	$\tau_3 [\text{ns}]$	$A_3 [\%]$	χ^2
$c=10^{-4} \text{ M}$								
0	500	2.26	48	6.25	9	4.78	43	1.13
	560	0.88	32	7.56	8	3.68	60	1.21
$5 \cdot 10^{-4}$	500	2.48	46	6.64	14	4.55	40	1.07
	560	1.04	30	7.15	14	3.95	56	1.11
10^{-3}	500	2.72	45	6.61	17	4.98	38	1.13
	560	1.21	30	7.59	24	3.37	46	1.27
$2.5 \cdot 10^{-3}$	500	2.91	46	6.15	24	4.65	30	1.31
	560	1.19	34	7.74	26	3.89	40	1.06
$5 \cdot 10^{-3}$	500	2.95	45	6.26	30	4.12	25	1.14
	560	1.26	30	7.89	34	3.33	36	1.19
$c=2.5 \cdot 10^{-4} \text{ M}$								
0	500	1.98	41	6.29	6	4.26	53	1.21
	560	0.81	31	7.95	8	3.56	61	1.06
$5 \cdot 10^{-4}$	500	2.32	38	6.78	12	4.78	50	1.13
	560	0.98	32	7.21	13	3.36	55	1.17
10^{-3}	500	2.56	35	6.54	21	4.54	44	1.34
	560	1.15	30	6.99	19	3.89	51	1.21
$2.5 \cdot 10^{-3}$	500	2.54	32	7.06	28	4.25	40	1.17
	560	1.21	29	7.21	25	3.87	46	1.06
$5 \cdot 10^{-3}$	500	2.38	38	6.45	34	4.39	28	1.19
	560	1.17	34	7.78	38	3.35	28	1.23
$c=5 \cdot 10^{-4} \text{ M}$								
0	500	1.94	36	6.48	7	4.56	57	1.04
	560	0.74	30	6.85	5	3.35	65	1.19
$5 \cdot 10^{-4}$	500	2.16	30	6.45	17	4.59	53	1.16
	560	0.89	27	7.51	13	3.40	60	1.08
10^{-3}	500	2.81	28	6.15	20	4.44	52	1.09
	560	1.21	28	7.18	26	3.25	56	1.24
$2.5 \cdot 10^{-3}$	500	2.89	32	6.18	28	4.25	40	1.17
	560	1.17	32	7.77	24	3.32	44	1.11
$5 \cdot 10^{-3}$	500	2.38	28	6.54	34	4.74	38	1.19
	560	1.59	25	7.23	38	3.78	37	1.07

Tab. 11 Czasy życia fluorescencji EDMAADCy ($c=10^{-4} \text{ M}$, $2,5 \cdot 10^{-4} \text{ M}$, 10^{-4} M) w roztworach DMSO–woda (4:1) zawierających różne stężenia $\gamma\text{-CD}$.

Zanik fluorescencji EDMAADCy zarejestrowany w roztworach DMSO–woda o różnym stężeniu $\gamma\text{-CD}$ można dopasować za pomocą funkcji trójwykładniczych. Trzy centra

fluorescencyjne i odpowiadające im składowe zaniku są związane z promienistą dezaktywacją konformeru charakteryzującego się wzajemną prostopadłością obu chromoforów (τ_1), „wypłaszczonej” formy **EDMAADCy** (τ_2) oraz kompleksu z międzycząsteczkowym wiązaniem wodorowym (τ_3). Z analizy otrzymanych danych eksperymentalnych (Tab. 11) jasno wynika, że wzrost stężenia γ -CD nie powoduje znaczących zmian w wartościach czasów życia fluorescencji (τ_1 , τ_2 , τ_3) natomiast obserwuje się wyraźne zmiany w udziałach procentowych poszczególnych składników. Brak zmiany wartości współczynnika A_1 wskazuje, że konformery charakteryzujące się wzajemną prostopadłością chromoforów nie inkludują do wnętrza nośnika. Wzrost wartości udziału procentowego drugiej składowej (A_2) wywołany wzrostem stężenia γ -CD, potwierdza, że forma planarna **EDMAADCy** oddziałuje z γ -CD tworząc kompleks inkluzyjny. Zaobserwowane zmiany w wartościach współczynnika A_3 wywołane wzrostem stężenia γ -CD (A_3 maleje w funkcji $[C_{\gamma\text{-CD}}]$) są zrozumiałe, gdyż wiązanie wodorowe jest rozrywane podczas wnikania **EDMAADCy** do wnętrza nośnika makrocyclicznego.

4.3 Stechiometria kompleksów inkluzyjnych **EDMAADCy**- γ -CD w roztworze DMSO i mieszaninie DMSO–woda

W celu ilościowego opisu oddziaływań między molekułą **EDMAADCy** a γ -cyklodekstrynami wykorzystano model *Benesi–Hildebranda* do określenia stechiometrii oraz stałych kompleksowania badanego układu. Analiza wartości parametrów K_1 i K_2 prowadzi do następujących wniosków:

- w przypadku molekuły **EDMAADCy** w obu badanych środowiskach w obecności γ -CD powstają w stanie wzbudzonym stabilne kompleksy o stechiometrii 1:1,
- otrzymane wartości K_1 w środowisku DMSO są o rząd wielkości większe niż stałe kompleksowania wyznaczone dla ośrodka DMSO–woda, co oznacza, że powstałe w stanie wzbudzonym kompleksy są bardziej trwałe w pierwszym z badanych układów,
- wartości stałych równowagowych zależą od długości fali światła wzbudzającego, tj. zmiana długości fali światła wzbudzającego z 360 nm na 400 nm powoduje wzrost wartości stałych równowagowych K_1 zarówno w czystym DMSO, jak i w mieszaninie DMSO–woda. To zachowanie jest zrozumiałe biorąc pod uwagę fakt, że rozkład przestrzenny konformerów jest inny w dwóch badanych układach (Tab. 12),

λ_{exc} [nm]	DMSO	DMSO-H ₂ O
	K_1 [M ⁻¹]	K_1 [M ⁻¹]
	c=5·10⁻⁴ M	
360	646	54
370	757	171
380	1075	217
390	1798	155
400	2121	179
	c=2.5·10⁻⁴ M	
360	362	60
370	493	156
380	521	164
390	587	179
400	1626	154
	c=10⁻⁴ M	
360	243	30
370	314	64
380	497	93
390	652	97
400	970	115

Tab. 12 Stałe równowagowe kompleksów EDMAADCy- γ -CD w stanie wzbudzonym wyznaczone na podstawie zależności *Benesi-Hildebranda* dla różnych długości fali światła wzbudzającego w środowisku DMSO i DMSO-woda w obecności γ -CD.

- stężenie molekuly **EDMAADCy** ma istotny wpływ na wartość stałej K_1 . Zmiany tej wartości wynikają z faktu, że wzrost stężenia **EDMAADCy** powoduje, że w zbiorze konforemerów o różnym kącie między D i A udział tych „wypłaszczonych” staje się coraz bardziej dominujący („*concentration-induced planarization*”). Należy również pamiętać, iż wcześniejsze badania jednoznacznie wykazały, że utworzenie kompleksu z konformerem „wypłaszczonym” jest bardziej prawdopodobne niż z cząsteczką, w której oba chromofory są ustawione prostopadłe względem siebie.

Rozdział 5

Oddziaływanie pochodnych benzoesu metylu z albuminą surowicy bydlęcej (BSA)

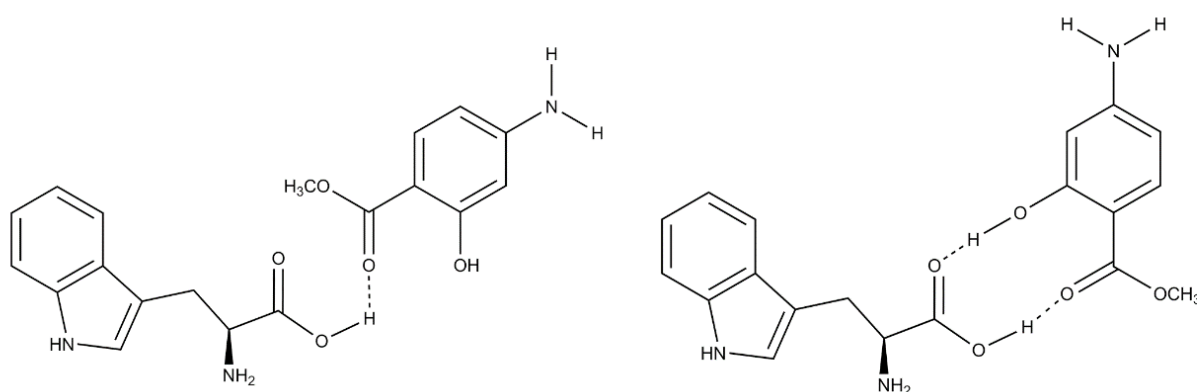
Podkreślono już wcześniej, że badanie oddziaływań leków z różnymi biomolekułami cieszy się niesłabnącym zainteresowaniem wśród wielu grup farmaceutów, biochemików czy medyków [1,3,15]. Poznanie i zrozumienie procesów oddziaływania lek–biomolekuła jest niezmiernie ważne z punktu widzenia odpowiedniej skuteczności podanego medykamentu. Badania eksperymentalne i teoretyczne wykazały, że albuminy pochodzenia zwierzęcego m.in. albumina wołowa są najczęściej stosowane, jako białka modelowe w ocenie interakcji z mniejszymi cząsteczkami organicznymi [47-49]. W celu pogłębienia wiedzy na temat natury i mechanizmów interakcji pomiędzy związkami organicznymi a białkami w niniejszej rozprawie doktorskiej skupiono się na wyjaśnieniu mechanizmów oddziaływań pomiędzy dwoma pochodnymi benzoesu metylu (I i II) a BSA. Realizacja powyższych celów wymagała zastosowania metod stacjonarnej i czasowo-rozdzielczej spektroskopii. Otrzymane wyniki zostały przedstawione w publikacji naukowej:

A5 K. Baranowska, M. Mońka, P. Bojarski, M. Józefowicz, *Insight into Molecular Interactions of Two Methyl Benzoate Derivatives with Bovine Serum Albumin*, International Journal of Molecular Sciences 22 (2021) 11705, IF₂₀₂₁ = 6.208, MNISW = 140.

4.1 Widma absorpcji i emisji oraz czasy życia fluorescencji układów dwuskładnikowych I-Trp, II-Trp, I-Tyr, II-Tyr, I-Phe i II-Phe

Biorąc pod uwagę fakt, że głównymi składnikami wnęk hydrofobowych albumin są reszty aminokwasowe takie jak: tryptofan (Trp), tyrozyna (Tyr) oraz fenyloalanina (Phe) oraz to, że białka zawdzięczają swoje właściwości fluorescencyjne obecność właśnie tych aminokwasów, przed przystąpieniem do właściwych badań oddziaływań pochodnych benzoesu metylu z białkiem BSA, w pierwszym etapie skupiono się nad badaniami mechanizmów odpowiedzialnych za oddziaływanie dwóch badanych związków organicznych z trzema kluczowymi aminokwasami.

Zmierzone widma absorpcji i emisji molekuł **I** i **II** w roztworach wodnych (bufor fosforanowy pH=7.4) istotnie zależą od stężenia tryptofanu, podczas gdy w przypadku układów **I-Tyr**, **II-Tyr**, **I-Phe** i **II-Phe** miareczkowanie aminokwasem nie powoduje wyraźnych zmian w stacjonarnych widmach absorpcji i emisji (brak utworzenia stabilnych kompleksów z tymi aminokwasami). Analiza zmian molowego współczynnika absorpcji oraz intensywności fluorescencji w oparciu o model *Benesi–Hildebranda*, sugeruje, że dla układów **I-Trp** i **II-Trp** zarówno w stanie podstawowym, jak i wzbudzonym tworzą się kompleksy z międzycząsteczkowym wiązaniem wodorowym o stechiometrii 1:1. Obecność punktów izobestycznych w widmach absorpcji i emisji jest również potwierdzeniem utworzenia takich kompleksów. Warto w tym miejscu podkreślić, że wzrost stężenia Trp powoduje zmniejszenie intensywności pasma emisyjnego ze stanu ESIPT dla molekuły **II**, co wskazuje na to, że podczas oddziaływania **II** z Trp istotną rolę odgrywają podstawniki -OH i/lub -COOCH₃ (Schemat 5).



Schemat 5 Możliwe struktury kompleksów utworzonych w wyniku oddziaływania tryptofanu z molekułą **II**.

Uzupełnieniem i pomocą w opisie mechanizmów odpowiedzialnych za oddziaływanie **I-Trp** i **II-Trp** były pomiary czasowo-rozdzielcze. Główne wnioski, które otrzymano na podstawie analizy czasów gaśnięcia fluorescencji **I** i **II** w roztworach wodnych (bufor fosforanowy pH=7.4) o różnym stężeniu Trp (Tab. 13):

- w przypadku molekuły **I** znajdującej się w czystym roztworze wodnym zanik emisji jest jednowykładniczy (τ_1 -emisja ze stanu LE) podczas gdy dla cząsteczki **II** obserwuje się zanik dwuwykładniczy (τ_1 -emisja ze stanu LE, τ_2 -emisja ze stanu ESIPT),
- wzrost stężenia Trp powoduje, że wartości współczynników A_1 i A_2 , opisujące udział procentowy składowej zaniku emisji LE (**I** i **II**) i ESIPT (**II**) maleją, z jednoczesnym

pojawieniem się w zaniku fluorescencji dodatkowej składowej τ_3 , której udział rośnie wraz ze wzrostem stężenia Trp. Powyższe zachowanie sugeruje, że czas zaniku τ_3 należy przypisać promienistej dezaktywacji kompleksu, który powstał pomiędzy pochodną benzoesanu metylu a tryptofanem (Tab. 13).

Molekuły	Stężenie Trp 10^{-5} [M]	τ_1 [ns]	A_1 [%]	τ_2 [ns]	A_2 [%]	τ_3 [ns]	A_3 [%]	r^2
I	0	0.19	100					0.97
	0.4	0.14	12			3.45	88	0.99
	0.8	0.12	6			3.34	94	0.99
	1.2	0.12	5			3.5	95	0.99
	1.6	0.13	3			3.5	97	0.99
	2.0	0.14	2			3.5	98	0.99
II	0	0.20	24	0.80	76			0.95
	0.4	0.13	4	0.65	15	3.46	81	0.98
	0.8	0.11	3	0.87	7	3.71	90	0.99
	1.2	0.16	1	0.61	3	3.49	96	0.96
	1.6					2.90	100	0.98
	2.0					2.98	100	0.98

Tab. 13 Czasy życia fluorescencji I i II w roztworach wodnych (bufor fosforanowy pH=7.4) o różnym stężeniu tryptofanu.

4.2 Widma absorpcji i emisji oraz czasy życia fluorescencji molekuł I i II w roztworach wodnych (bufor fosforanowy pH=7.4) o różnym stężeniu BSA

Po zapoznaniu się z podstawowymi charakterystykami spektroskopowymi układów dwuskładnikowych w przypadku których jednym ze składników była cząsteczka aminokwasu, który znajduje się w „kieszeniach” hydrofobowych BSA, w kolejnym etapie skupiono się na wyjaśnieniu na poziomie molekularnym międzycząsteczkowych oddziaływań między dwoma pochodnymi benzoesanu metylu a białkiem pochodzenia zwierzęcego (BSA). Wyniki uzyskane z miareczkowania spektrofotometrycznego i fluorometrycznego wykazały, że zarówno w stanie podstawowym, jak i wzbudzonym w wyniku oddziaływania międzymolekularnego tworzy się kompleks między I i BSA oraz II i BSA. Analiza ilościowa danych spektroskopowych wykorzystująca model *Benesi–Hildebranda* wykazała, że podobnie, jak to miało miejsce w przypadku układów I-Trp, II-Trp, powstałe kompleksy charakteryzują się stechiometrią 1:1. Warto również w tym miejscu zauważyć, że w przypadku molekuły II wzrost stężenia BSA powoduje stopniowy spadek intensywności pasma fluorescencji ze stanu ESIPT. Zachowanie takie sugeruje, że ma miejsce specyficzne oddziaływanie między II i BSA, w które

zaangażowane są grupy -OH i/lub -COOCH₃ molekuły **II** (brak możliwości zajęcia procesu fotoindukowanego wewnątrzcząsteczkowego przeniesienia protonu).

Aby uzyskać informację na temat dynamiki procesów zachodzących w badanych układach w stanie wzbudzonym zmierzono widma czasowo-rozdzielcze oraz dokonano analizy krzywych zaniku fluorescencji. Krzywe zaniku fluorescencji badanych pochodnych benzoesanu metylu w obecności BSA wykazują pewne podobieństwa do krzywych otrzymanych w układach **I-Trp** i **II-Trp**, mianowicie:

- w przypadku molekuły **I** w obecności BSA zanik emisji jest dwuwykładniczy, podczas gdy dla układu **II-BSA** jest on bardziej skomplikowany i krzywą zaniku można dobrze dopasować stosując funkcję trójwykładniczą,
- zmiany jakie zauważono w udziałach procentowych poszczególnych składowych wywołane wzrostem stężenia BSA t.j. spadek względnego udziału procentowego składowych zaniku czasów τ_1 i τ_2 z jednoczesnym wzrostem współczynnika A_3 pozwalają na następujące wyjaśnienie pochodzenia wszystkich składowych. Pierwsza składowa τ_1 związana jest z emisją molekuł **I** i **II** ze stanu lokalnie wzbudzonego (LE), drugą składową τ_2 można przypisać emisji ze stanu ESIPT (molekuła **II**). Ostatnia składowa (τ_3) odpowiada emisji kompleksów inkluzyjnych **I-BSA** i **II-BSA** (Tab. 14).

Badania czasowo-rozdzielcze pozwoliły również na poszerzenie wiedzy na temat procesów dynamiki solwatacji wybranych pochodnych benzoesanu metylu w wodnych roztworach zawierających cząsteczki BSA. Jak już wspomniano w rozdziale 3, relaksacja solwatacyjna w jednorodnych rozpuszczalnikach zachodzi w skali subpikosekundowej, podczas gdy ulega ona wyraźnemu spowolnieniu, gdy badana cząsteczka zostanie „uwięziona” we wnęce makrocyklicznego nośnika. W celu określenia czasu relaksacji solwatacyjnej i porównania dynamiki solwatacji w obecności wybranych nośników chemicznych (kukurbit[7]uryle i sulfonowe kaliks[6]areny) z układami, w skład których wchodzi albuminy BSA przeanalizowano czasową ewolucję położenia maksimum pasma fluorescencji. Na podstawie procedury zaproponowanej przez *Maroncelliego* i *Fleminga* dla obu badanych molekuł skonstruowano funkcję $c(t)$, której zanik można dobrze dopasować stosując funkcje dwuwykładnicze (szybka składowa $\tau_1(\text{solv.})$ (**I**) = 430 ps (2%), $\tau_1(\text{solv.})$ (**II**) = 520 ps (3%) i wolna składowa $\tau_2(\text{solv.})$ (**I**) = 4.07 ns (98%), $\tau_2(\text{solv.})$ (**II**) = 5.25 ps (97%)). Wyznaczono również średni

czas relaksacji solwatacyjnej: $\langle \tau(\text{solv.}) \text{ (I)} \rangle = 4.06 \text{ ns}$, $\langle \tau(\text{solv.}) \text{ (II)} \rangle = 5.24 \text{ ns}$. Analiza uzyskanych wyników wyraźnie wskazuje, że proces solwatacji wybranych pochodnych benzoesu metylu w obecności nośników chemicznych jest znacznie różny od sytuacji, gdy w sąsiedztwie molekuly luminezującej znajduje się albumina. Stwierdzono, że średni czas relaksacji solwatacyjnej w układzie molekula-białko jest o około rząd wielkości dłuższy niż w układzie z nośnikami makrocyclicznymi (CB[7] i SCA[6]). Na podstawie otrzymanych wyników można wnioskować, że cząsteczka wody jest „silniej” uwięziona wewnątrz hydrofobowych wnęk BSA, niż w we wnęce CB[7] czy SCA[6], co skutkuje wydłużeniem czasu solwatacji.

Molekuły	Stężenie BSA 10^{-5} [M]	$\tau_1 \text{ [ns]}$	$A_1 \text{ [%]}$	$\tau_2 \text{ [ns]}$	$A_2 \text{ [%]}$	$\tau_3 \text{ [ns]}$	$A_3 \text{ [%]}$	r^2
I	0	0.19	100					0.98
	0.1	0.20	7			4.49	93	0.99
	0.2	0.30	3			5.36	97	0.99
	0.4	0.30	2			5.18	98	0.99
	0.6	0.32	1			5.57	99	0.99
	0.8					5.51	100	0.99
	1.4					5.69	100	0.99
	2.0					5.78	100	0.99
II	0	0.20	24	0.80	76			0.95
	0.1	0.21	14	1.15	15	5.79	71	0.99
	0.2	0.19	5	1.19	17	5.56	78	0.99
	0.4	0.19	2	1.00	9	5.71	89	0.99
	0.6	0.18	1	1.00	6	5.23	93	0.99
	0.8					5.58	100	0.99
	1.4					5.52	100	0.99
	2.0					5.84	100	0.99

Tab. 14 Czasy życia fluorescencji I i II w roztworach wodnych (bufor fosforanowy pH=7.4) o różnym stężeniu BSA.

4.3 Mechanizm wygaszania fluorescencji

Bardzo użyteczną metodą badania oddziaływań białek ze związkami organicznymi jest metoda oparta na pomiarach wygaszania fluorescencji białek w wyniku interakcji z molekułą organiczną. W celu określenia charakteru wygaszania fluorescencji BSA przez molekuly I i II (statyczny bądź dynamiczny) oraz wyznaczenia stałej K_{SV} (*Sterna-Volmera*), przeprowadzono analizę wyników spektroskopowych otrzymanych przy użyciu stacjonarnej i rozdzielonej w czasie spektroskopii emisyjnej posługując się równaniem *Sterna-Volmera*. Na podstawie analizy danych spektroskopowych otrzymanych w różnych temperaturach (wartość stałej K_{SV} maleje wraz ze wzrostem temperatury) stwierdzono, że proces wygaszania ma charakter

statyczny oraz we wszystkich temperaturach powstają kompleksy o stechiometrii 1:1. W celu potwierdzenia statycznego charakteru mechanizmu wygaszania fluorescencji BSA wywołanego obecnością molekuly organicznej zmierzono krzywe zaników fluorescencji albuminy w obecności molekuł I i II. Otrzymane wyniki wskazują, że zmiany stężenia pochodnych benzoesanu metylu nie wpływają na wartość średniego czasu życia, co potwierdza statyczny mechanizm wygaszania.

Aby poprzeć powyższe wnioski analizę danych otrzymanych w różnych temperaturach przeprowadzono z wykorzystaniem zależności *Scatcharda*. Wyznaczone wartości stałej wiązania (K_b) oraz liczby miejsc wiążących dla cząsteczek białka (n) są w bardzo dobrej zgodności z wartościami otrzymanymi z zależności *Benesi-Hildebranda* i *Sterna-Volmera*.

Wartości parametrów termodynamicznych procesu kompleksowania pochodnych benzoesanu metylu z BSA wyznaczone z zależności *van't Hoffa* przyczyniły się do lepszego zrozumienia mechanizmów oddziaływań pochodnych benzoesanu metylu z BSA. Z otrzymanych parametrów termodynamicznych zestawionych w Tab. 15 wynika, że:

- proces kompleksowania pochodnych benzoesanu metylu z albuminą w roztworach wodnych jest egzotermiczny, a czynnikiem decydującym o sile napędowej procesu jest entalpia,
- w oddziaływaniu pomiędzy I i II a BSA główną rolę odgrywają wiązania wodorowe i słabe siły *van der Waalsa*.

Molekuły	Temp. [K]	$K_{SV} 10^4 [M^{-1}]$	$K_{BH} 10^4 [M^{-1}]$	$K_b 10^4 [M^{-1}]$	n	$\Delta H [kJ]$	$\Delta S [J]$	$\Delta G [kJ]$
I	278	2.4	2.6	3.5	1.07	-25.66	-4.86	-24.308
	288	2.1	2.4	2.7	1.04			-24.260
	298	1.9	2.0	2.1	1.00			-24.211
	303	1.5	1.3	1.3	0.93			-24.187
II	278	2.6	3.6	3.5	1.07	-25.12	-2.17	-24.512
	288	2.2	3.0	3.0	1.05			-24.491
	298	1.8	2.7	2.7	1.02			-25.469
	303	1.5	1.8	1.3	0.99			-25.458

Tab. 15 Stała wygaszania *Sterna-Volmera* (K_{SV}), stałe wiązania wyznaczone metodami *Benesi-Hildebranda* (K_{BH}) i *Scatcharda* (K_b) oraz parametry termodynamiczne (liczba wiązania (n), zmiany entalpii (ΔH), entropii (ΔS) i energia swobodna Gibbsa (ΔG) procesu wiązania) w różnych temperaturach.

Podsumowanie

Niniejsza praca doktorska zawiera wyniki badań, których celem było scharakteryzowanie oddziaływań molekularnych pomiędzy wybranymi molekułami organicznymi wykazującymi zjawisko fotoindukowanego wewnątrzcząsteczkowego przeniesienia ładunku (cztery pochodne benzoenu metylu oraz pochodna bifenylu) a związkami makrocyclicznymi, które dzięki swej budowie są zdolne do tworzenia kompleksów inkluzyjnych oraz zrozumienie mechanizmów oddziaływań pomiędzy dwoma pochodnymi benzoenu metylu a albuminą surowiczą wołową. Do realizacji zamierzonych celów użyto metod stacjonarnej i rozdzielonej w czasie spektroskopii oraz spektroskopii magnetycznego rezonansu jądrowego.

Poniżej zestawiono najważniejsze wyniki i spostrzeżenia przedstawione w pracy doktorskiej:

- dla wszystkich badanych molekuł organicznych znajdujących się w rozpuszczalniku polarnym, protycznym, jakim jest woda występują oddziaływania specyficzne między molekułą luminezującą a molekułami rozpuszczalnika (międzycząsteczkowe wiązania wodorowe w stanie podstawowym i wzbudzonym),
- analiza wyników eksperymentalnych wsparta obliczeniami kwantowo-chemicznymi pokazała, że w przypadku pochodnych benzoenu metylu (I i II) znajdujących się w mieszaninie rozpuszczalników THF-H₂O występują dwa konkurencyjne procesy: zjawisko solwatacji preferencyjnej oraz tworzenie różnych kompleksów z cząsteczkami wody. W sytuacji gdy ułamek molowy polarnej składowej jest mniejszy od 0.9 tylko pojedyncza cząsteczka wody jest przyłączona do badanego związku („*monohydrated complex*”), podczas gdy dla ułamka molowego wody $x_{H_2O} > 0.9$ możliwe jest powstanie kompleksów, w których dwie cząsteczki wody są połączone z badanymi molekułami („*dihydrated complex*”),
- w przypadku badanych pochodnych benzoenu metylu stabilne kompleksy w stanie podstawowym powstają w obecności β -CD, γ -CD, CB[7] oraz SCA[6], podczas gdy dla układu z α -CD nie znaleziono przesłanek potwierdzających powstanie trwałego kompleksu. Analiza danych doświadczalnych jednoznacznie wskazuje, że kompleksy inkluzyjne między badanymi molekułami organicznymi a CB[7] powstają w stanie

podstawowym, z tym że występuje zasadnicza różnica w geometrii i trwałości utworzonych kompleksów (I-CB[7] i III-CB[7] *versus* II-CB[7] i IV-CB[7]),

- analiza danych doświadczalnych uzyskanych przy użyciu metod stacjonarnej i rozdzielonej w czasie spektroskopii wskazuje, że w stanie wzbudzonym powstają kompleksy inkluzyjne między molekułami I-IV a wszystkimi badanymi związkami makrocyklicznymi. W przypadku molekuł typu ESIPT (II i IV) utworzenie kompleksu powoduje izolację molekuly od środowiska wodnego, co prowadzi do uaktywnienia procesu fotoindukowanego wewnątrzcząsteczkowego przeniesienia protonu w stanie wzbudzonym. Dynamika fotoindukowanych procesów dla molekuł typu TICT różni się w zależności od rodzaju nośnika makrocyklicznego użytego do badań,
- analiza danych eksperymentalnych wskazuje, że dla molekuł I i II w obecności CD, CB[7] i SCA[6] w stanie wzbudzonym tworzą się kompleksy o stechiometrii 1:1. W przypadku molekuł III i IV zaobserwowano, że w obecności CB[7] powstają kompleksy o stechiometrii 1:1, a dla układów z obecnością CD i SCA[6] oprócz kompleksów o stechiometrii 1:1 występują kompleksy o stechiometrii 1:2,
- spośród wszystkich badanych układów w stanie podstawowym największą komplementarnością, a co za tym idzie największą trwałością charakteryzują się układy z kukurbit[7]urylami, zaś najmniej stabilne są kompleksy z cyklodekstrynami. Tę samą prawidłowość obserwuje się dla kompleksów w stanie wzbudzonym,
- w wodnych roztworach zawierających SCA[6] i CB[7] proces relaksacji solwatacyjnej jest co najmniej o dwa (SCA[6]) lub trzy (CB[7]) rzędy wolniejszy od sytuacji, gdy molekula luminezująca znajduje się w ośrodku jednorodnym. Stwierdzono również, że średni czas relaksacji solwatacyjnej w układzie molekula-białko jest o około rząd wielkości dłuższy niż w układzie z nośnikami makrocyklicznymi (CB[7] i SCA[6]),
- badania przeprowadzone przy użyciu spektroskopii magnetycznego rezonansu jądrowego (^1H NMR) pozwoliły uzyskać wgląd w strukturę kompleksów inkluzyjnych oraz ustalić geometrię badanych układów,
- w przypadku molekuly **EDMAADCy** w obu badanych środowiskach w obecności γ -CD powstają w stanie wzbudzonym stabilne kompleksy o stechiometrii 1:1. Wartości stałych równowagowych zależą od stężenia molekuly oraz długości fali światła wzbudzającego. Zachowanie to jest zrozumiałe biorąc pod uwagę fakt, że już w stanie podstawowym związek **EDMAADCy** tworzy układ spektroskopowo niejednorodny,

- analiza ilościowa danych spektroskopowych wykazała, że zarówno w przypadku układów I-Trp, II-Trp, jak i I-BSA, II-BSA powstają kompleksy, które charakteryzują się stechiometrią 1:1. Co więcej w specyficzne oddziaływanie między II i Trp oraz II i BSA zaangażowane są grupy OH i/lub $-\text{COOCH}_3$ molekuly II (brak możliwości zajścia procesu fotoindukowanego wewnątrzcząsteczkowego przeniesienia protonu).

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Spectroscopic studies of inclusion complexation between ortho derivatives of *p*-methylaminobenzoate and α - and γ -cyclodextrins

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ABSTRACT

Using the steady-state and time-resolved spectroscopic techniques and quantum-chemical calculations, photophysical and photochemical properties of the two ortho derivatives of *p*-methylaminobenzoate (methyl *o*-methoxy *p*-methylaminobenzoate (**I**) and methyl *o*-hydroxy *p*-methylaminobenzoate (**II**)) have been studied in binary mixture THF-H₂O and aqueous solutions containing different concentrations of α - and γ -CD. Nonlinear solvatochromic shifts of the absorption and fluorescence bands were observed for both fluorophores in a mixture of polar aprotic (THF) and polar protic (H₂O) solvents. This nonlinearity has been explained in terms of the non-specific (dielectric enrichment of the solvent around the polar solute) and specific (hydrogen bond) solute-solvent interactions. Spectroscopic measurements were used to investigate the role of H-bonding solute-solvent interactions, and the excited-state intramolecular proton transfer process in the formation of inclusion complexes between fluorophore and cyclodextrins. The obtained results were used to calculate, according to Benesi-Hildebrand's plot and nonlinear least-squares regression analysis, equilibrium constants of the fluorophore-cyclodextrin inclusion complexes. Performed analysis indicates also that both 1:1 and 1:2 inclusion complexes were formed between studied compounds and α - and γ -cyclodextrins.

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1. Introduction

It is well-known that an intramolecular and/or intermolecular proton transfer is one of the most fundamental reactions in biological, physical and chemical systems [1–5]. The mechanism of this phenomenon has become the subject of a widely spread discussion among many scientific groups, right after the Weller's discovery of dual emission in the fluorescence spectrum of salicylic acid and methyl salicylate [6]. This process, known as an excited state intramolecular proton transfer (ESIPT), has been extensively investigated not only from a purely theoretical point of view (i.e. comprehending the mechanisms of processes taking place in an examined compounds with proton donor and/or acceptor sites, as well as explaining the mechanisms of interaction between examined compounds and their protic environment), but also because of the possibility to use such ESIPT molecules in a variety of applications, such as fluorescent sensors, probes for biological environments, laser dyes, UV photostabilizers, filtered materials, molecular memory storage devices, fluorescent probes, polymer protectors, metabolic process of living systems, fluorescent solar concentrators, organic light-emitting devices [7–13].

Simultaneously to the steady-state and time-resolved spectroscopic studies of different ESIPT fluorophores dissolved in neat, homogeneous

environments, in recent years, considerable attention has been focused on understanding and controlling supramolecular interactions between organic molecules (e.g., potential drugs) and well-recognized macrocyclic hosts: cyclodextrins, cucurbit[*n*]urils, crown ethers, calixarenes and cyclophanes [14–17]. Both in photochemistry and pharmaceutical chemistry there has been increasing interest focused on understanding and characterize non-specific and specific interactions occurring between the fluorophore in the ground/excited state and microenvironment, as well as potential applications of cyclodextrin-based systems. In the light of the presented information, one can completely understand the fact that the family of naturally occurring oligosaccharides, cyclodextrins, has great potential in the delivery and controlled release of drugs. The hydrophobic cavity with hydrophilic external walls make them suitable and fascinating systems not only for use as drug carriers, but also for the study of interactions between the investigated dyes in the ground and/or excited states and cyclodextrins i.e., the stoichiometry and the ground and excited-state equilibrium constants of the inclusion complexes.

In the last few years, our special attention has been paid to the description of the mechanisms of interaction between some methyl benzoate derivatives and their environment (neat solvents and binary solvent mixtures) [18–24]. It has been shown that photophysical and photochemical properties of these molecules are largely influenced by the nature of the substituents of the phenyl ring. Moreover, it is clear from the steady-state and time-resolved spectroscopic measurements,

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as well as semiempirical quantum-chemical calculations that methyl *p*-amino and *p*-dimethylaminobenzoate and their ortho (—OH, —OCH₃) derivatives undergo, in the excited state, conformational changes caused by inter- and intramolecular proton transfer reactions and/or twisted intramolecular charge transfer (TICT) processes.

As it was mentioned, the excited-state intramolecular proton transfer process in different molecules dissolved in neat liquid medium has been the subject of very extensive experimental and theoretical research for several decades [1–6]. Relatively less attention has been given to the study of the role of the excited-state inter- and/or intramolecular proton transfer in inclusion complexes formed between fluorophore and cyclodextrins. Taking into account above, in the present paper, we seek to gain a deeper insight into the spectroscopic characterization of two dyes in aqueous solutions containing different concentrations of α - and γ -CD. The first one dissolved in aprotic solvent exhibits only locally excited fluorescence (molecule **I**), whereas the second one exhibits dual fluorescence i.e., emission from the locally excited state and the intramolecular proton transfer state (molecule **II**). Therefore, the steady-state and time-resolved studies were used to obtain reliable information about intermolecular and intramolecular interactions causing host-guest type inclusion complexes.

2. Experimental details

The molecules under study have been synthesized and purified by Gormin et al. [25–27]. The purity of synthesized dyes were controlled by thin layer chromatography. The measurements showed that studied compounds is high purity. Both α -CD and γ -CD were purchased from Aldrich Chemical Co. and used without further purification.

Steady-state absorption and fluorescence measurements at room temperature were carried out using a computer-controlled Shimadzu UV-2401 PC spectrophotometer and Shimadzu RF-5301 PC spectrofluorometer. Time-resolved emission spectra of the sample were measured applying streak camera (C4334-01 Hamamatsu) and 2501 S spectrograph (Bruker Optics). Solid state Nd: YAG laser (PL 2143A/SS EKSPLA) and optical parametric generator (PG 401/SH EKSPLA) were used as an excitation light pulses source [28].

All calculations concerning the spectroscopic properties of molecules under study have been performed using the CAChe WS 5.04 program. The geometrical structure of the investigated molecules has been determined using the PM3 semiempirical molecular orbital method at the Restricted Hartree Fock (RHF) level including single excitation configuration interaction (CIS). The PM3 method based on neglect of diatomic differential overlap (NDDO) approximation takes into account valence electrons only and for simplicity some of the two electron integrals are omitted. The ZINDO semiempirical method has been used in calculations of electronic singlet Franck–Condon (FC) state energies for UV-visible spectra. In the calculations of oscillator strengths of the transition between the corresponding states the wave functions used included all electronic states, which are generated by single excitation of electrons in the highest occupied (HOMO) and lowest unoccupied (LUMO) molecular orbitals.

3. Results and discussion

3.1. Absorption spectra in the binary THF-H₂O solvent mixture and their analysis on the basis of the quantum-chemical calculations

Fig. 1 shows the absorption spectra of **I** and **II** in a mixture of THF-H₂O at different water concentration keeping a constant solute concentration of $c = 5 \cdot 10^{-5}$ M. As can be seen, for both molecules, the absorption spectrum in neat THF and H₂O possesses two main bands: strong long-wavelength band in region of 260–340 nm, and weaker short-wavelength in 220–250 nm. The folded structure of the long-wavelength absorption band points the presence of two electronic transitions ($S_0 \rightarrow S_1$ and $S_0 \rightarrow S_2$). The peculiarities of these bands (for

methyl benzoate derivatives in vapour-phase) have been thoroughly described in [18–22]. Finally, one the basis of the experimental results and quantum-chemical calculations, it has been shown that the long-wavelength absorption band of the studied dyes consists of two overlapping components.

It is also clear from Fig. 1 that the shape, absorption maxima positions and molar absorption coefficient of the long-wavelength absorption spectrum of **I** and **II** in protic water are different than these in aprotic THF. The absorption band shifts to the red with increasing concentration of a water in a mixture of polar aprotic (THF) and polar protic (H₂O) solvents. Furthermore, the relative contributions of two lowest-energy transitions ($S_0 \rightarrow S_1$, $S_0 \rightarrow S_2$) depend strongly on the concentration of polar protic component in the binary mixture. For both studied molecules, the value of the molar absorption coefficient of the long-wavelength absorption band, corresponding to $S_0 \rightarrow S_1$ transition, increases with increasing x_{H_2O} . This behavior is accompanied by decrease of the short-wavelength absorption band ($S_0 \rightarrow S_2$ transition). The mentioned changes are more pronounced in the absorption spectrum of **I** in comparison to the molecule **II**. It should be noted that, for both investigated dyes, the absorption spectra in THF-H₂O solvent mixtures containing less than about 0.9 mole fraction of water component differ considerably from those obtained at higher water concentration ($x_{H_2O} > 0.9$). Furthermore, when the mole fraction of water is increased from 0 to 0.9, an isosbestic point can be clearly seen in normalized absorption bands (see Fig. 1). The appearance of isosbestic point indicates the formation of hydrogen-bond complex of **I** and **II** with water molecules in the ground state. In order to analyze quantitatively the solvatochromic shifts of the absorption ($S_0 \rightarrow S_1$, $S_0 \rightarrow S_2$ transitions) and fluorescence spectra as a function of the mole fraction of polar protic solvent, Fig. 2 shows normalized shifts plotted against the H₂O concentration. It is clear from Fig. 2 that for both dyes the position of the two long-wavelength absorption bands ($S_0 \rightarrow S_1$, $S_0 \rightarrow S_2$) evolves continuously only up to $x_{H_2O} = 0.9$ and this simple dependence breaking down at higher water concentration. Analyzing the data assembled in Fig. 1, in connection with the graphical presentation of the normalized solvatochromic shifts of two long-wavelength absorption bands (corresponding to $S_0 \rightarrow S_1$ and $S_0 \rightarrow S_2$ transitions, see Fig. 2), it follows that the nature of absorbing centers is different in the two discussed ranges of H₂O concentration (0–0.9 and 0.9–1.0).

As it was mentioned earlier, the solvent-induced shift of the absorption and fluorescence spectra arises from either universal (non-specific) and/or specific (hydrogen bond) solute-solvent interactions. Taking into account that studied systems contain a polar molecule (**I** and **II**) in a mixture of aprotic and protic solvents (THF-H₂O), in addition to non-specific (dipole-dipole) interactions, hydrogen bonding between the solute and solvent molecules can also occurs. Thus, the absorption spectra of **I** and **II** in a mixture of polar aprotic THF and protic H₂O can consist of electronic transitions of different centers absorbing at different wavelengths (i.e., neutral molecule and hydrogen-bonded complexes with H₂O molecules). In order to understand the nature of the solute-solvent interactions in the ground state (i.e., the influence of hydrogen bonds on the absorption spectra) a series of quantum-chemical calculations were carried out. In the lowest panels of Fig. 1, the bars under the absorption curves give the theoretical electronic state energy values and corresponding oscillator strengths of the neutral investigated dyes (**I** and **II**) in THF, as well as different hydrogen-bond complexes of **I** (i.e., **Ia**, **Ib**) and **II** (i.e., **IIa**, **IIb**, **IIc**) with H₂O. Taking into account our previous findings (i.e., fact that methyl *p*-dimethylaminobenzoate and its two ortho derivatives in a hydrochloric acid solution exist as a mixture of neutral, mono-, and dicationic forms) [21], it is clear that the investigated molecules possess the functional groups which are the likely sites for intermolecular hydrogen bonding with water molecules. To understand how water molecule interacts with investigated dyes we considered a number of possible complexes between **I** or **II** and H₂O molecule. Special attention has been given to the possible interaction of water molecule with active centers of

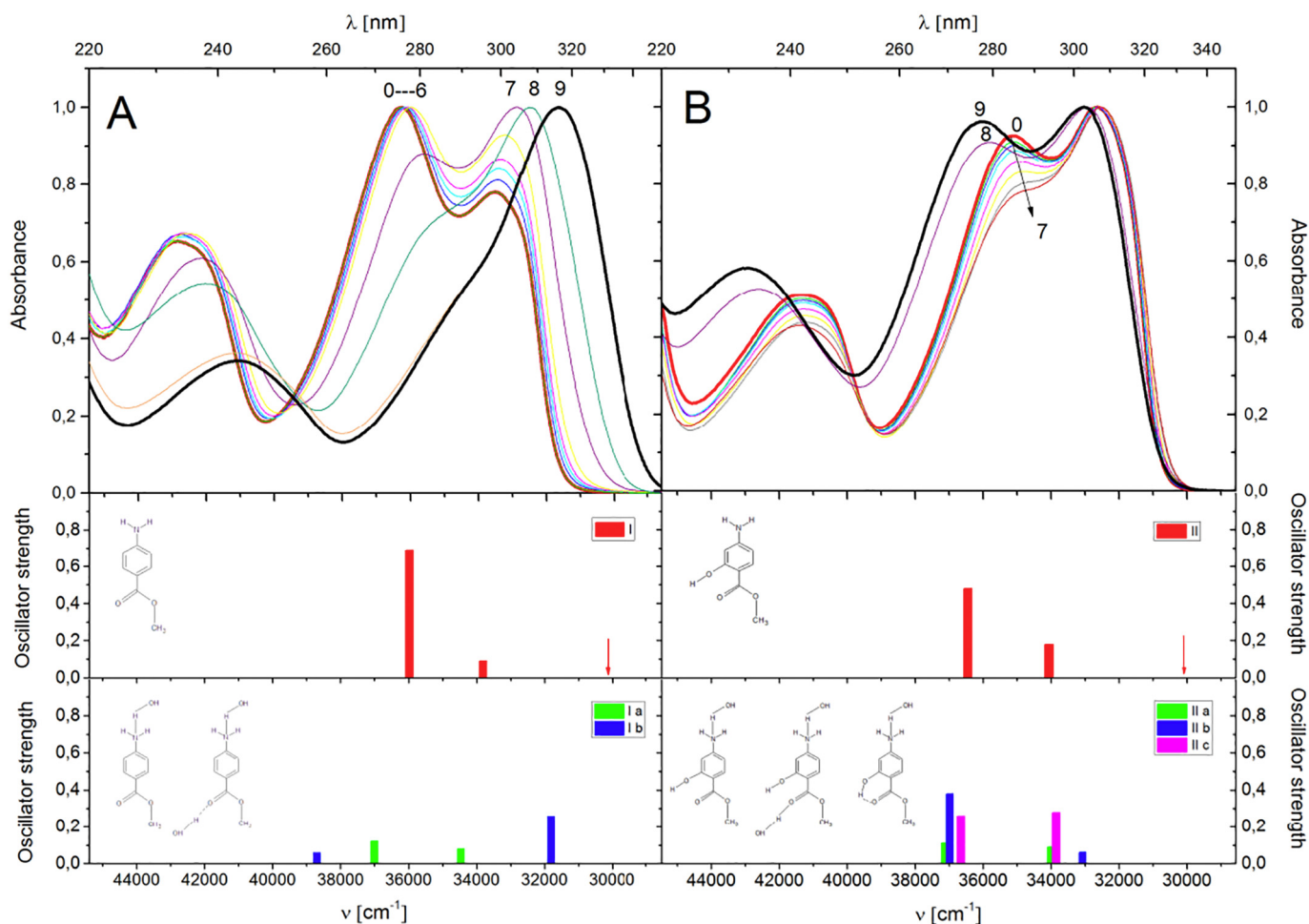


Fig. 1. The absorption spectra of **I** (A) and **II** (B) in neat solvents of THF (0) and H₂O (9) and their mixtures at different mole fraction, x_p , of H₂O.

fluorophore i.e., —NH₂ and —COOCH₃ groups. The structures of the possible complexes between **I** or **II** and water molecule are presented in Fig. 1 (lower panels). As can be seen, the water molecule was added to the investigated dyes only at the locations considered important for hydrogen bond formation. It is worth noting that real situation in THF-H₂O system can be more complex but the simple complex considered can give a first insight into the specific solute-solvent interaction in the ground state.

On the basis of the quantum-chemical calculation results, presented in Table 1 and Fig. 1, one can state that in the binary THF-H₂O solution, a specific interactions i.e., hydrogen-bonding between **I** or **II** and water molecules, are occurring in addition to the well-known dipole-dipole interactions. As it was mentioned earlier, for both studied molecules the long-wavelength absorption bands become more and more red-shifted on increasing the water concentration. This bathochromic shift, as well as the gradual changes in the value of the molar absorption coefficient of the long-wavelength absorption band are understandable in terms of formation of different water complexes e.g., monohydrated complex **Ia**, **IIa** (see Fig. 1) and dihydrated complex (H-bonding between H₂O and —NH₂ and —COOCH₃ groups) **Ib**, **IIb**, **IIc**. Naturally, the participation of monohydrated and dihydrated complexes of the investigated dyes in the absorption spectrum is higher at higher water concentrations. In order to obtain more insight into hydrogen bond formation between solute and solvent molecules the spectral evolution should be analyzed in two ranges of mole fraction of H₂O: $0 < x_{H_2O} < 0.9$ and $x_{H_2O} > 0.9$. Analyzing the spectral behavior of **I** and **II** in THF-H₂O solvent mixtures containing <0.9 mole fraction of water (i.e., appearance of the isosbestic point in the absorption spectra) in

connection with quantum-chemical calculations, it follows that an equilibrium is established between two absorbing species: free molecule (**I** or **II**) and monohydrated form of the parent molecule (**Ia** or **IIa**). Very complex spectral features observed at higher H₂O concentrations ($x_{H_2O} > 0.9$) are understandable taking into account not only appearance of new electronic transitions of monohydrate complexes but mainly dihydrated forms of the parent molecules (**Ib**, **IIb**, **IIc**).

3.2. Steady-state fluorescence spectra in the binary THF-H₂O solvent mixture

Fig. 3 presents the normalized fluorescence spectra of **I** and **II** in neat THF and H₂O and their mixtures at different mole fraction of water. In polar aprotic THF the emission spectrum of **I** possesses a single fluorescence band (locally excited), whereas fluorescence spectrum of **II** consists of two partially overlapping bands i.e., short-wavelength (locally excited) fluorescence, and intramolecular proton transfer band. The locally excited fluorescence band of **I** and **II** shows the same general dependence on increasing the water concentration as observed for absorption spectra in THF-H₂O mixture i.e., the nonlinear, bathochromic shift (see Fig. 2). As can be seen in Fig. 3, for molecule **I**, upon increasing x_{H_2O} the position of LE fluorescence maximum is red-shifted and simultaneously a new long-wavelength band centered at around 450 nm appears. This indicates that the hydrogen-bond complex or complexes of **I** and water molecule exist in the excited state. The fluorescence behavior of **I** in THF-H₂O mixture cannot be explained by only two emitting centers over the whole range of x_{H_2O} because isoemissive point is evident at mole fraction of water from 0 to 0.4, and it disappears at higher polar-

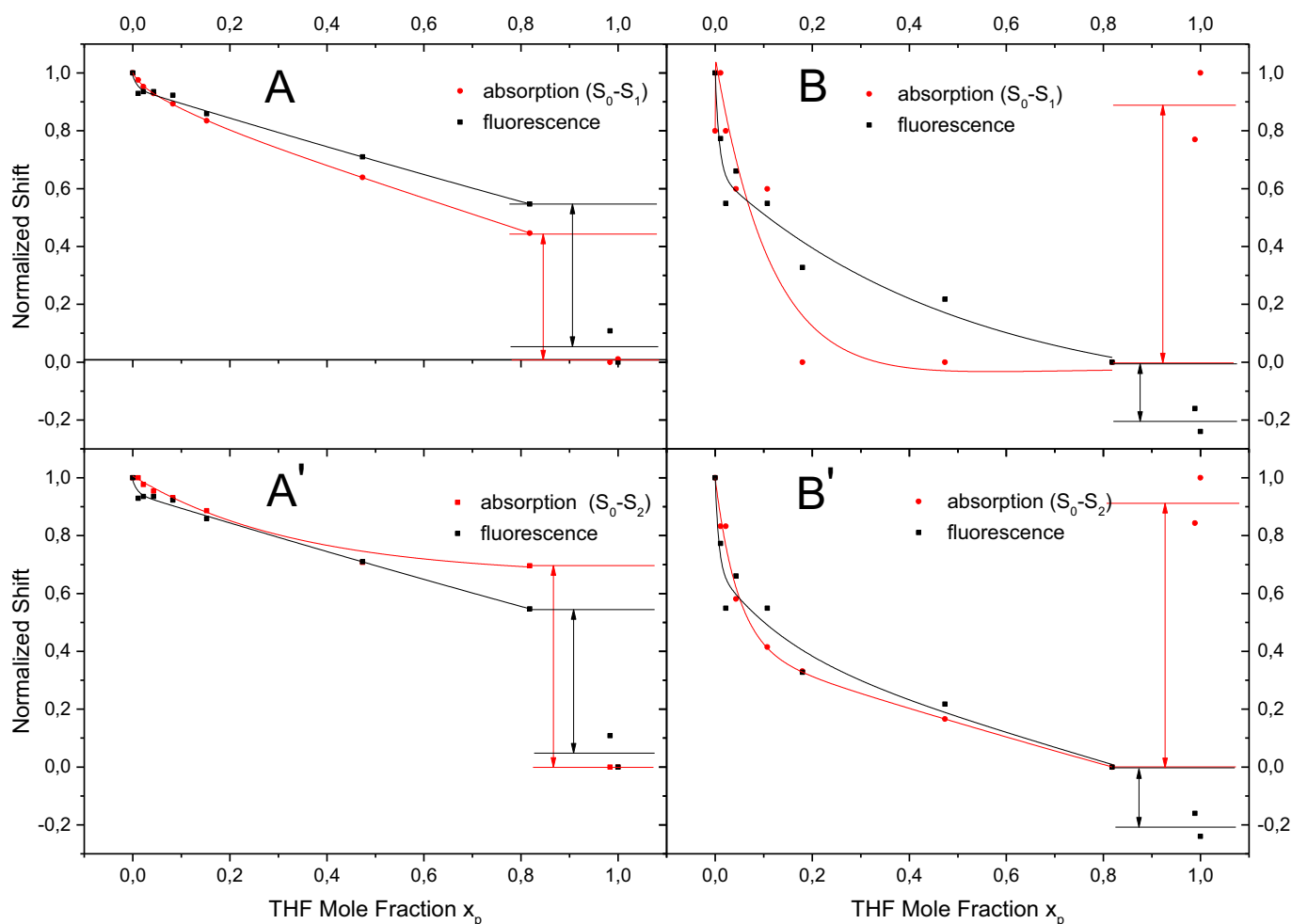


Fig. 2. Normalized shift of the absorption ($S_0 \rightarrow S_1$ and $S_0 \rightarrow S_2$ transitions) and short-wavelength fluorescence bands maxima of **I** (A, A') and **II** (B, B') determined for different mole fraction, x_p , of H_2O .

protic solvent concentration ($x_{H_2O} > 0.4$). Thus, the occurrence of an isoemissive point in the fluorescence bands suggests the existence of two types of emissive species (free molecule and hydrogen-bond complex) only up to $x_{H_2O} = 0.4$.

Fluorescence spectra of **II** in mixed binary solvents undergo also complex changes on adding water to tetrahydrofuran. While the gradual red-shift of the LE band was observed with increase in water concentration, the maximum position of the ESIPT band is practically of

the solvent polarity independent. On the other hand, it is important to note that the ESIPT fluorescence full-width at half maximum value increases by about 25% when the x_{H_2O} is increased from 0 to 1. This behavior is understandable taking into account that at higher water concentrations, the molecule **II** in the excited state exists as a mixture of neutral, mono- and dihydrated forms.

3.3. Absorption and fluorescence spectra in aqueous solution with α - and γ -cyclodextrin

The effect of increasing α - and γ -cyclodextrin concentration on absorption spectra of **I** and **II** is shown in Figs. 4(A, B) and 5(A, B). As can be seen the observed changes in the spectral behavior are very different in the two analyzed systems (α -CD and γ -CD). As the concentration of α -CD increases (Fig. 4B) the absorption maximum position of **II** is very slightly red-shifted with a small, gradual change in absorbance, whereas for molecule **I** practically no spectral changes occurred with increasing α -CD concentration. Similar behavior was observed for methyl *o*-hydroxy *p*-dimethylamino benzoate in α -CD [23], which confirms that no complexation for molecule **I** and methyl *o*-hydroxy *p*-dimethylamino benzoate with α -CD has occurred. On the other hand, for both investigated dyes upon increasing the concentration of γ -CD, the value of the molar absorption coefficient of the long-wavelength absorption band distinctly decreases. This behavior is understandable taking into account that the cavity of γ -CD is larger than that of α -CD and γ -CD could more effectively interacts with both investigated molecules causing host-guest type inclusion complexes in the ground state.

Table 1

Theoretical energy values of the singlet excited states, E_i , and corresponding oscillator strengths, f_i , of the investigated dyes.

Compound	State	$E_i(cm^{-1})/\lambda(nm)$	f_i
I	$S_{n,\pi}$	29,395/340.2	0.0003
	S_1	33,807/295.8	0.09
	S_2	35,971/278.0	0.69
Ia	S_1	34,459/290.2	0.08
	S_2	37,010/270.2	0.12
Ib	S_1	31,807/314.4	0.26
	S_2	38,700/258.4	0.06
II	$S_{n,\pi}$	29,129/343.3	0.0005
	S_1	34,060/293.6	0.18
	S_2	36,443/274.4	0.48
IIa	S_1	33,944/294.6	0.09
	S_2	37,065/269.8	0.11
IIb	S_1	33,069/302.4	0.06
	S_2	36,982/270.4	0.38
IIC	S_1	33,852/295.4	0.28
	S_2	36,657/272.8	0.26

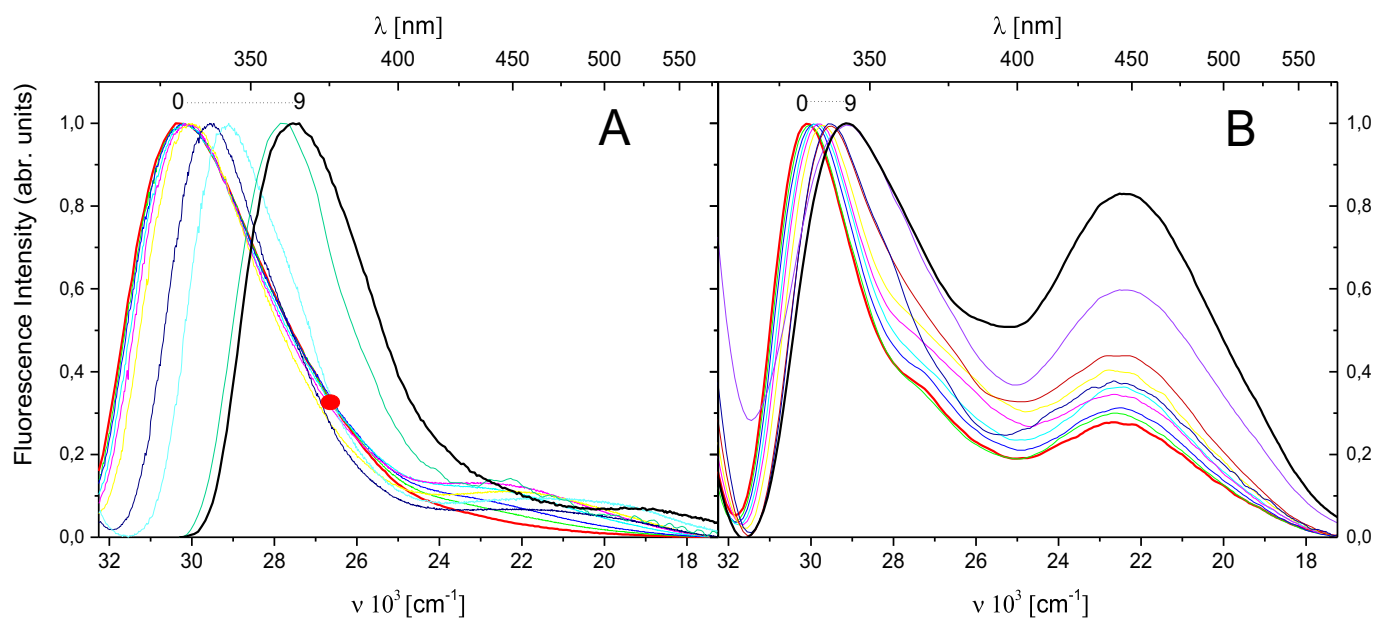


Fig. 3. The normalized fluorescence spectra of I (A) and II (B) in neat solvents of THF (0) and H₂O (9) and their mixtures at different mole fraction, x_p , of H₂O.

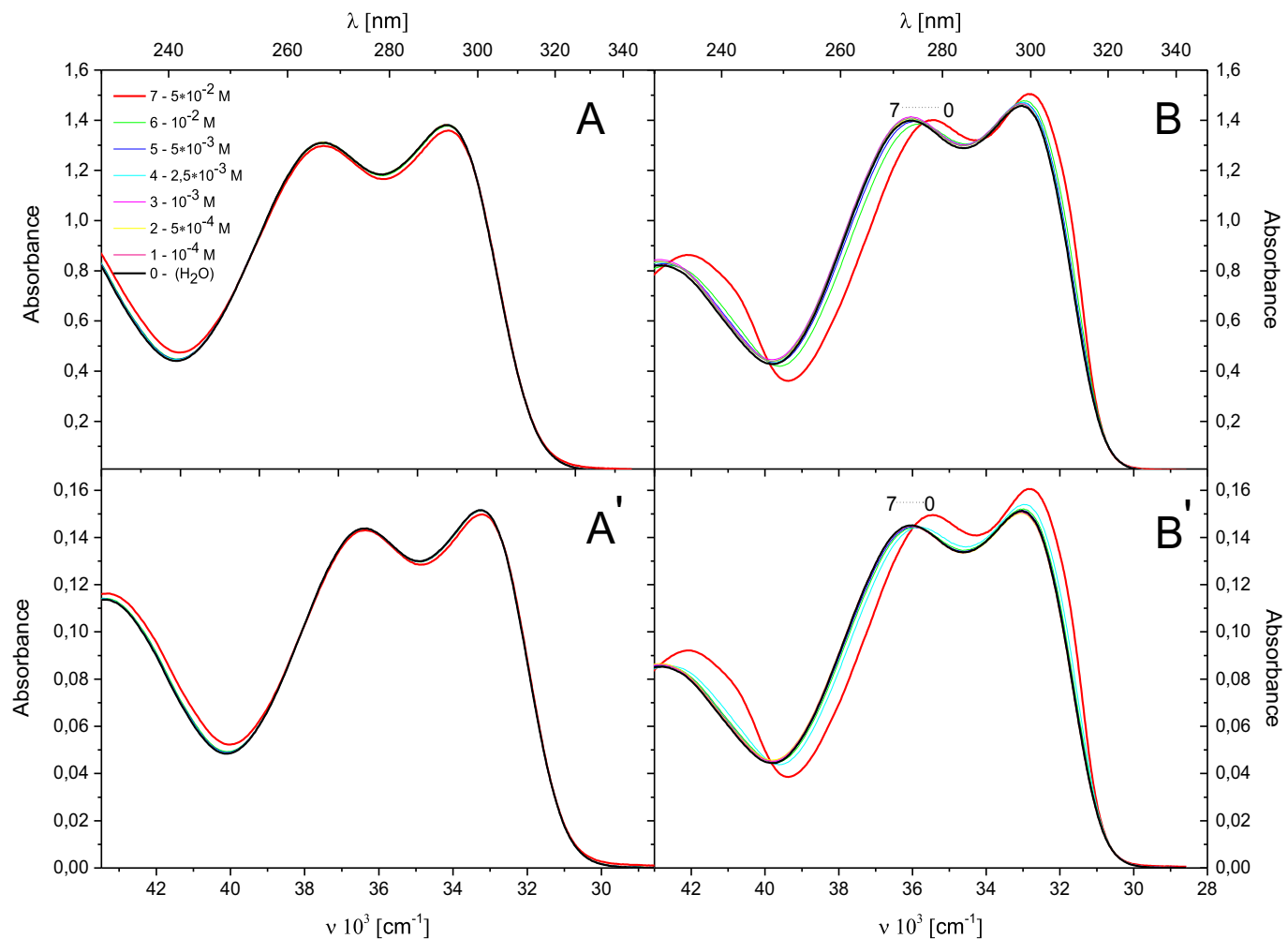


Fig. 4. Absorption and normalized absorption spectra of I (A, A') and II (B, B') in aqueous solutions containing different concentrations of α -CD (0–0 M, $1 \cdot 10^{-4}$ M, $2.5 \cdot 10^{-4}$ M, $3 \cdot 10^{-3}$ M, $4 \cdot 2.5 \cdot 10^{-3}$ M, $5 \cdot 5 \cdot 10^{-3}$ M, $6 \cdot 10^{-2}$ M, $7 \cdot 5 \cdot 10^{-2}$ M).

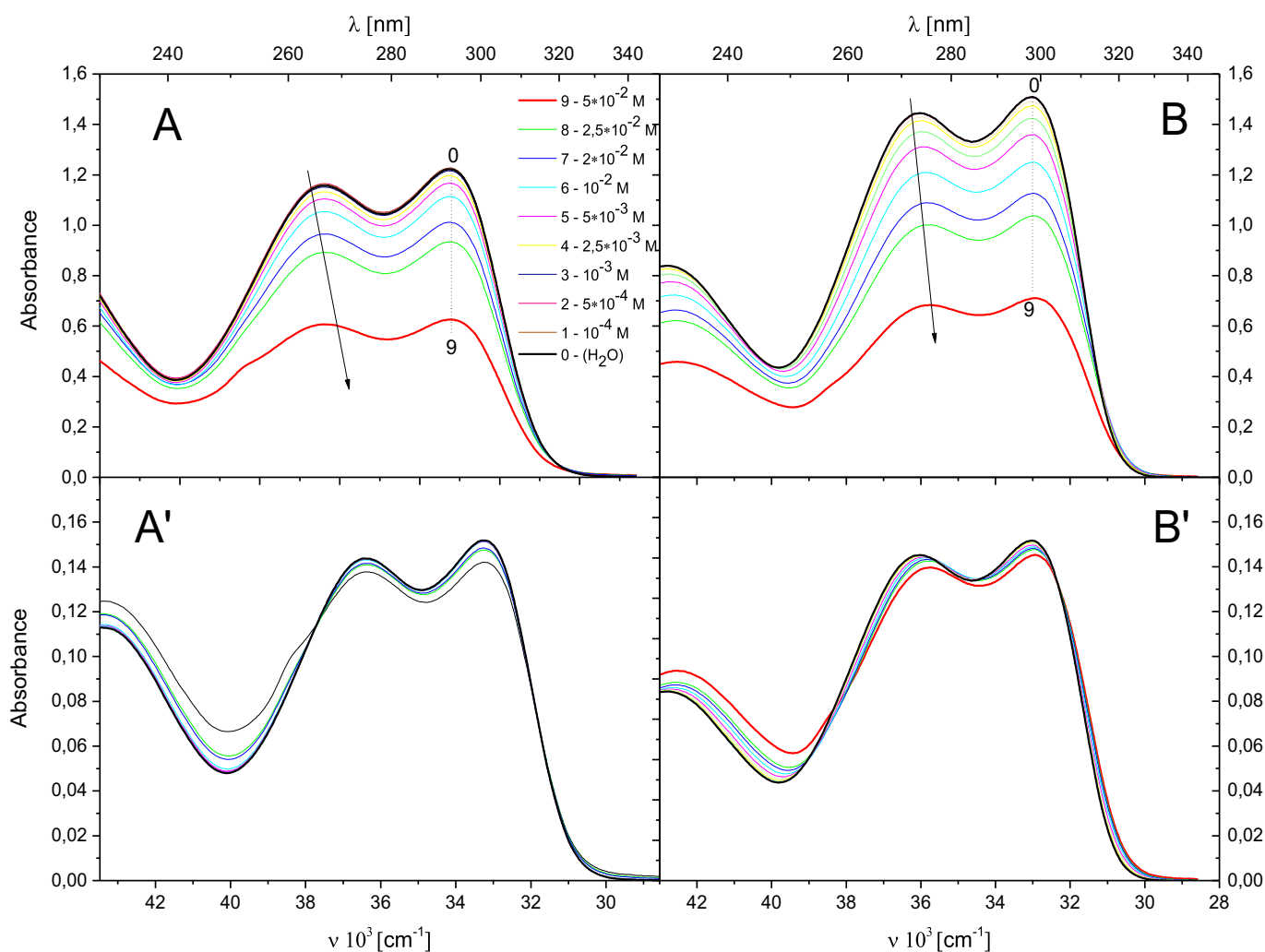


Fig. 5. Absorption and normalized absorption spectra of **I** (A, A') and **II** (B, B') in aqueous solutions containing different concentrations of γ -CD (0–0 M, $1 \cdot 10^{-4}$ M, $2 \cdot 5 \cdot 10^{-4}$ M, $3 \cdot 10^{-3}$ M, $4 \cdot 2 \cdot 5 \cdot 10^{-3}$ M, $5 \cdot 5 \cdot 10^{-3}$ M, $6 \cdot 10^{-2}$ M, $7 \cdot 2 \cdot 10^{-2}$ M, $8 \cdot 2 \cdot 5 \cdot 10^{-2}$ M, $9 \cdot 5 \cdot 10^{-2}$ M).

In order to clarify the role of CD in the spectroscopic properties of the molecules under study in the ground state, all absorption spectra were normalized by scaling the area under the spectrum to be equal to unity (see Figs. 4(A', B') and 5(A', B')). The occurrence of an isosbestic points in the normalized absorption spectra of **I** and **II** in γ -CD, as well as for **II** in α -CD suggests that an equilibrium is established between two types of absorbing species in the studied medium. For the molecule **I** in α -CD, there is no clear isosbestic point in the normalized absorption spectra which rule out the formation of well-defined 1:1 (**I**-(α -CD)) complex.

Fig. 6A and B show a typical example of the α -cyclodextrin effect on the fluorescence spectrum of the investigated dyes. Because the fluorescence spectra of **I** and **II** in γ -CD are very similar to those in α -CD they are not presented here. As it was mentioned earlier, and as can be seen in Fig. 6, the emission spectrum of **I** in H_2O possesses two fluorescence bands: short-wavelength – locally excited fluorescence band and much weaker, the long-wavelength band results from the emission of intermolecular hydrogen-bond complexes i.e., mono- and dihydrated forms of the molecule **I**. On the other hand, fluorescence spectrum of **II** in H_2O consists of three partially overlapping bands: LE, ES IPT bands and emission of H-bonded complexes. For both studied dyes in α - and γ -CD the locally excited fluorescence band blueshifts slightly and the fluorescence intensity increases as the concentration of cyclodextrins increases. Similar behavior was observed for the long-wavelength fluorescence band i.e., LW fluorescence component becomes more intense.

Observed blueshift in the LE fluorescence band indicates that both studied molecules are experiencing an microenvironment that is much less dipolar than water. The blue-shifts of LE fluorescence band caused by increasing CD concentration along with the increased of fluorescence intensity are due to inclusion of **I** and **II** in the hydrophobic cavities of α - and γ -CD.

To further characterize the cyclodextrin-induced changes in the fluorescence spectra of **I** and **II** each of the area under the fluorescence curves were normalized to unity. In this case, each the fluorescence spectrum corresponds to the emission of the same number of emitting centers (see Fig. 6A' and B'). As can be seen in Fig. 6A' and B' the normalized fluorescence spectra of **I** and **II** show a systematic changes with gradual addition of α -CD showing an isosbestic point. Similar behavior was observed for fluorescence spectra of the studied dyes in γ -CD. These spectral changes with the clean isosbestic point seem to indicate the formation of well-defined host-guest inclusion complexes between α - and γ -CD and molecules under study in the electronic excited state.

3.4. Stoichiometry of inclusion complexes

Steady-state and time-resolved spectroscopic measurements can be used for determination of stoichiometry and equilibrium constants of the inclusion complexes in the ground and excited states. For a simple 1:1 inclusion complex between fluorophore (F) and cyclodextrins (CD) in the excited ($F^* + CD \rightarrow (F - CD)^*$)

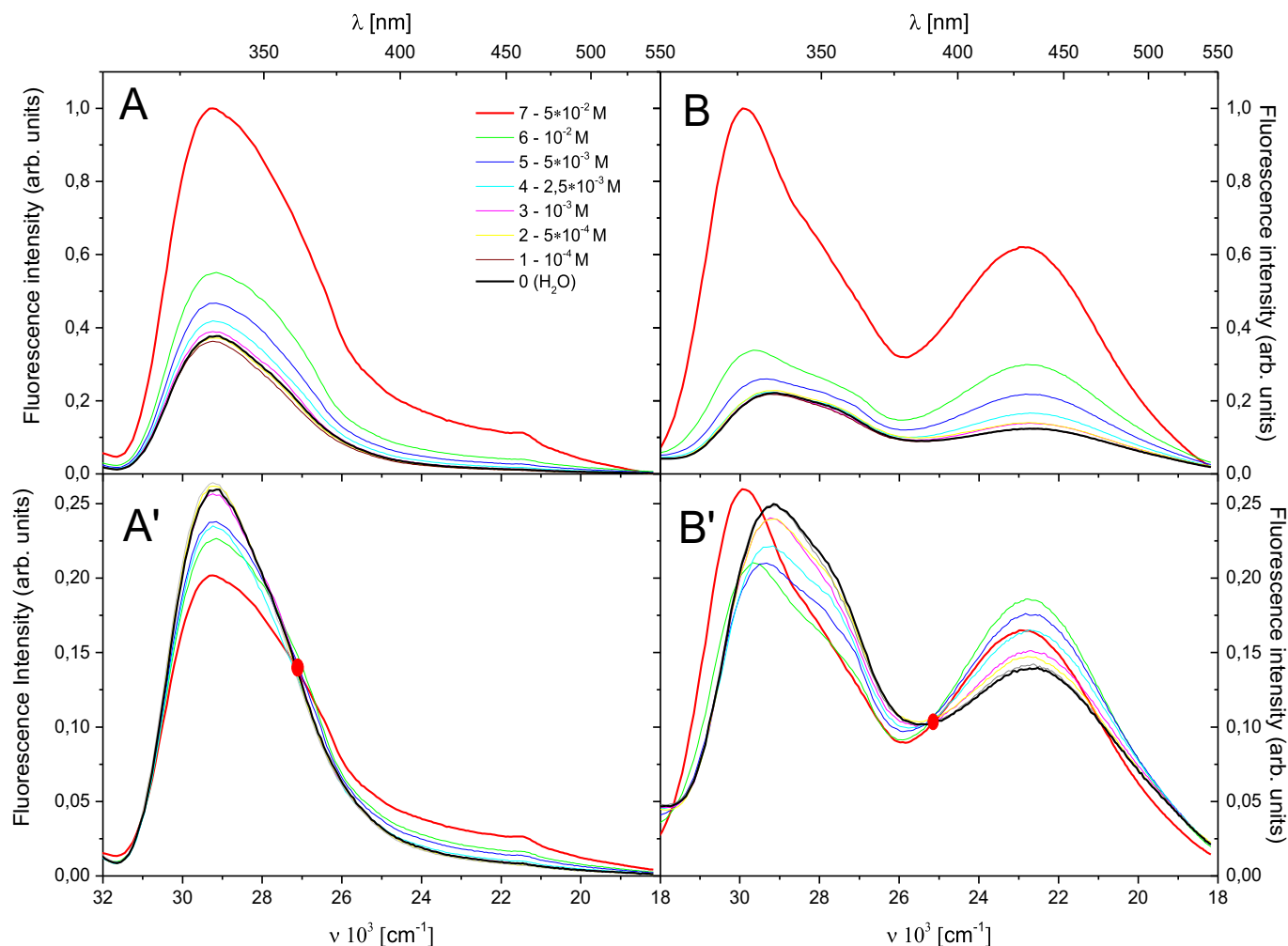


Fig. 6. Fluorescence and normalized fluorescence spectra of I (A, A') and II (B, B') in aqueous solutions containing different concentrations of α -CD (0–0 M, $1 \cdot 10^{-4}$ M, $2 \cdot 5 \cdot 10^{-4}$ M, $3 \cdot 10^{-3}$ M, $4 \cdot 2 \cdot 5 \cdot 10^{-3}$ M, $5 \cdot 5 \cdot 10^{-3}$ M, $6 \cdot 10^{-2}$ M, $7 \cdot 5 \cdot 10^{-2}$ M).

or ground ($F + CD \rightarrow F - CD$) states, $I - I_0$ or $A - A_0$ versus CD concentration ($[CD]$) should fit the Benesi–Hildebrand equation [29–31]:

$$\frac{1}{I - I_0} = \frac{1}{K_1^{ex}(I_1 - I_0)} \cdot \frac{1}{[CD]} + \frac{1}{I_1 - I_0}, \quad (1a)$$

$$\frac{1}{A - A_0} = \frac{1}{K_1^{gr}(A_1 - A_0)} \cdot \frac{1}{[CD]} + \frac{1}{A_1 - A_0}, \quad (1b)$$

where I_0 (A_0), I_1 (A_1) and I (A) are the fluorescence intensities (or absorbances) of the studied molecule in water (in the absence of CD), in 1:1 complex (when all of molecules is complexed) and at the particular concentration of CD. The equilibrium constants K_1^{gr} and K_1^{ex} are expressed as:

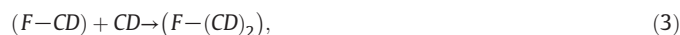
$$K_1^{gr} = \frac{[F - CD]}{[F][CD]}, \quad K_1^{ex} = \frac{[(F - CD)^*]}{[F^*][CD]} \quad (2)$$

where $[F]$ ($[F^*]$), $[CD]$, $[F - CD]$ ($[(F - CD)^*]$) are the stoichiometric concentration of the fluorophore in the ground (excited) state, cyclodextrins and 1:1 inclusion complex in the ground (excited) state.

Nevertheless, one has to remember that, in many cases, the plot of $1/(I - I_0)$ (or $1/A - A_0$) versus $1/[CD]$ (see Eqs. (1a) and (1b)) can be drawn on the whole studied range of cyclodextrin concentrations not as a single straight line but as two straight lines. In this case, the initial linear portion, at high $[CD]$, might contain equilibrium constant (K_2) for the

formation of 1:2 inclusion complex between fluorophore and cyclodextrins, while the final linear portion, at low $[CD]$, might contain K_1 value.

For 1:2 inclusion complexes between fluorophore (F) and cyclodextrins (CD) the equilibrium can be written as [29–31]:



where

$$K_2 = \frac{[(F - (CD)_2)]}{[(F - CD)][CD]}, \quad (4)$$

and $[(F - (CD)_2)]$ is stoichiometric concentration of 1:2 inclusion complex. For this equilibrium the following relationship can be obtained:

$$\frac{1}{I - I_0} = \frac{1}{(I_2 - I_0)K_2[CD]^2} + \frac{1}{I_2 - I_0}. \quad (5)$$

It was shown by Nigam and Durocher [32–34] that graphical method (Benesi–Hildebrand plot) does not allow the determination of equilibrium constants properly. They pointed out that spectroscopic data obtained at low CD concentrations are widely spaced as compared to high-concentration values. Consequently, the slope of the line is very sensitive to the y value of the data points having the largest x value [32]. Taking into account above, the graphical method based on Benesi–Hildebrand plot provides an estimates rather than proper

equilibrium constants of the inclusion complexes. The nonlinear least-squares regression analysis is an excellent instrument to determine more reliable equilibrium constant value. For a low cyclodextrins concentration (i.e., when only 1:1 complex is formed), the spectroscopic data should be fitted using nonlinear regression program into following equation [32–34]:

$$I = \frac{I_0 + I_1 K_1 [CD]}{1 + K_1 [CD]} \quad (6)$$

Here we would like to note that, nonlinear regression requires preliminary parameters estimates (K_1 , I_1) which can be determined from the linear regression approach i.e., Benesi–Hildebrand plot (see Eq. (1a)).

In a series of papers [32–37] were shown that, if consider a combination of 1:1 and 1:2 inclusion complexes in studied system, more reliable values of equilibrium constants can be obtained from the following equation:

$$I = \frac{I_0 + I_1 K_1 [CD] + I_2 K_1 K_2 [CD]^2}{1 + K_1 [CD] + K_1 K_2 [CD]^2} \quad (7)$$

Knowing the initial values of K_1 , K_2 , I_0 , I_1 , I_2 estimated from Eqs. (1a), (5) and (6), we are able to calculate two equilibrium constants (K_1 and K_2) using nonlinear regression program. Furthermore, taking into account the mentioned above considerations, the graphical method based on Benesi–Hildebrand plot provides a qualitative rather than quantitative rationalization of the experimental results (preliminary parameters).

As it was mentioned in Section 3.3, the α -cyclodextrin-induced changes in the absorption spectra of **I** and **II** are not very pronounced. Therefore, we applied discussed above procedures to calculate ground-state equilibrium constants of the inclusion complexes in aqueous solutions with γ -CD, only. As can be seen in Fig. 7, in the γ -CD system, the graphs of $1/(A-A_0)$ versus $1/[\gamma\text{-CD}]$ demonstrate linear variation with good correlation coefficient ($r = 0.99$, for molecule **I** and, $r = 0.97$ for molecule **II** in γ -CD). This finding confirms the formation of 1:1 inclusion complex between both investigated molecules and γ -CD in the ground state. The K_1^{gr} values obtained from the Benesi–Hildebrand plot are: 32 M^{-1} for molecule **I** (with experimental error being 3) and 66 M^{-1} for molecule **II** (with experimental error 7). Further, using the initial values determined from the linear regression approach, nonlinear regression procedure (see Eq. (6)) was applied in

order to determine K_1^{gr} value. The K_1^{gr} values of **I** and **II** determined by two methods differ only by about 16% and 3%, respectively (see Table 2). It should be noted that, the experimental error in determination of the K_1^{gr} value using Benesi–Hildebrand plot does not differ from this calculated using nonlinear least-squares regression analysis and equals $\sim 10\%$. It is also interesting to note that in aqueous solutions of γ -CD, the K_1^{gr} value determined for molecule **I** differs significantly from this calculated for **II** i.e., the ratio of the ground-state equilibrium constant of **II** and **I** ($K_1^{\text{gr}}(\text{II})/K_1^{\text{gr}}(\text{I})$) is about 2. This indicates that molecule **II** forms in the ground state a stronger inclusion complex of 1:1 stoichiometry as compared to molecule **I**.

To understand the nature of complexation of the investigated dyes in the excited state with α - and γ -CD i.e., the stoichiometry and excited-state equilibrium constants of the inclusion complexes, first of all, double reciprocal Benesi–Hildebrand plot has been drawn (see Fig. 8). It can be seen from Fig. 8 that only the plot of $1/(I-I_0)$ as a function of $1/[CD]$ indeed exhibits a straight line (with a good correlation coefficient $r = 0.99$) for molecule **I** in α -CD system. For both studied molecules in γ -CD, as well as for **II** in α -CD the Benesi–Hildebrand plot can be drawn not as a single straight line but as two straight lines, indicating 1:2 complexations between the guest and the host. As it was mentioned earlier, the two parameters: K_1 and I_1 estimated from the linear regression method (Benesi–Hildebrand plot for low CD concentrations) are employed as initial values. Next, using the initial values (K_1 , I_1) the nonlinear regression procedure (see Eq. (6)) was applied in order to determine K_1 value. Finally, the more reliable values of K_1 and K_2 were calculated (on the basis of the drawings presents in Fig. 9) using Eq. (7). It is worth nothing that, in this calculation, the initial value of K_2 was estimated from Eq. (5) at high CD concentrations. The initial and final values of the excited-state equilibrium constants K_1 and K_2 estimated for both molecules in α - and γ -CD are listed in Table 2.

Analyzing the data assembled Table 2, it can be clearly stated that excited-state K_1 and K_2 values are different when using the two methods (Benesi–Hildebrand (BH) plot and nonlinear regression (NL) procedure) as described above. This confirms Durocher's earlier assumptions that Benesi–Hildebrand plot does not allow the determination of equilibrium constants properly and provides a estimates rather than proper values. Furthermore, it is also evident that the values of equilibrium constants of **II** determined by these two method differ significantly ($K_1^{\text{NL}}(\text{II})/K_1^{\text{BH}}(\text{II}) = 0.36$, $K_2^{\text{NL}}(\text{II})/K_2^{\text{BH}}(\text{II}) = 0.14$ in α -CD and $K_1^{\text{NL}}(\text{II})/K_1^{\text{BH}}(\text{II}) = 2.03$, $K_2^{\text{NL}}(\text{II})/K_2^{\text{BH}}(\text{II}) = 0.32$ in γ -CD), whereas smaller differences were observed for **I** ($K_1^{\text{NL}}(\text{I})/K_1^{\text{BH}}(\text{I}) \approx 0.93$, also in α -CD and $K_1^{\text{NL}}(\text{I})/K_1^{\text{BH}}(\text{I}) \approx 1.5$, $K_2^{\text{NL}}(\text{I})/K_2^{\text{BH}}(\text{I}) \approx 0.78$ in γ -CD). This large difference

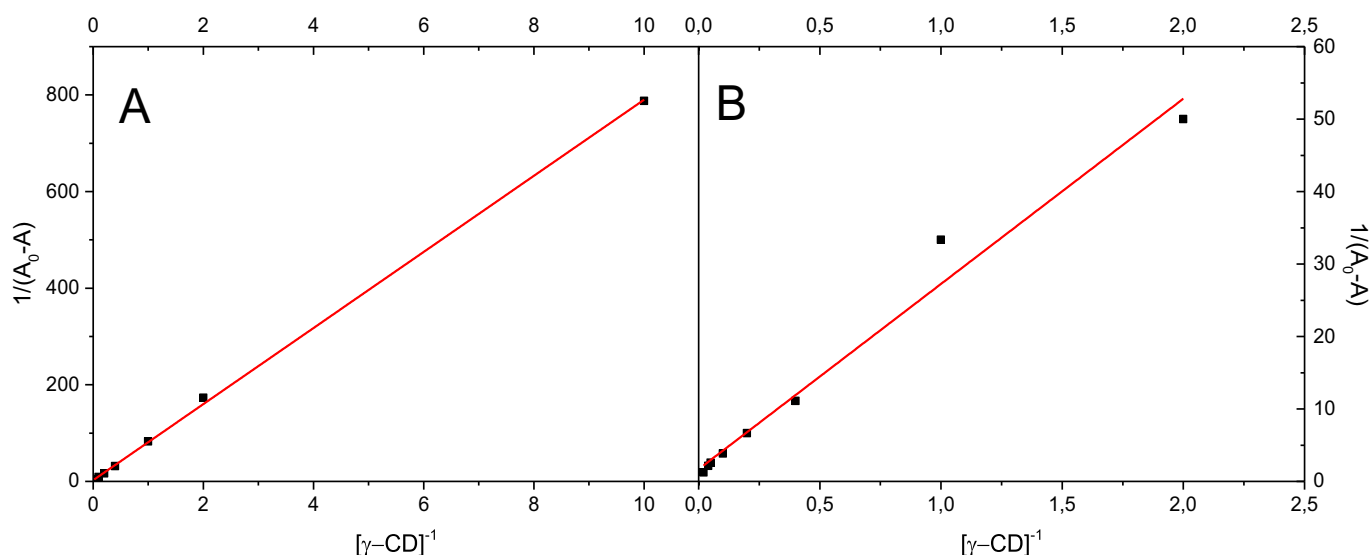


Fig. 7. The Benesi–Hildebrand dependence ($1/(A-A_0)$ vs. $[\gamma\text{-CD}]^{-1}$) for **I** (A) and **II** (B) in aqueous solutions containing different concentrations of γ -CD.

Table 2
Equilibrium constants K_1 (M^{-1}) and K_2 (M^{-1}) of **I** and **II** in α - and γ -CD.

Molecule	Medium	K_1	K_2
I	α -CD	–	–
		$(15 \pm 2)^c$	–
		$(14 \pm 2)^d$	–
γ -CD	$(32 \pm 3)^a/(38 \pm 2)^b$	–	
	$(3.0 \pm 0.8)^c$	$(16 \pm 2)^c$	
	$(4.5 \pm 0.5)^d$	$(12 \pm 1)^d$	
II	α -CD	–	–
		$(587 \pm 63)^c$	$(35 \pm 5)^c$
		$(210 \pm 20)^d$	$(5 \pm 2)^d$
	γ -CD	–	$(11 \pm 2)^e$
		$(66 \pm 7)^a/(68 \pm 6)^b$	–
		$(187 \pm 80)^c$	$(47 \pm 20)^c$
	$(379 \pm 50)^d$	$(15 \pm 2)^d$	
	–	$(15 \pm 3)^e$	

^a Determined by Benesi–Hildebrand method provided by Eq. (1b).

^b Determined by the nonlinear least-squares regression provided by Eq. (6).

^c Determined by Benesi–Hildebrand method provided Eqs. (1a) and (5).

^d Determined by the nonlinear least-squares regression provided by Eq. (7).

^e Determined by lifetime method provided by Eq. (8).

between K_1 and K_2 values for **II** determined using two methods can be understandable taking into account that ESIPT process in molecule **II** is accompanied by more complex spectral features than in molecule **I**

which exhibits only locally excited fluorescence. Such differences in the calculated values of equilibrium constants were also observed for fluorenone (FI) and 4-hydroxyfluorenone (4HOFl) in protic solvents [35]. It has been shown that the hydrogen-bond complexes of FI and 4HOFl are of a different nature i.e., proton-transfer relay chain is formed between oxygen atom and the functional hydroxyl group of 4HOFl.

The analysis of the K_1 and K_2 values collected in Table 2, gives reliable information about stability of 1:1 and 1:2 inclusion complexes. It is clear that molecule **II** forms a stronger inclusion complexes of 1:1 stoichiometry as compared to molecule **I** ($K_1(\text{II}) = 210$ vs. $K_1(\text{I}) = 14$ in α -CD, $K_1(\text{II}) = 379$ vs. $K_1(\text{I}) = 4.5$ in γ -CD). On the other hand, at high CD concentrations, 1:2 inclusion complexes between studied molecules and γ -cyclodextrins are the same stabilized ($K_2(\text{II}) = 15$ vs. $K_2(\text{I}) = 12$). Summarizing the findings obtained from the nonlinear regression analysis, it must be also stated that interactions causing host-guest type inclusion complex are strongest in γ -CD in comparison to α -CD system (see Table 2). This finding is understandable taking into account that the cavity of γ -CD is larger than that of α -CD and γ -CD could more effectively interact with the investigated dyes.

3.5. Fluorescence decay times in aqueous solutions with α - and γ -CD

It is important to remember that the analysis of the fluorescence decay curves of the molecule in aqueous solutions containing different concentrations of cyclodextrins gives reliable information about intermolecular interactions causing host-guest type inclusion complexes.

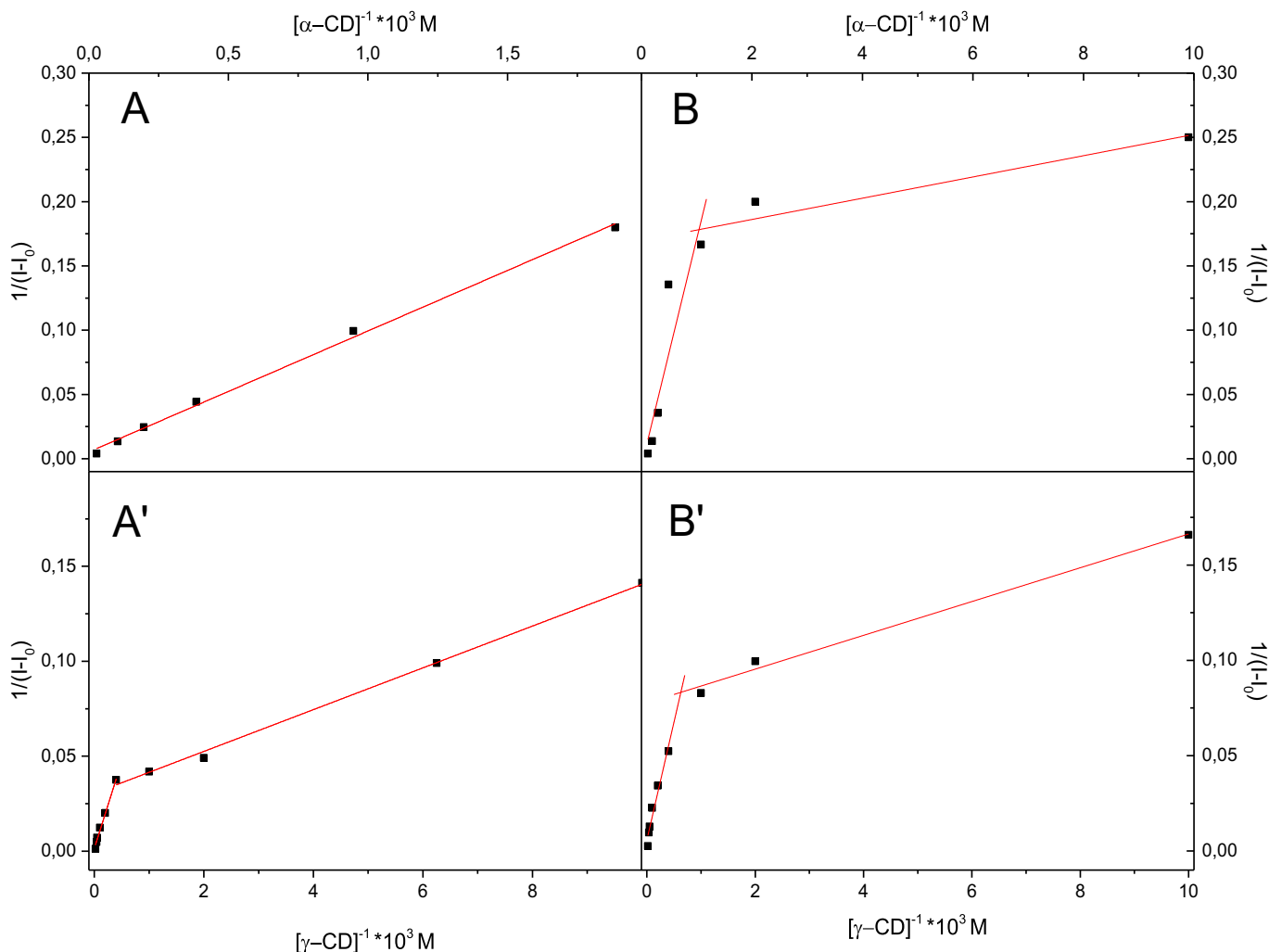


Fig. 8. Double-reciprocal plot for molecule **I** (A, A') and **II** (B, B') complexed to α -CD (A, B) and γ -CD (A', B').

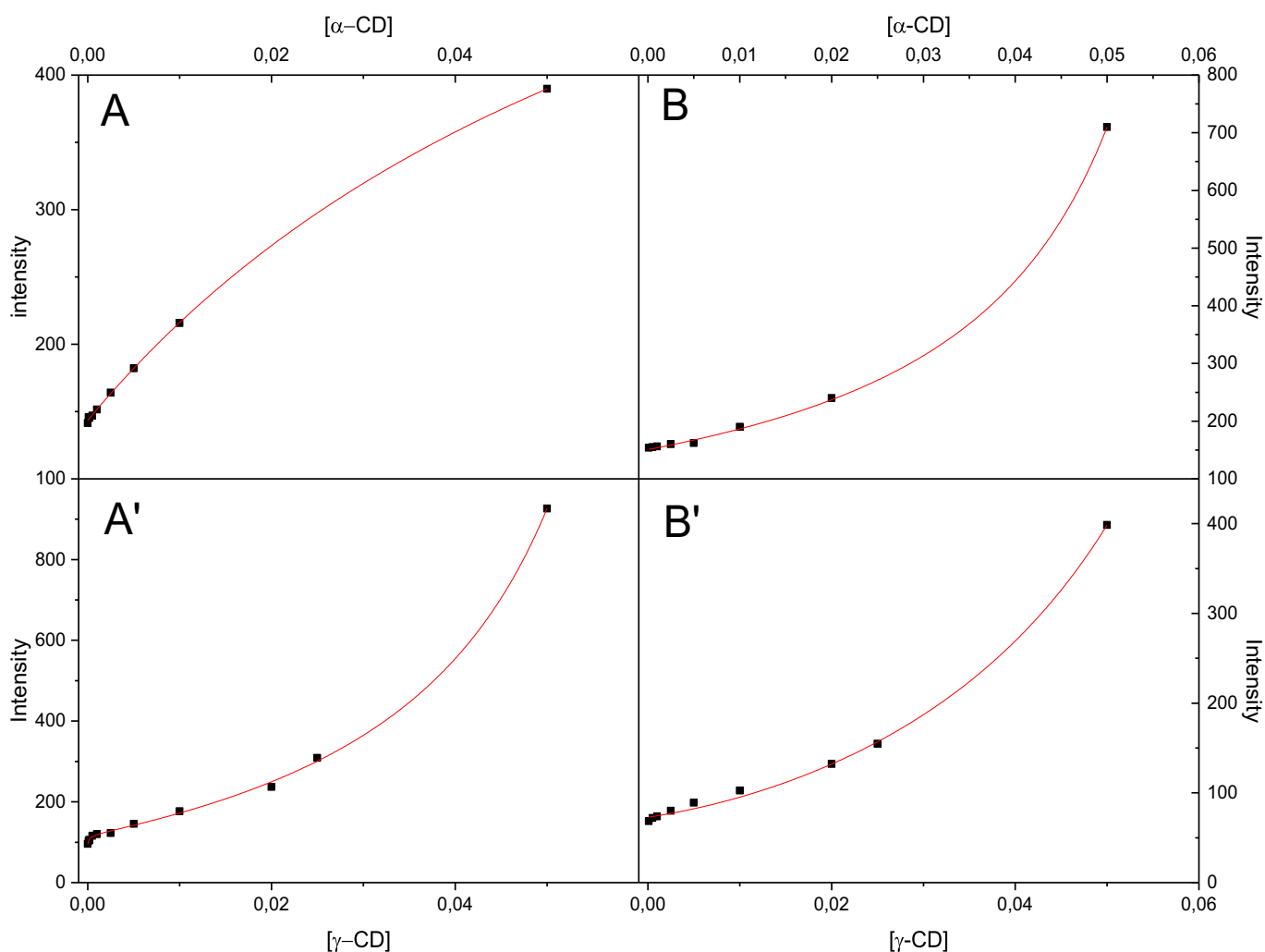


Fig. 9. Plots of the fluorescence intensity versus $[\alpha\text{-CD}]$ (A, B) and $[\gamma\text{-CD}]$ (A', B') for molecule **I** (A, A') and **II** (B, B').

The time-resolved emission spectra were investigated and from them fluorescence decay times of **I** and **II** in aqueous solutions with α - and γ -CD (at different concentrations) were calculated. The excitation was performed at 290 nm. Fluorescence kinetics of **I** and **II** in α - and γ -CD (at low CD concentrations ranging from 0 to 0.005 M) can be reasonably well fitted by biexponential decays with the shorter component of about 60 ps (for molecule **I**), and about 50 ps (for molecule **II**), and longer one of 150–300 ps for **I** and 120–320 ps for **II** (see Table 3). It is important to note that significant deviation of the data from biexponential functions was observed for **I** in γ -CD and for **II** in α - and γ -CD at higher cyclodextrin concentrations (ranging from 0.01 to 0.05 M). Therefore, we carried out triple-exponential analysis, which leads to a large improvement in the χ^2 value. The pre-exponential coefficient (describing the contribution of the i th fluorescence decay component to the total emission), B_i , and decay time of the i th component, τ_i , are shown in Table 3. The average value of χ^2 , describing the goodness of the fitting procedure, for all decay curves equals $\chi^2 = 1.2 \pm 0.2$.

On examining the fluorescence decay times and pre-exponential factors obtained for both investigated dyes in neat water and in α - and γ -CD at the lowest concentrations of cyclodextrin, as well as, based on the fluorescence lifetimes available in literature for these molecules in polar protic solvents [23–27], it is evident that the fast decay component (τ_1) originates from the emission of the excited hydrogen-bonded complex (molecule-H₂O). Furthermore, based on the quantum-chemical calculations, it can be clearly stated that dihydrated form of the parent molecule plays a dominant role in the emission of

excited molecule **I** or **II** in neat water (see Fig. 1 and Table 3). For all studied systems, the pre-exponential factor describing the contribution of the fast component (B_1) decreases with increasing CD concentrations, whereas B_2 factor related to longer component (τ_2) presents the opposite behavior. Taking into account that intermolecular (solute-solvent) hydrogen bonding is reduced upon encapsulation of the studied dye inside CD cavities, the longer lifetime component can be assigned to the emission of the molecule which from 1:1 type inclusion complexes. This suggestion is further supported by the fact that the value of τ_2 decay time correlates well with lifetime of the molecules in neat aprotic solvents [23–27]. Thus, quantitative analysis of the fluorescence kinetics of **I** and **II** in α - and γ -CD confirms conclusion drawn from the evolution of the steady-state spectra i.e., fact that at lowest concentration of cyclodextrin, the contribution from 1:2 complex is negligible. On the other hand, for **I** in γ -CD and **II** in α - and γ -CD in the presence of the highest CD concentration (ranging from 0.01 M to 0.05 M) the fluorescence decay profiles are very well fitted by three-exponential functions, with lifetime component τ_3 contributing quite significantly. For molecule **II** in α - and γ -CD, the τ_3 values differ from τ_2 values by a factor of about two, whereas for **I** in γ -CD the τ_2 and τ_3 values are essentially the same (~200 ps). Taking into account that the intramolecular proton transfer fluorescence of **II** in neat aprotic solvents decays with lifetime of about 0.5 ns, it is clear that τ_3 component observed for **II** in α - and γ -CD at high CD concentration is associated to the fluorescence from the ESIPt state. At high CD concentrations, molecule **II** is entrapped in the two cyclodextrins and new 1:2 inclusion complex is formed. In

Table 3
Fluorescence lifetimes of **I** and **II** in aqueous solutions containing different concentrations of α - and γ -CD.

Medium	Concentration [M]	τ_1 [ps]	B_1 [%]	τ_2 [ps]	B_2 [%]	τ_3 [ps]	B_3 [%]	χ^2	
I									
α -CD	0	60	92	240	8	0	0	1.17	
	0.0005	70	84	185	16	0	0	1.24	
	0.001	94	82	280	18	0	0	1.14	
	0.005	66	74	300	26	0	0	1.22	
	0.01	80	58	260	42	0	0	1.18	
	0.02	70	40	305	60	0	0	1.14	
	0.04	64	21	227	79	0	0	1.04	
	0.05	82	12	284	88	0	0	1.17	
	γ -CD	0	54	88	180	12	0	0	1.11
		0.0005	44	83	146	17	0	0	1.08
0.001		80	74	220	26	0	0	1.21	
0.005		84	78	256	22	0	0	1.14	
0.01		62	66	184	30	120	4	1.16	
0.02		80	58	180	26	212	16	1.21	
0.04		58	32	200	43	147	25	1.18	
0.05		72	22	164	43	206	35	1.24	
II									
α -CD		0	34	88	120	12	0	0	1.27
	0.0005	48	74	205	26	0	0	1.12	
	0.001	32	68	148	32	0	0	1.21	
	0.005	24	64	160	36	0	0	1.14	
	0.01	62	44	180	52	420	4	1.16	
	0.02	38	36	212	48	512	16	1.08	
	0.04	72	24	148	51	447	25	1.21	
	0.05	54	18	160	47	506	35	1.16	
	γ -CD	0	92	64	180	8	0	0	1.27
		0.0005	84	58	226	16	0	0	1.12
0.001		72	54	320	28	0	0	1.14	
0.005		71	46	268	29	0	0	1.21	
0.01		63	33	200	35	460	2	1.16	
0.02		48	28	358	40	520	12	1.13	
0.04		35	20	404	33	600	32	1.08	
0.05		24	34	380	28	580	38	1.19	

such CD internal cavity the intermolecular hydrogen bonding interactions are inhibited and process of excited-state intramolecular proton transfer may occurs. In the case of **II**- γ -CD system at the highest concentrations of cyclodextrin solution also three emissive species contribute to the total emission, which confirms the steady-state experiments indicating the formation of inclusion complexes of different stoichiometry (1:1 and 1:2).

It is worth nothing that Jobe and co-workers [36] have shown that the equilibrium constants can be also determined from time-resolved measurements. According to their work, the equilibrium constant K_2 can be written as:

$$K_2 = \frac{[F(CD)_2]}{[FCD][CD]} = \frac{B_3}{(B_1 + B_2)[CD]} = \frac{B_3}{(1 - B_3)[CD]}, \quad (8)$$

and K_2 value can be calculated from the slope of the plot of $B_3 / (1 - B_3)$ versus $[CD]$. For all studied systems, the $B_3 / (1 - B_3)$ follows a linear dependence on the CD concentrations. Equilibrium constants determined by the time-resolved method are presented in Table 2. As can be seen in Table 2, the equilibrium constant K_2 determined by the nonlinear regression procedure correlates well with the alternatively calculated on the basis of the decay times measurements. Thus, two independent

methods confirm the formation of 1:2 inclusion complexes between **I** and γ -CD, as well as **II** and α -CD or γ -CD in the excited state.

4. Conclusions

The steady-state and time-resolved spectroscopic studies demonstrate that hydrogen-bonding solute-solvent interactions as well as excited-state intramolecular proton transfer process play a significant role in formation of inclusion complexes between investigated fluorophores and α - and γ -CD. A general conclusion from this study is that nonlinear least-squares regression analysis provides an excellent method characterizing interactions causing host-guest type inclusion complexes. Performed analysis indicates that both 1:1 and 1:2 inclusion complexes were formed between studied compounds and α - and γ -cyclodextrins.

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Preferential encapsulation of different conformers of ethyl 5-(4-dimethylaminophenyl)-3-amino-2,4-dicyanobenzoate in γ -cyclodextrins

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ABSTRACT

This work represents a continuation of our systematic spectroscopic studies of organic donor (D)-acceptor (A) molecule - ethyl 5-(4-dimethylaminophenyl)-3-amino-2,4-dicyanobenzoate (EDMAADCy) - which undergoes excited-state intramolecular charge transfer (ICT) process. The relation between concentration-dependent spectral behaviour and molecular conformations of EDMAADCy has been studied using steady-state and time-resolved spectroscopic techniques and quantum-chemical calculations. The observation- and excitation-wavelength dependence of the fluorescence excitation and emission spectra, as well as fluorescence decay profiles observed at different solute concentrations were interpreted in terms of concentration-induced planarization model. Then, investigated dye has been employed as a guest to probe ICT within the interior of the host γ -cyclodextrins (γ -CD) in DMSO and DMSO-water binary mixture. The obtained spectroscopic results were used to calculate, according to Benesi-Hildebrand's plot, equilibrium constants of the EDMAADCy- γ -CD inclusion complexes. Finally, performed analysis indicates also that specific solute-solvent interaction (hydrogen bonds) occurring in the DMSO-water system has a significant influence on the stability of inclusion complexes.

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1. Introduction

It is well-known that interaction between a luminescent molecule and its microenvironment has an important meaning in photophysics, photochemistry and related sciences [1–3]. In the past few decades, there has been considerable interest in the photophysical and photochemical properties of donor (D)-acceptor (A) organic molecules with possible rotation of the fragments. The interest to the D-A system is not only academic (excited-state intramolecular electron transfer belongs to the most fundamental process in chemistry and biochemistry) [3–5], but also is due to their potential applications in material science (non-linear optical materials), photonic technologies (optical computing, optical switching, second harmonic generators (SHG)) and fluorescent probes [6–9].

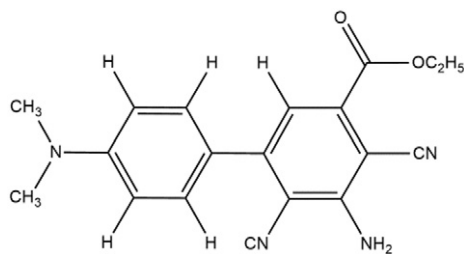
Biphenyl derivative, ethyl 5-(4-dimethylaminophenyl)-3-amino-2,4-dicyanobenzoate (EDMAADCy) (see Scheme 1), organic molecule containing separate electron donor and electron acceptor groups belongs to a class of D-A molecules that undergo intramolecular charge

transfer process upon excitation into the singlet excited state. On the basis of the analysis of the obtained spectroscopic data and semiempirical quantum-chemical calculations, we have shown [9–13] that the fluorescence spectra of EDMAADCy in medium-polar solvents consist of two bands: the locally-excited, short-wavelength (SW) emission - corresponding to the molecular conformation in which the donor and acceptor moieties are orthogonal to each other and the long-wavelength (LW) emission - assumed to originate from the singlet excited state with charge transfer (ICT) character and almost planar geometry. Because spectroscopic properties of the investigated dye are very sensitive to the microenvironment, it has been exploited to study various inhomogeneous microenvironments such as polymeric systems, rigid glass [9,10]. It was shown that ICT fluorescence full-width at half maximum shows a linear dependence on the time of methyl methacrylate polymerization, which predestines the EDMAADCy molecule to be used as a fluorescent probe for monitoring the polymerization process.

In recent years, considerable attention has been focused on understanding and controlling supramolecular interactions between some organic and inorganic molecules and well-recognized macrocyclic hosts: cyclodextrins, cucurbit[n]urils, crown ethers, calixarenes and cyclophanes [14–17]. Systematic investigations of the host-guest type inclusion complexes are very interesting issue not only from a purely

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Scheme 1. Chemical structure of ethyl 5-(4-dimethylaminophenyl)-3-amino-2,4-dicyanobenzoate (EDMAADCy).

theoretical point of view (i.e. explaining the role of supramolecular assemblies in the photophysics of studied dye) but also because macrocyclic compounds are widely applied in various fields (medicine, nanomachines, smart materials, pharmaceutical chemistry, analytical chemistry, food technology, chemical synthesis, and catalysis) [18–21]. Recently, our group has contributed to this field of interest by introducing the role of specific solute-solvent interactions (H-bonding) and excited-state intramolecular charge (proton and electron) transfer process in the formation of inclusion complexes between fluorophore and cyclodextrins [22–24]. Although the interactions of cyclodextrins with D-A molecules have been reported, the cyclodextrin effect on molecular systems with inhomogeneous distribution of different conformers in the ground and excited states (each possessing different absorption and relaxation properties) have received less attention. Taking into account above, in the present paper, the effect of γ -cyclodextrins (γ -CD) on both emission modes: locally excited (LE) and intramolecular charge transfer (ICT) of the fluorescence spectrum of EDMAADCy in DMSO and DMSO-water binary mixture has been investigated using steady-state and time-resolved fluorescence techniques. Because the main purpose of this work was to investigate the influence of molecular conformation of the investigated D-A dye on the formation of inclusion complexes with cyclodextrins, the basic, concentration-dependent luminescent characteristics (absorption, fluorescence excitation, and emission spectra, as well as fluorescence decay times) were measured in DMSO and DMSO-water binary mixture in the presence of γ -CD. The relation between concentration-dependent spectral behaviour and molecular conformations of EDMAADCy was interpreted in terms of concentration-induced planarization model.

2. Experimental details

The method of synthesis of ethyl 5-(4-dimethylaminophenyl)-3-amino-2,4-dicyanobenzoate (EDMAADCy) is based on modified, previously described, the procedure given by Milart and Sepi \acute{o} [25] and it has been described elsewhere [11]. The solvents used (dimethyl sulfoxide (DMSO) and doubly distilled and deionized water (H_2O)) for spectroscopic studies were of the highest grade commercially available and were used without further purification. Cyclodextrins were purchased from Aldrich Chemical Co. and used without further purification.

Steady-state absorption and fluorescence measurements at room temperature were carried out using a computer-controlled Shimadzu UV-2401 PC spectrophotometer and Shimadzu RF-5301 PC spectrofluorometer. Fluorescence measurements were carried out using the triangular cuvette with frontal excitation to minimize the effect of reabsorption. The emission was observed perpendicular to the direction of the exciting beam. Thus the errors due to fluorescence reabsorption were reduced in a way that mathematical corrections were superfluous. The fluorescence lifetimes were measured using an Edinburgh Instruments, single-photon counting system (FLS920P spectrometer). The apparatus utilizes for the excitation a picosecond diode laser generating pulses of about 58 ps at 373 nm. Short laser pulses in combination with a fast microchannel plate photodetector and ultrafast electronics make a successful analysis of fluorescence decay signals with a

resolution of few picoseconds possible. The fluorescence light was monitored at the magic angle in respect to the polarized exciting beam.

All quantum-chemical calculations were performed using CAChe WS 5.04 program. The minimalized ground-state energy and geometrical spatial structure as a function of rotation coordinate were calculated using the PM3 semiempirical molecular orbital method at the Restricted Hartree-Fock (RHF) level, including single excitation configuration interaction (CIS). The configuration interaction matrix was compared with all electronic states taking into account valence electrons only. The ZINDO/S method has been used in calculations of the singlet Franck-Condon (FC) state energies for the UV-Visible spectra corresponding to transition moments and oscillator strengths *f*.

3. Results and discussion

The analysis of the concentration-dependent spectroscopic properties of investigated D-A biphenyl derivative in different microenvironments should begin by recalling that geometrical conformation of EDMAADCy corresponding to the energy minimum in S_0 state is not planar. The ground-state rotational potential is essentially flat in the range from 60° to 120° with an energy minimum for almost perpendicular conformation ($\varphi = 105^\circ$) that is only 0.07 eV below the conformation with $\varphi = 60^\circ$. It causes that EDMAADCy forms typically spectrally an inhomogeneous system i.e., conformers having different geometry can absorb and emit at different energies [11]. We have recently shown that the fluorescence spectra of the investigated fluorophore are strongly solvent dependent. In medium-polar solvents, fluorescence spectrum consists of two bands: short-wavelength (SW), which corresponds to the emission from the locally excited state (perpendicular conformation) and long-wavelength (LW) which originates from emission of solvent-relaxed ICT state (almost coplanar conformation), whereas in non-polar and strongly polar environments, fluorescence spectrum possesses only a single, broad short-wavelength band. Our previous investigations and detailed analysis of literature reports unanimously point that the observed locally-excited fluorescence in non-polar and strongly polar solvents originates mainly from the emission of the excited ethyl 3-amino-2,4-dicyanobenzoate (acceptor moiety) [9–13].

Since the main purpose of this work was to investigate the influence of molecular conformation of EDMAADCy on the formation of inclusion complexes with cyclodextrins, the basic luminescent characteristics (steady-state absorption, fluorescence excitation, and emission spectra, as well as fluorescence decay times) were measured in DMSO and, because of the limited solubility of EDMAADCy in water, in DMSO-water (4:1 ratio) binary mixture. The first system comprises the strongly polar DMSO. In this medium the fluorescence spectrum possesses a single, broad short-wavelength band originates from the emission of molecular conformation in which the donor and acceptor moieties are orthogonal to each other. The second system - solvent mixture - contains water molecules which are capable of forming hydrogen bonds with the investigated dye. In this binary mixture, the fluorescence spectrum consists of two bands at room temperature. The short-wavelength band corresponds to the emission from the locally excited state (mainly emission of the excited acceptor moiety) whereas the long-wavelength band is related to emission of EDMAADCy conformers with strong charge-transfer character and planar geometry. This allows us to examine the nature of complexation in systems where only perpendicular, as well as perpendicular and planar conformers, occur.

3.1. Steady-state and time-resolved spectroscopic studies in DMSO

Fig. 1 shows the absorption (Fig. 1A) and fluorescence excitation spectra (recorded by detection at $\lambda_{obs} = 475$ nm (Fig. 1B) and 510 nm (Fig. 1C)) at different solute concentration (from $5 \cdot 10^{-6}$ to $5 \cdot 10^{-4}$ M) in polar DMSO. Additionally, to facilitate the analysis of experimental results, all fluorescence excitation spectra were normalized by scaling the

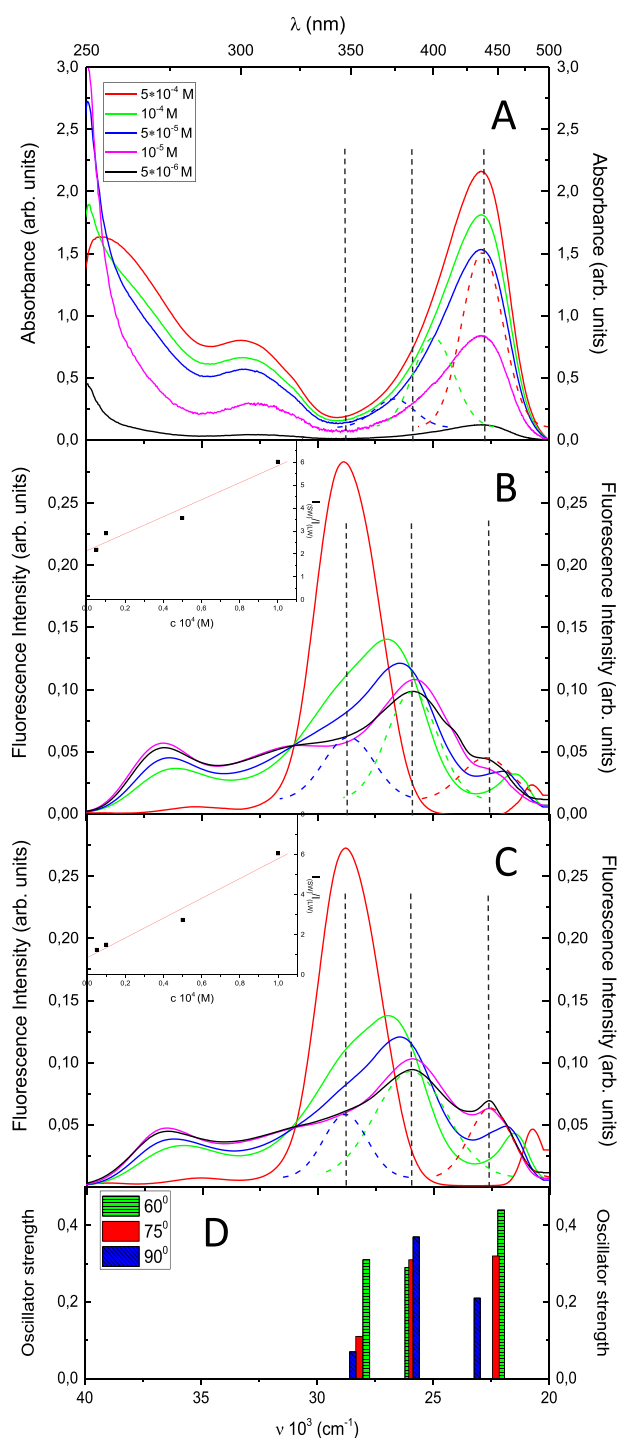


Fig. 1. Absorption (A) and normalized fluorescence excitation spectra of EDMAADcy recorded by detection at $\lambda_{\text{obs}} = 475$ nm (B) and $\lambda_{\text{obs}} = 510$ nm (C) in DMSO at different solute concentrations: $c = 5 \cdot 10^{-6}$ M, 10^{-5} M, $5 \cdot 10^{-5}$ M, 10^{-4} M, $5 \cdot 10^{-4}$ M. The bars presented in panel D give the theoretical electronic state energy values and corresponding oscillator strengths for different EDMAADcy molecule conformations ($\varphi = 60^\circ$, 75° and 90°). Insert - Plot of the fluorescence intensity of the SW and LW fluorescence excitation bands ($I_{\text{SW}}/I_{\text{LW}}$) versus solute concentration.

area under the spectrum to be equal to unity. Then, each of excitation spectra corresponds to the emission of the same number of emitting EDMAADcy molecules.

The broad, structureless long-wavelength absorption band in DMSO occurs between 325 and 500 nm with λ_{max} at about 440 nm. Concentration-dependent measurements did not show any significant changes in the steady-state absorption spectra, whereas the strong

dependence of the fluorescence excitation spectrum on both concentration and observation wavelengths was observed. As can be seen in Fig. 1, the concentration- and observation-wavelength-dependent fluorescence excitation spectra in DMSO in the range above 325 nm consist of two overlapping bands centered at about 340–380 nm (SW band) and 440–470 nm (LW band). Furthermore, the folded structure of the band, SW fluorescence excitation band clearly suggests that the analyzed band consists of two electronic transitions. It is also clear from Fig. 1 that detection at blue (475 nm) and red (510 nm) edges of the emission spectrum resulted in the changes in the relative intensities of these two fluorescence excitation bands. When the observation wavelength is increased from 475 nm to 510 nm, the participation of the long-wavelength components (~ 440 nm) in the total excitation spectrum visibly increases. Such correlation is not observed only at high dye concentrations i.e., for $c \geq 5 \cdot 10^{-4}$ M. In this case, the fluorescence excitation spectrum is independent on the observation wavelength. These results confirm our previous findings that for EDMAADcy (at solute concentrations $c < 5 \cdot 10^{-4}$ M) a distribution of spatial conformers (lie in difference of rotational angles between donor and acceptor moieties) is always present [11].

It is important to note here, the fluorescence excitation spectra also strongly depend on the solute concentration. In order to clarify the role of solute concentration in photophysics of the studied dye, the spectral evolution is analyzed in the concentration range from $5 \cdot 10^{-6}$ to $5 \cdot 10^{-4}$ M. To further characterize the folded structure of obtained concentration-dependent spectra, we used a linear combination of Gaussian functions to decompose the absorption and fluorescence excitation (recorded by detection at 475 nm and 510 nm) spectra into three Gaussian profiles. Fig. 1 shows the example of such deconvolution indicating the presence of three electronic transitions ($S_0 \rightarrow S_1$, $S_0 \rightarrow S_2$ and $S_0 \rightarrow S_3$) centered at about 430, 380 and 340 nm. Analyzing the concentration-dependent fluorescence excitation spectra obtained at two different observations, it is found that upon increasing the solute concentration from $5 \cdot 10^{-6}$ to $5 \cdot 10^{-4}$ M, the intensity of SW band (consists of two electronic transitions $S_0 \rightarrow S_2$ and $S_0 \rightarrow S_3$) continuously increases and at the same time its maximum becomes more and more blue-shifted. Finally, at high solute concentration, the band centered at around 340 nm becomes dominant. This behavior is accompanied by decrease of the intensity of LW excitation band (centered at around 430 nm) with a simultaneous red-shift of its maximum. In order to better visualize the obtained changes in concentration-dependent excitation spectra, the ratio of the fluorescence intensity of the LW and SW fluorescence excitation bands ($I_{\text{exc}}(\text{SW})/I_{\text{exc}}(\text{LW})$) is presented in the insert in Fig. 1. As can be seen, the ratio of $I_{\text{exc}}(\text{SW})/I_{\text{exc}}(\text{LW})$ follows a linear dependence on the solute concentration ranging between $5 \cdot 10^{-6}$ to $5 \cdot 10^{-4}$ M. The above results, combined with our previous studies (steady-state, time-resolved spectroscopic measurements in different solvents and quantum-chemical calculations), confirm that systematic changes in the relative intensities of these two bands are caused by slight changes in the distribution of spatial conformers [9–13].

Fig. 2 presents the concentration and excitation-wavelength dependence of fluorescence spectra of EDMAADcy in strongly polar DMSO. In order to facilitate analysis of spectral behavior and to clarify the role of the solvent polarity in the photophysics of studied biphenyl derivative, fluorescence spectra registered for two different excitation wavelengths ($\lambda_{\text{exc}} = 380$ and 440 nm) in medium-polar ethyl acetate are given for comparison. As can be seen in Fig. 2, the emission spectrum in DMSO possesses a single, broad fluorescence band. Moreover, at the lowest solute concentration ($c = 10^{-5}$ M) the emission spectrum in DMSO is quite similar to the short-wavelength spectrum observed in medium polar solvents (see Fig. 2). A scrupulous analysis of the emission spectra presented in Fig. 2 allows to state that the fluorescence intensity distribution not only depends on the excitation wavelength (as the excitation wavelength is shifted to the red, the position of the fluorescence maximum is also red-shifted), but also on the dye concentration. Upon

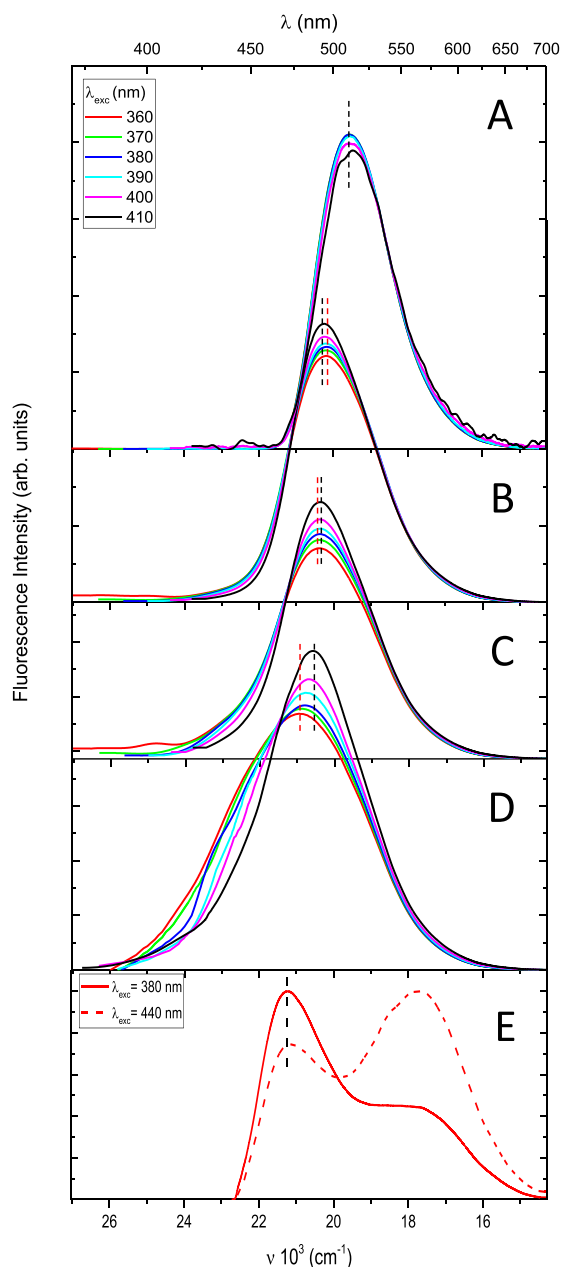


Fig. 2. Normalized fluorescence spectra of EDMAADCy in DMSO at different solute concentrations: $c = 5 \cdot 10^{-4}$ M (A), 10^{-4} M (B), $5 \cdot 10^{-5}$ M (C) and 10^{-5} M (D) as a function of λ_{exc} . Fluorescence spectra of the studied compound in ethyl acetate for two different λ_{exc} (380 nm and 440 nm) are given for comparison.

increasing the EDMAADCy concentration, the fluorescence maximum position is shifted to the longer wavelengths. Moreover, progressive increase of the solute concentration leads to decreasing of the full-width at half-maximum ($\Delta\nu_F^{FWHM}$) of the emission band. When the solute concentration increased from $5 \cdot 10^{-6}$ to $5 \cdot 10^{-4}$ M the $\Delta\nu_F^{FWHM}$ value decreases from 3500 to 2250 cm^{-1} . In order to make these behaviors more evident the position of the fluorescence maximum versus the excitation wavelengths and fluorescence full-width at half maximum versus solute concentration are shown in Fig. 3. Since the band broadening can rise from the distribution of spatial conformers, the very sharp decrease of the $\Delta\nu_F^{FWHM}$ value at high solute concentration indicates that heterogeneity of the spectral centers significantly depends on the solute concentration i.e., an increase of the solute concentration leads to the system becoming more spectrally homogeneous. This is also confirmed by observed the excitation-wavelength dependence of fluorescence

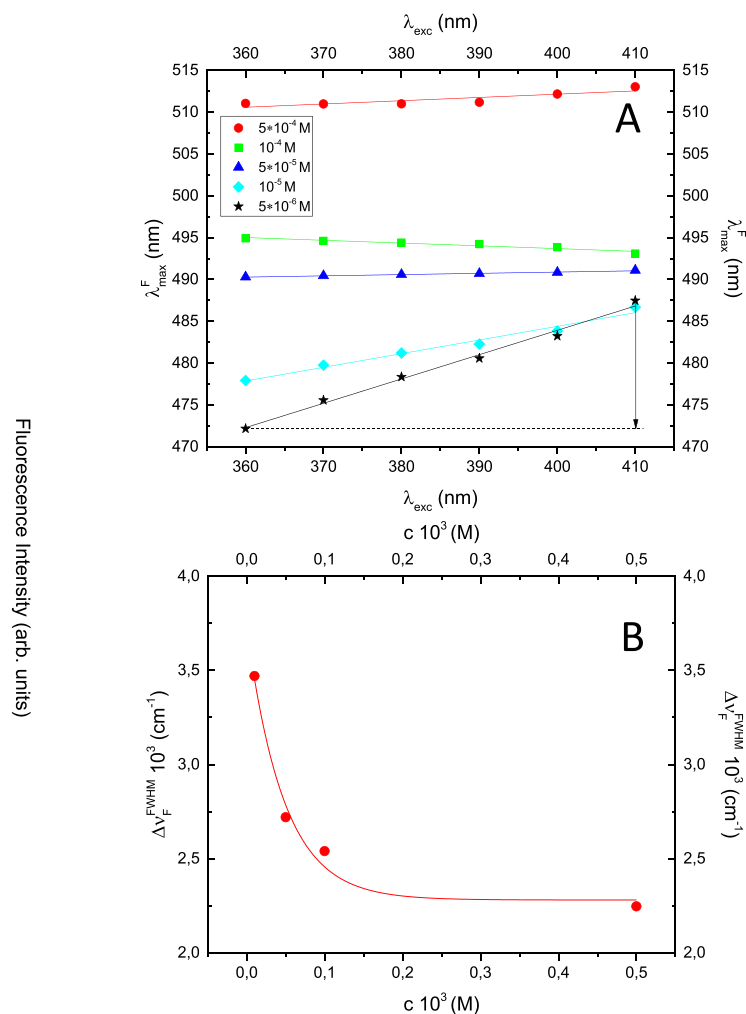
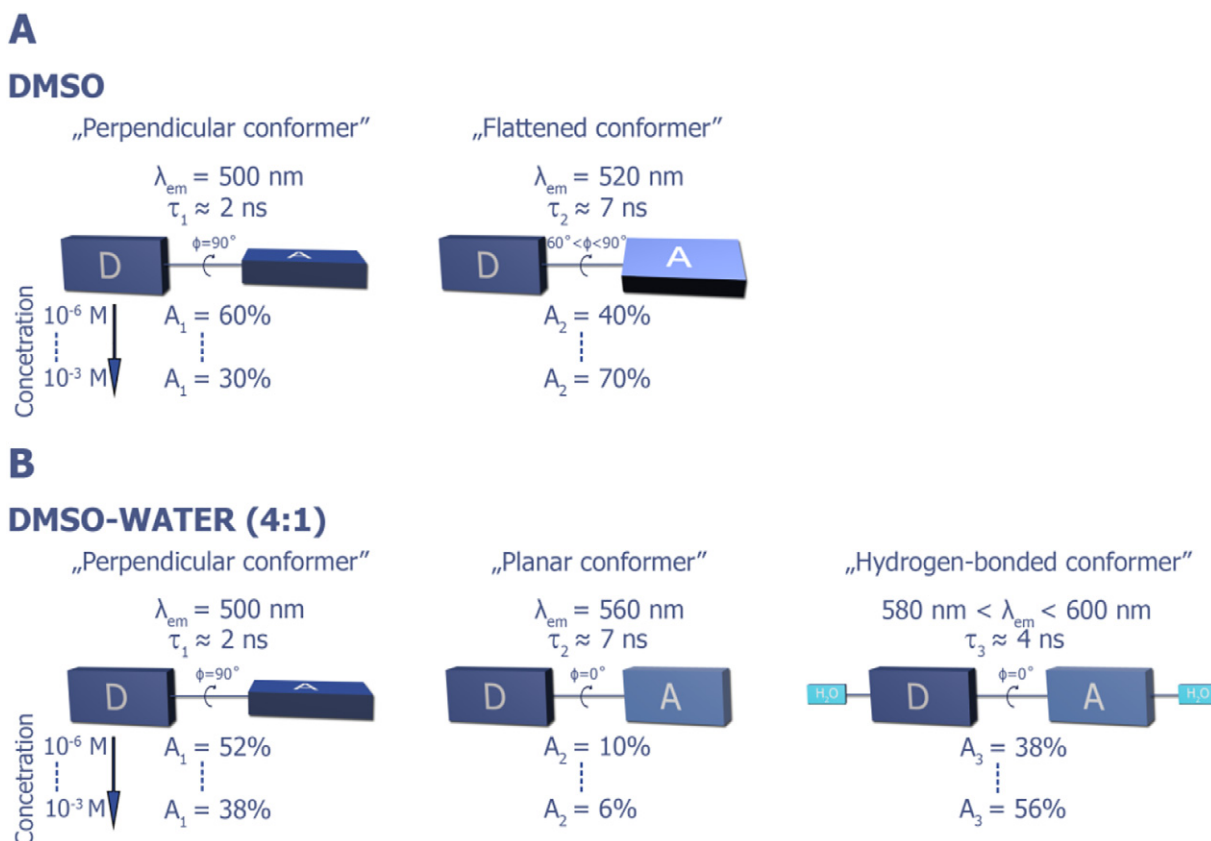


Fig. 3. The excitation-wavelength dependence of the fluorescence intensity maximum position of EDMAADCy in DMSO at different solute concentrations (A). Fluorescence full-width at half maximum ($\Delta\nu_F^{FWHM}$) as a function of solute concentration (B).

spectra i.e., the position of the maximum of emission band shows dependence on the excitation wavelengths only at the low EDMAADCy concentrations (at the high dye concentrations ($c > 10^{-4}$ M), the ν_F^{max} does not depend on the excitation wavelength (see Fig. 3A)).

Thus, the observation- and excitation-wavelength dependences of the steady-state fluorescence excitation and emission spectra, in connection with our previous quantum-chemical calculations [9–13], confirm that for investigated molecule in DMSO a distribution of spatial conformers is also present. When the excitation is carried out at the maximum of the absorption band, the conformers having perpendicular geometry are excited. As the λ_{exc} is gradually shifted toward longer wavelengths, the flattened conformers ($60^\circ < \varphi < 90^\circ$) are also selectively excited. Moreover, the analysis of the obtained concentration-dependent excitation and emission spectra suggests that at higher solute concentrations greater participation of flattened conformers occurs.

Spectral changes, commonly observed as a function of a concentration, are generally interpreted in terms of the intra- and intermolecular effects (specific aggregation, excimer formation and/or intramolecular planarization) [26–31]. While it is possible that most examples of spectral behaviors of organic D-A compounds reported in the literature involve planarization and aggregation, their relative contributions have remained a matter of speculation. Conformational changes should result in relatively small spectral shifts retaining high emission yields and vibrational structure, whereas aggregation effects should give rise to more shifted, excimer-like emission, with a loss of vibrational structure



Scheme 2. Schematic representation of concentration effect on the geometrical structure of EDMAADcy in DMSO (A) and DMSO-water (B) systems.

and shorter lifetimes that result from selfquenching [26–31]. Very interesting interpretation of the concentration-induced spectral changes of 1,4-bis(9-ethynylantraceny)benzene was offered by Garcia-Garibay et al. [29–31]. They showed that the wavelength dependence of the fluorescence excitation and emission spectra observed at different concentrations of 1,4-bis(9-ethynylantraceny)benzene in THF were due to concentration-induced planarization of studied dye rather than formation of observable cofacial excimers. Relatively disordered aggregates lead to planarization of the phenyleneethylene moieties long before a structurally demanding and spectroscopically observable cofacial interaction may occur [30].

To test whether the concentration-dependent changes in the fluorescence excitation and emission spectra of EDMAADcy in DMSO originate from the coexistence of the investigated dye in different geometries a series of conformational, semiempirical quantum mechanical calculations were carried out. We performed theoretical calculations on the investigated D-A molecule with fixed angle between the donor and acceptor moieties. Three electronic transitions (up to $30,000 \text{ cm}^{-1}$, $S_0 \rightarrow S_1$, $S_0 \rightarrow S_2$ and $S_0 \rightarrow S_3$) and their oscillator strengths (see bars under the Fig. 1D) have been calculated for three different EDMAADcy conformations ($\phi = 90^\circ$, 75° and 60°) in DMSO using CNDO/S-CI method. Analyzing the steady-state spectroscopic data assembled in Fig. 1A, B, C in connection with the graphical presentation of the theoretical calculations (Fig. 1D) and our previous finding that the ground-state rotational potential is essentially flat in the range from 60° to 120° , one can state that the observation- and excitation-wavelength dependence of the excitation and fluorescence spectra of EDMAADcy in DMSO is caused by the presence of various conformers having different geometries ($60^\circ < \phi < 90^\circ$) with different absorption and emission properties. The observed concentration-dependent spectral behavior of the studied molecule in DMSO i.e., the red-shift of the LW fluorescence excitation band located at around 440 nm, as well as the gradual increase in the fluorescence intensity of the SW band

located at about 340 nm caused by increasing solute concentration are understandable in terms of formation of different EDMAADcy conformers going from mainly twisted ($\phi = 90^\circ$) (low solute concentration) to flattened ($\phi = 60^\circ$) (high solute concentration) - concentration-induced planarization (see Scheme 2A). As can be seen in Fig. 1D, the positions of the $S_0 \rightarrow S_1$, and $S_0 \rightarrow S_3$ transitions undergo a pronounced red-shift on changing the angle between the donor and acceptor moieties from $\phi = 90^\circ$ to 60° with a simultaneous increase of their oscillator strength value. Naturally, the participation of the flattened conformers in the excitation spectrum is higher at higher solute concentration. Moreover, the fluorescence excitation spectrum

Table 1

Fluorescence lifetimes of EDMAADcy in DMSO and DMSO-water (4:1) systems at different solute concentrations.

Solute concentration (M)	λ_{obs} (nm)	τ_1 (ns)	A_1 (%)	τ_2 (ns)	A_2 (%)	τ_3 (ns)	A_3 (%)	χ^2
DMSO								
$5 \cdot 10^{-6}$	470	2.71	60	6.83	40	–	–	1.27
	510	1.92	54	6.04	46	–	–	1.14
$5 \cdot 10^{-5}$	470	2.68	54	7.14	46	–	–	1.31
	510	2.13	39	5.92	61	–	–	1.08
$5 \cdot 10^{-4}$	470	2.92	37	6.34	63	–	–	1.13
	510	2.51	32	5.93	68	–	–	1.17
DMSO-H ₂ O (4:1)								
$5 \cdot 10^{-5}$	470	2.14	52	7.21	10	4.48	38	1.08
	550	0.84	38	6.82	4	3.56	58	1.31
10^{-4}	470	2.41	46	6.11	8	4.53	46	1.27
	550	0.68	34	7.38	10	3.53	56	1.10
$2.5 \cdot 10^{-4}$	470	2.04	40	6.21	9	4.21	53	1.07
	550	0.71	30	7.25	6	3.23	64	1.11
$3.5 \cdot 10^{-4}$	470	2.38	41	6.83	5	4.37	54	1.30
	550	0.53	28	7.18	9	3.36	63	1.18
$5 \cdot 10^{-4}$	470	1.91	38	6.23	6	4.46	56	1.11
	550	0.63	29	6.93	3	3.37	68	1.08

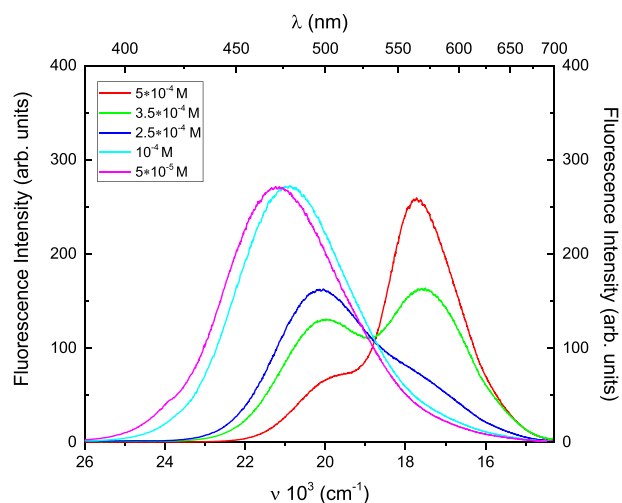


Fig. 4. Fluorescence spectra of EDMAADcy in DMSO-water (4:1) system at different solute concentrations from $5 \cdot 10^{-5}$ to $5 \cdot 10^{-4}$ M upon excitation at 360 nm.

(independent on the observation wavelength) registered at high solute concentrations ($c > 10^{-4}$ M), can be assigned mainly to the emission of flattened EDMAADcy conformers ($\varphi = 60^\circ$). This suggestion is further supported by the fact that for $c > 5 \cdot 10^{-4}$ M, the fluorescence spectrum is independent on the excitation wavelength i.e., an increase of the

solute concentration leads to the system becoming more spectrally homogeneous.

In order to understand the nature of concentration-dependent fluorescence spectra of investigated D-A molecule in DMSO, we have carried out time-resolved measurements. It is well-known that conformational changes should have significant contributions to the decay profiles registered at different solute concentrations. The fluorescence decays of the EDMAADcy were measured upon excitation at 370 nm and detection at two different wavelengths (short-wavelength – 470 nm and long-wavelength - 510 nm) and they were fitted to a biexponential decays with the shorter component τ_1 (1.92–2.92 ns) and the longer one τ_2 (5.92–7.14 ns). The participation of the time decay components of two emitting centers in the total emission distinctly depends on the solute concentration as well as detection wavelength. The data for various solute concentrations are collected in Table 1. According to our previous papers [9–13], we can state that the shorter decay component originates from the emission of perpendicular conformer ($\varphi = 90^\circ$), whereas the τ_2 decay time is attributed to the flattened conformer emission. Upon increasing the solute concentration from $5 \cdot 10^{-6}$ to $5 \cdot 10^{-4}$ M, the preexponential factor describing the contribution of fast decay component decreases from 60% to 37% for detection at 470 nm and from 54% to 32% for detection at 510 nm. In the meantime, the preexponential factor describing the contribution of slow decay component increases from 40% to 63% for detection at 470 nm and from 46% to 68% for detection at 510 nm.

Quantitative analysis of the kinetics confirms the conclusions drawn from the evolution of concentration-dependent steady-state excitation and emission spectra described above i.e., fact that at lowest solute

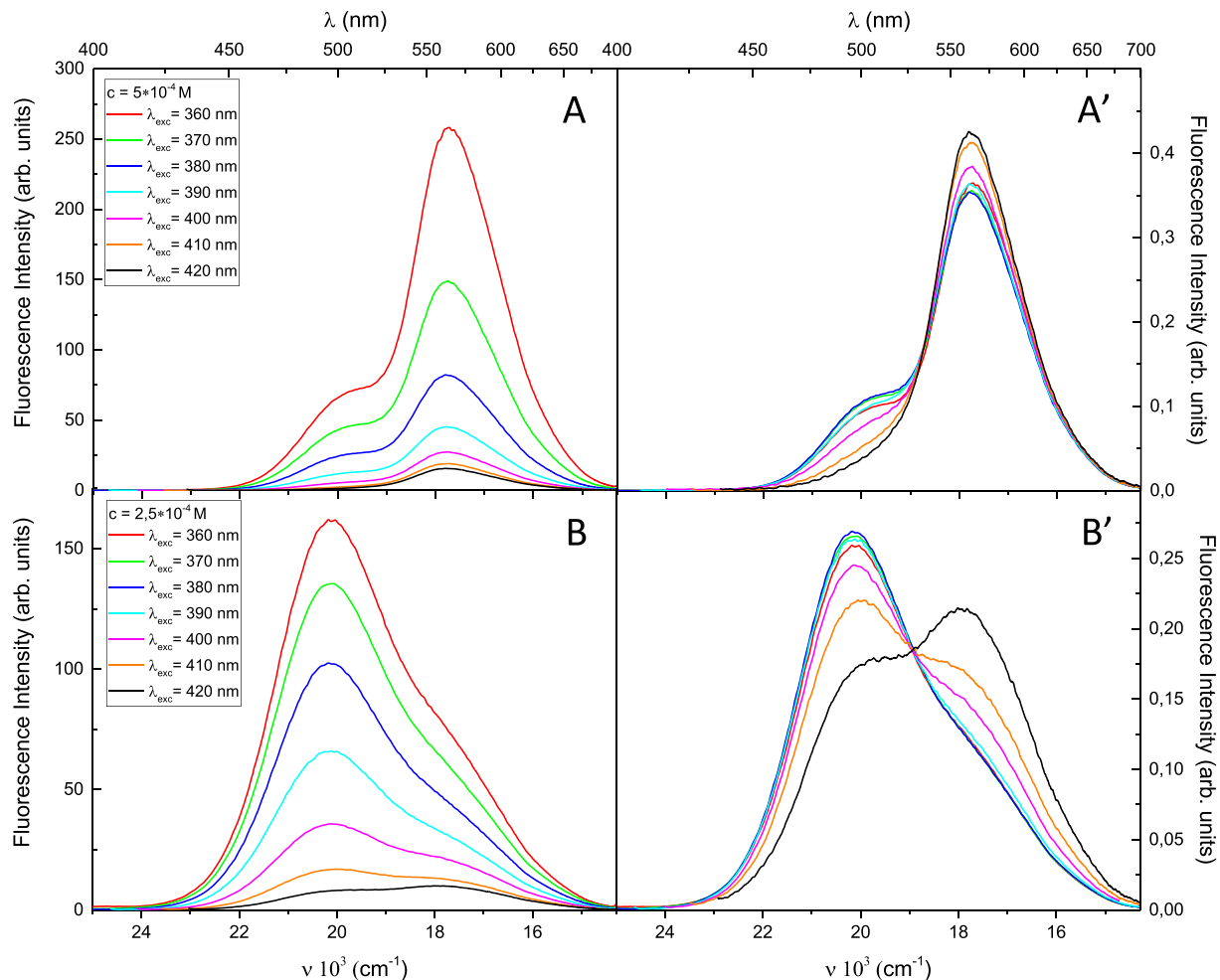


Fig. 5. Excitation-wavelength dependence of fluorescence (A, B) and normalized fluorescence spectra (A', B') of EDMAADcy in DMSO-water (4:1) system determined at two different solute concentrations.

concentration emission originates from radiative deactivations of molecules in which two moieties (donor and acceptor) are orthogonal to each other (mainly) and flattened conformers, whereas an increase of the solute concentration leads to concentration-induced planarization process (geometrical structures of EDMAADCy going from mainly twisted to mainly flattened) (see Scheme 2A). As illustrated in Scheme 2A, we propose (support for this hypothesis was also found by an analysis of quantum-chemical calculations) that relatively disordered aggregates may lead to planarization of the donor and acceptor moieties long before a structurally demanding and spectroscopically observable cofacial interaction may occur.

3.2. Steady-state and time-resolved spectroscopic studies in DMSO-water binary mixture

Fig. 4 shows fluorescence spectra of EDMAADCy in DMSO-H₂O (4:1) at different solute concentrations upon excitation at 360 nm. For dilute solution ($c = 5 \cdot 10^{-5}$ M) of the investigated dye, the fluorescence spectrum has emission peak at around 460 nm accompanied by a weak shoulder at around 560 nm. Upon increasing solute concentration, the maximum of the SW emission band shifts to the red with a simultaneous decreasing of its intensity. This behavior is accompanied by an increase of the intensity of LW emission band. Noticed spectral dependences can be also understandable in terms of concentration-induced planarization model i.e., at high concentrations the distance between EDMAADCy molecules becomes closer enough to the significant solute-solute intermolecular interactions causing planarization of D-A dye. This is also confirmed by the observed concentration-dependent fluorescence excitation spectra, recorded at different detection wavelengths (not presented here), which show the same general dependence on increasing solute concentration as observed for DMSO medium.

It is worth noting that fluorescence spectra of EDMAADCy in DMSO-H₂O are also strongly excitation-wavelength dependent. As can be seen in Fig. 5, when the excitation wavelength is increased from 360 nm to 420 nm, the fluorescence intensity strongly decreases. Moreover, analyzing the normalized fluorescence spectra (Fig. 5A' and B'), it is evident that upon increasing excitation wavelength SW fluorescence band decreases with a simultaneous increase of LW band. Additionally, isosbestic point, indicating the presence of equilibrium between two conformers, is observed. The above results point that the investigated biphenyl derivative in DMSO-H₂O established also spectroscopically inhomogeneous system attributed to the emission from LE and ICT states, wherein ICT state (long-wavelength fluorescence band) may be formed either by further geometrical changes of the locally excited conformer ($\varphi = 90^\circ$) or directly by the excitation of planar conformers.

In order to clarify the role of solute-solute intermolecular interactions in the photophysics of EDMAADCy, we also measured the fluorescence decays in DMSO-H₂O (4:1 (v:v) ratio) at different solute concentrations (from $5 \cdot 10^{-5}$ to $5 \cdot 10^{-4}$ M) (Table 1). As can be seen, in a binary solvent mixture the fluorescence decay data can be reasonably well fitted by three-exponential decays with two shorter components (τ_1 and τ_3) with principal contributions (A_1 and A_3) and the longer one τ_2 having only a meager amplitude. The shorter component τ_1 is close to the fluorescence lifetime of perpendicular conformer in DMSO, whereas decay time τ_2 is similar to the flattened conformer emission in DMSO. Since the spectroscopic properties of EDMAADCy dissolved in binary mixture of polar aprotic (DMSO) and polar protic (water) should be dependent on the general (dipole-dipole) and specific (hydrogen bonds) solute-solvent interactions, it is quite reasonable to assume that τ_3 decay component is attributed to the hydrogen-bonded complex (EDMAADCy-H₂O). Moreover, the pre-exponential factor A_3 , which signifies the hydrogen-bond complexes, shows progressive increase with solute concentration along with decrement in the amplitude of perpendicular form (A_1). This behavior is also understandable in terms of the concentration-induced planarization of the

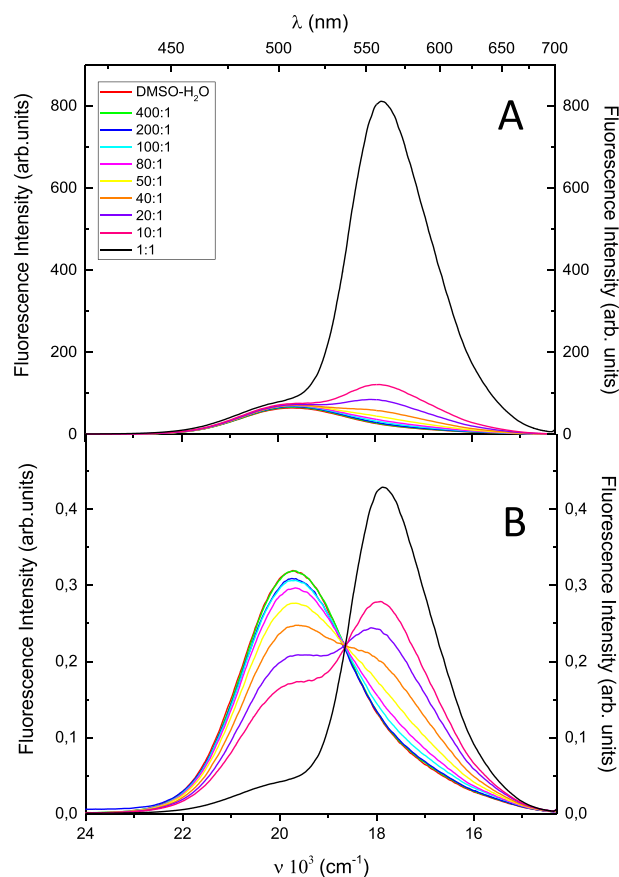


Fig. 6. Fluorescence (A) and normalized fluorescence (B) spectra of EDMAADCy ($c = 5 \cdot 10^{-4}$ M) in mixtures of DMSO-water at different mole fraction of water.

studied D-A molecule. Therefore, the fluorescence behavior in DMSO-H₂O mixture can be explained by the coexistence of three emitting centers: perpendicular and planar (negligible contribution) conformers and hydrogen-bonded complex with H₂O molecules (relative contribution is quite significant and increases with increasing solute concentration) (see Scheme 2B).

To differentiate between the non-specific and specific (H-bonding) solute-solvent interactions, the steady-state and time-resolved spectroscopic measurements in DMSO-water at different mole fractions of water were measured. The fluorescence spectra of EDMAADCy ($c = 5 \cdot 10^{-4}$ M) in mixtures of DMSO-water at different H₂O concentrations are shown in Fig. 6. Upon increasing water concentration the position of the short-wavelength fluorescence maximum is slightly red-shifted and simultaneously a new, broad long-wavelength band centered at around 560 nm appears. At high water concentration the LW fluorescence band, which is assigned to the emission of planar, hydrogen-

Table 2

Fluorescence lifetimes of EDMAADCy in binary mixtures of DMSO-water at different water concentrations.

DMSO:H ₂ O (v:v)	λ_{obs} (nm)	τ_1 (ns)	A_1 (%)	τ_2 (ns)	A_2 (%)	τ_3 (ns)	A_3 (%)	χ^2
$c = 2.5 \cdot 10^{-4}$ M								
10:1	470	2.11	44	7.38	21	4.25	35	1.24
	550	1.38	29	8.35	18	4.21	53	1.18
8:1	470	1.86	38	6.73	8	4.78	54	1.09
	550	0.76	31	7.24	8	3.71	61	1.21
5:1	470	1.64	35	7.71	9	4.38	56	1.17
	550	0.93	26	7.81	4	3.13	70	1.31
4:1	470	2.04	40	6.21	7	4.21	53	1.11
	550	0.71	30	7.25	6	3.23	64	1.20

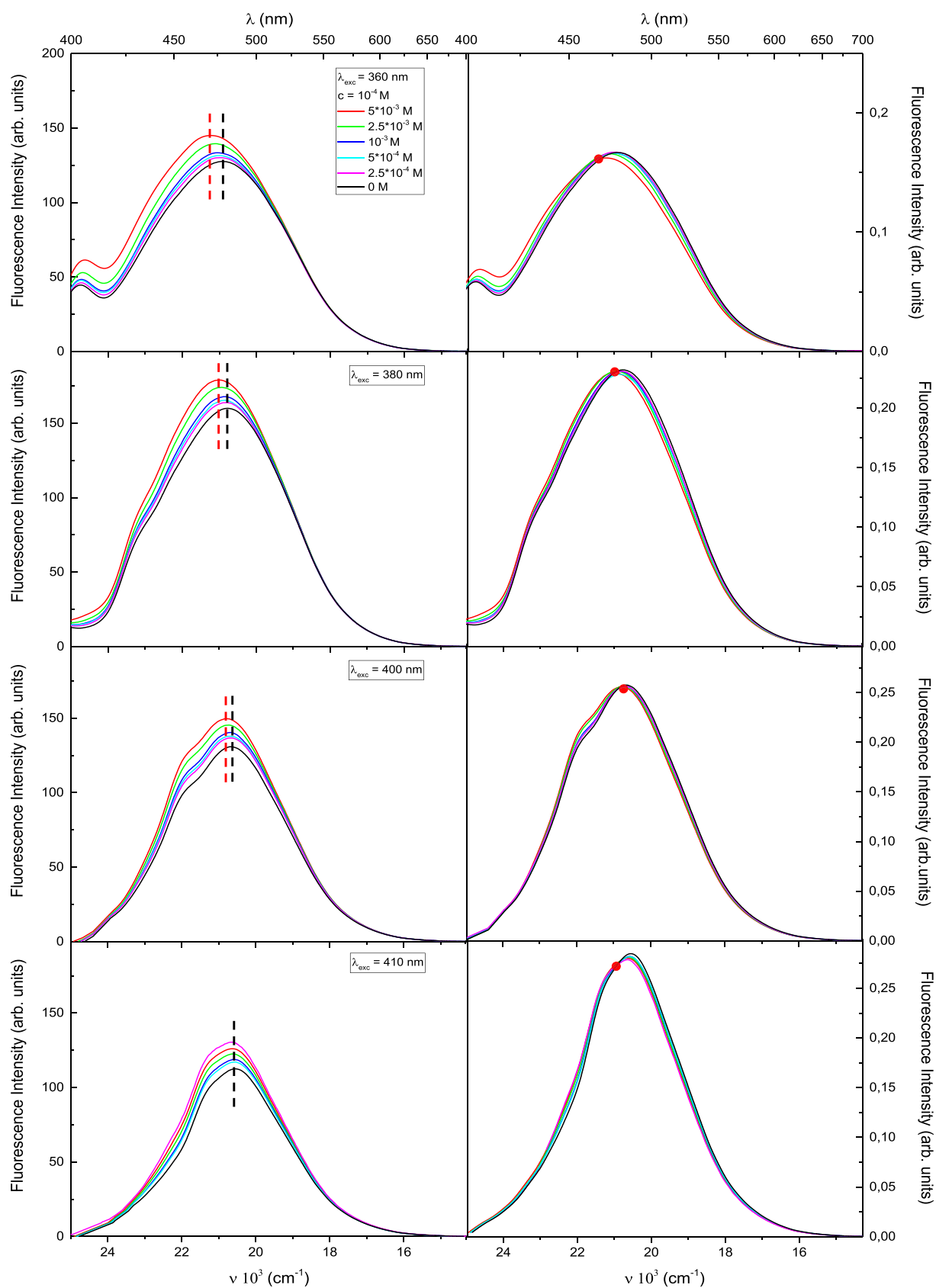


Fig. 7. Excitation-wavelength dependence of fluorescence (left panel) and normalized (right panel) fluorescence spectra of EDMAADCy ($c = 10^{-4}$ M) in DMSO containing different concentrations of γ -CD: 0 M, $2.5 \cdot 10^{-4}$ M, $5 \cdot 10^{-4}$ M, 10^{-3} M, $2.5 \cdot 10^{-3}$ M, $5 \cdot 10^{-3}$ M.

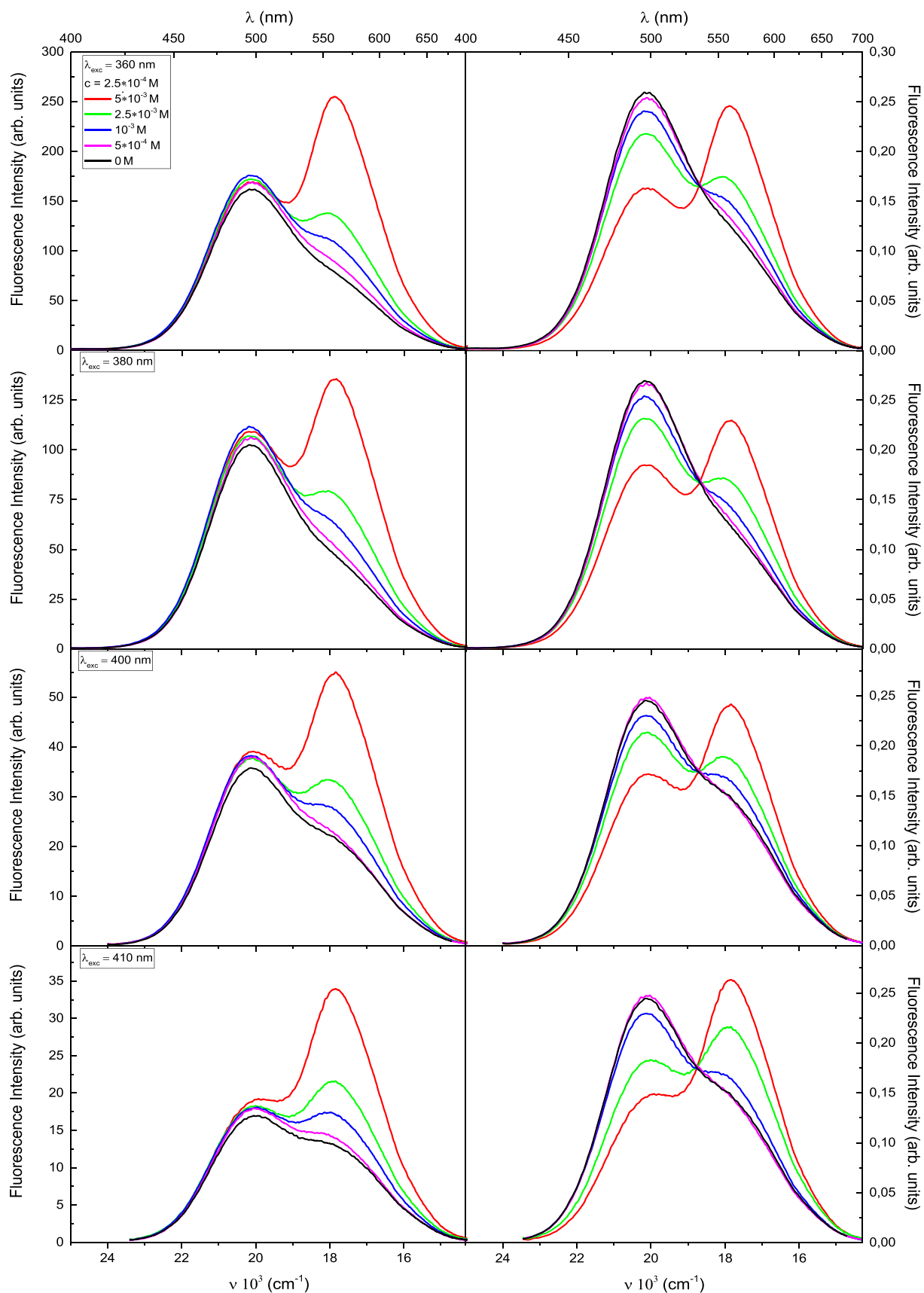


Fig. 8. Excitation-wavelength dependence of fluorescence (left panel) and normalized (right panel) fluorescence spectra of EDMAADCy in DMSO-water system containing different concentrations of γ -CD: 0 M, $5 \cdot 10^{-4}$ M, 10^{-3} M, $2.5 \cdot 10^{-3}$ M, $5 \cdot 10^{-3}$ M.

bonded complex of EDMAADcy with water molecules, dominates. An isosbestic point can be clearly seen between SW and LW normalized bands, which suggests that equilibrium is established between two types of emitting species in the investigated medium.

In order to understand processes taking part in DMSO-H₂O system time-resolved spectroscopic measurements have been applied. Fluorescence decays of the EDMAADcy in DMSO-water at different mole fractions of water were measured upon excitation at 370 nm and detection at the wavelengths where either SW or LW emission is prevailing (i.e., at 470 and 550 nm, respectively). Fluorescence kinetics in binary mixtures of DMSO-H₂O at different water concentrations were deconvoluted to the best fit of a sum of three exponentials (Table 2). As can be seen, upon increasing the mole fraction/concentration of polar protic component in the binary mixture, all fluorescence decay components remain almost the same values within experimental error, whereas the relative amplitude of decay components strongly depends on the $x_{\text{H}_2\text{O}}$. From the comparison of the decay times of investigated dye in DMSO and DMSO-H₂O, as well as based on the fluorescence lifetimes available in literature for EDMAADcy and EMAADcy in different solvents [9–13], it can be stated that in binary mixture τ_1 component originates from the emission of locally-excited perpendicular conformer, whereas the τ_2 decay time, which contribution A_2 is minor and decreasing with increasing water concentrations, is attributed to the planar conformer emission. It is well-known that the solute-solvent hydrogen bonding interactions play a predominant role at higher water concentration, therefore, the τ_3 lifetime component (with increasing contribution at higher water concentration) can be assigned to the emission of the molecule which form hydrogen-bond complex.

Taking into account that: (i) as the solute concentration is increasing LW fluorescence band (corresponding to emission of conformers with donor and acceptor groups coplanar) grows in; (ii) fluorescence intensity distribution of the LW band of EDMAADcy in DMSO-H₂O is broad and described by a classical Gaussian shape; (iii) three fluorescence decay times are required to satisfactorily reproduce the experimental decay data in DMSO-H₂O, one can state that concentration-induced planarization model with the presence of hydrogen-bonded ICT conformers can be assumed for the explanation of observed phenomena (see Scheme 2B). Thus, concentration-dependent processes responsible for the observed spectroscopic behavior in DMSO-H₂O system were identified as radiative deactivations of (1) locally-excited molecule (perpendicular conformation); (2) ICT molecule with strong charge-transfer character and planar geometry; (3) hydrogen-bond complex of EDMAADcy and water.

3.3. Steady-state and time-resolved spectroscopic studies in DMSO and DMSO-water in the presence of γ -cyclodextrins

Because EDMAADcy- γ -CD complexes are barely soluble in water, steady-state and time-resolved spectroscopic studies have been conducted in DMSO and mixture of DMSO and water (4:1). On addition of γ -CD to DMSO and DMSO-H₂O solution of EDMAADcy, practically no absorption spectral changes are observed. Therefore, we do not use these experimental data to calculate of the equilibrium constants in the ground state. Fig. 7 shows the excitation-wavelength dependence of fluorescence spectra of the investigated molecule in DMSO ($c = 10^{-4}$ M) as a function of γ -CD concentration. In DMSO solution with different concentrations of γ -CD, for all studied excitation wavelengths (360, 380, 400 and 410 nm), the LW fluorescence band is absent and only broad, SW emission band occurs. Moreover, gradual addition of γ -CD to DMSO solution of EDMAADcy (in the studied solute concentration range from $5 \cdot 10^{-6}$ to $5 \cdot 10^{-4}$ M) led to insignificant increase in fluorescence intensity of SW band along with blue shifts in the position of the fluorescence maximum (~ 10 nm) (Fig. 7). Such shift can be interpreted in terms of inclusion complex formation resulting in a reduced polarity upon encapsulation of the D-A molecule inside CD

Table 3

Fluorescence lifetimes of EDMAADcy ($c = 10^{-4}$ M) in DMSO containing different concentrations of γ -CD.

$C_{[\gamma\text{-CD}]}$ (M)	τ_1 (ns)	A_1 (%)	τ_2 (ns)	A_2 (%)	χ^2
0	2.76	64	7.34	36	1.31
$2.5 \cdot 10^{-4}$	2.63	63	7.61	37	1.06
$5 \cdot 10^{-4}$	2.54	61	7.01	39	1.11
10^{-3}	2.88	60	7.44	40	1.14
$2.5 \cdot 10^{-3}$	3.02	59	6.84	41	1.23
$5 \cdot 10^{-3}$	2.66	57	7.32	43	1.17

cavities. The formation of well-defined host-guest inclusion complexes between γ -CD and conformers having perpendicular geometry is also reflected in the occurrence of the sharp isosbestic point in normalized fluorescence spectra (Fig. 7).

Interestingly, the spectral changes of EDMAADcy in mixture of DMSO and water (4:1) containing different concentrations of γ -CD are different to those observed in DMSO solution with γ -CD. As can be seen in Fig. 8, upon addition of γ -CD, the LW emission band which is assigned to the emission from the planar form of EDMAADcy is greatly enhanced, while the intensity of the SW (locally-excited) fluorescence band remains relatively unchanged. The occurrence of an isosbestic point in the normalized fluorescence spectra is in clear agreement with the existence of two types of emissive species in the studied medium. The opposite spectral changes in DMSO and DMSO-water (4:1) solutions with different concentrations of γ -CD suggest that the process of formation of inclusion complex in DMSO and DMSO-H₂O is different in terms of geometrical structure of EDMAADcy and specific interactions between solute and solvent molecules. In other words, the geometrical structure of EDMAADcy and the presence of hydrogen-bond complex have a significant influence on the stoichiometry and strength of binding interactions of the molecule with cyclodextrins.

Table 4

Fluorescence lifetimes of EDMAADcy ($c = 10^{-4}$ M, $2.5 \cdot 10^{-4}$ M, $5 \cdot 10^{-4}$ M) in DMSO-water (4:1) system containing different concentrations of γ -CD.

$C_{[\gamma\text{-CD}]}$ (M)	λ_{obs} (nm)	τ_1 (ns)	A_1 (%)	τ_2 (ns)	A_2 (%)	τ_3 (ns)	A_3 (%)	χ^2
$c = 10^{-4}$ M								
0	500	2.26	48	6.25	9	4.78	43	1.13
	560	0.88	32	7.56	8	3.68	60	1.21
$5 \cdot 10^{-4}$	500	2.48	46	6.64	14	4.55	40	1.07
	560	1.04	30	7.15	14	3.95	56	1.11
10^{-3}	500	2.72	45	6.61	17	4.98	38	1.13
	560	1.21	30	7.59	24	3.37	46	1.27
$2.5 \cdot 10^{-3}$	500	2.91	46	6.15	24	4.65	30	1.31
	560	1.19	34	7.74	26	3.89	40	1.06
$5 \cdot 10^{-3}$	500	2.95	45	6.26	30	4.12	25	1.14
	560	1.26	30	7.89	34	3.33	36	1.19
$c = 2.5 \cdot 10^{-4}$ M								
0	500	1.98	41	6.29	6	4.26	53	1.21
	560	0.81	31	7.95	8	3.56	61	1.06
$5 \cdot 10^{-4}$	500	2.32	38	6.78	12	4.78	50	1.13
	560	0.98	32	7.21	13	3.36	55	1.17
10^{-3}	500	2.56	35	6.54	21	4.54	44	1.34
	560	1.15	30	6.99	19	3.89	51	1.21
$2.5 \cdot 10^{-3}$	500	2.54	32	7.06	28	4.25	40	1.17
	560	1.21	29	7.21	25	3.87	46	1.06
$5 \cdot 10^{-3}$	500	2.38	38	6.45	34	4.39	28	1.19
	560	1.17	34	7.78	38	3.35	28	1.23
$c = 5 \cdot 10^{-4}$ M								
0	500	1.94	36	6.48	7	4.56	57	1.04
	560	0.74	30	6.85	5	3.35	65	1.19
$5 \cdot 10^{-4}$	500	2.16	30	6.45	17	4.59	53	1.16
	560	0.89	27	7.51	13	3.40	60	1.08
10^{-3}	500	2.81	28	6.15	20	4.44	52	1.09
	560	1.21	28	7.18	26	3.25	56	1.24
$2.5 \cdot 10^{-3}$	500	2.89	32	6.18	28	4.25	40	1.17
	560	1.17	32	7.77	24	3.32	44	1.11
$5 \cdot 10^{-3}$	500	2.38	28	6.54	34	4.74	38	1.19
	560	1.59	25	7.23	38	3.78	37	1.07

In order to clarify the role of supramolecular assemblies in the photophysics of EDMAADCy, the fluorescence decays in DMSO and DMSO-water (4:1) solutions with different concentrations of γ -CD were measured at wavelengths where either SW or LW emission is prevailing (i.e., at 470 nm and 500 nm, 560 nm in DMSO and DMSO-H₂O, respectively). In addition, the effect of EDMAADCy concentration on the fluorescence kinetics was investigated in DMSO-water solution with different concentrations of γ -CD.

The fluorescence decay curves of the investigated D-A molecule in DMSO in the presence of γ -CD can be satisfactorily deconvoluted by biexponential decays (Table 3). The obtained biexponential decays are distinguished by a faster component τ_1 (~3 ns), which bears a resemblance to that of the perpendicular conformer, and a slower decay component τ_2 (~7 ns), which contribution A_2 increasing with increasing γ -CD concentration along with a concerted decrement in A_1 . This behavior suggests that the faster and slower components in fluorescence decay have been assigned to the emission of free (uncapsulated) molecule

having perpendicular geometry and flattened molecule encapsulated in CD cavities.

As mentioned earlier, in DMSO-H₂O binary mixture the fluorescence decay data can be fitted by a sum of three exponential functions. From the data collected in Table 4, it is evident that when γ -CD have been added to the EDMAADCy solution at its various concentrations, there are not significant changes in the fluorescence lifetime values (τ_1 , τ_2 and τ_3) at both detection wavelengths (500 and 560 nm), whereas the factors describing the contribution of the *i*th fluorescence decay component to the total emission (A_1 , A_2 and A_3) undergo very pronounced changes on adding γ -CD. The preexponential factor A_3 (describing the contribution of decay component assigned to the emission of the hydrogen-bond complex) decreases with increasing γ -CD concentration, whereas A_2 factor presents the opposite behavior. It is important to note here that A_1 component originates from the emission of locally-excited perpendicular conformer does not depend (in the error limit of its determination) on the γ -CD concentration.

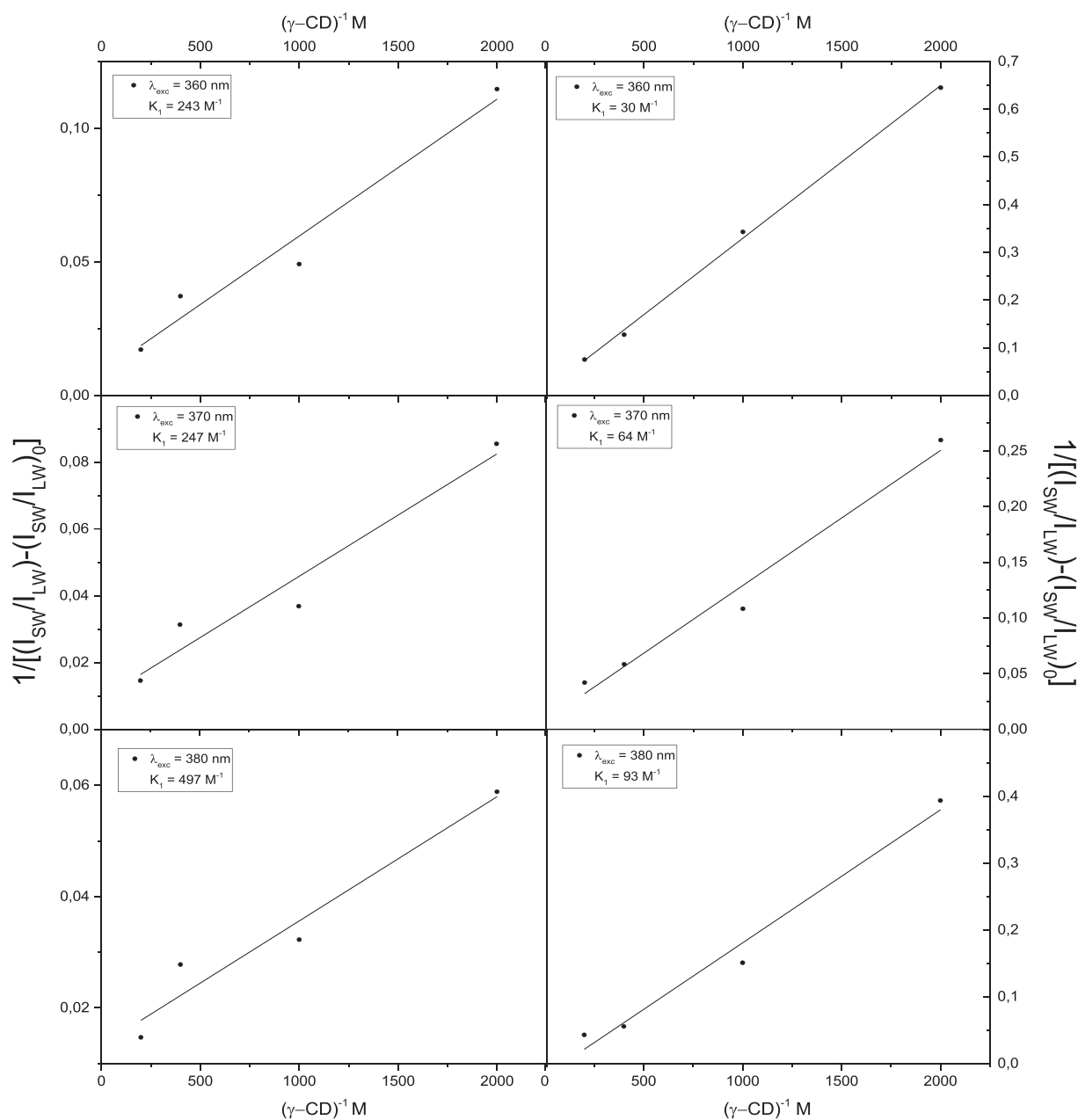


Fig. 9. Modified Benesi-Hildebrand dependence $1/[(I_{SW}/I_{LW}) - (I_{SW}/I_{LW})_0]$ versus $(\gamma\text{-CD})^{-1}$ for EDMAADCy in DMSO (left panel) and DMSO-water (right panel) systems containing different concentrations of γ -CD.

Observed dependencies indicate that intermolecular (solute-solvent) hydrogen bonding is reduced upon encapsulation of EDMAADCy inside CD cavity (decrease of A_3 factor with an increase of γ -CD concentration), and τ_2 lifetime component can be assigned to the emission of the planar molecule which forms 1:1 type inclusion complexes (relative contribution of A_2 increases with increasing γ -CD concentration).

3.4. Stoichiometry of inclusion complexes

The presence of an isosbestic point in the fluorescence spectra of EDMAADCy in both studied systems is indicative of the formation of well-defined 1:1 inclusion complex. To quantitatively determine the strength of binding interactions of the molecule with cyclodextrins and to confirm the stoichiometry of the formed inclusion complexes, the emission data have been analyzed by the modified Benesi-Hildebrand equation [32–34]:

$$\frac{1}{(I_{SW}/I_{LW}) - (I_{SW}/I_{LW})_0} = \frac{1}{K_1((I_{SW}/I_{LW})_1 - (I_{SW}/I_{LW})_0)} \cdot \frac{1}{[CD]} + \frac{1}{((I_{SW}/I_{LW})_1 - (I_{SW}/I_{LW})_0)}$$

where K_1 is equilibrium constant, I_{SW} and I_{LW} are the intensity of the short-wavelength (locally-excited) and long-wavelength (intramolecular charge transfer) fluorescence bands, respectively. $(I_{SW}/I_{LW})_0$, $(I_{SW}/I_{LW})_1$ and (I_{SW}/I_{LW}) are the fluorescence intensities of the studied molecule in DMSO or DMSO-H₂O in the absence of CD, in 1:1 complex (when all of molecules are complexed) and at the particular concentration of CD.

As can be seen in Fig. 9, for both investigated systems, the graphs of $1/((I_{SW}/I_{LW}) - (I_{SW}/I_{LW})_0)$ versus $1/[CD]$ (dependence obtained on the basis of an analysis of the emission spectra recorded for different excitation wavelengths) show linear dependence with good correlation factor ($r > 0.9$), confirming the formation of 1:1 stoichiometry. It is also clearly seen that K_1 values obtained from the slope and intercept of the plots are also strongly solute concentration and excitation-wavelength dependent (Table 5). Analyzing the data assembled in Table 5, it can be clearly stated that:

- the equilibrium constant determined in DMSO is approximately one order of magnitude larger than that of the inclusion complex in binary mixture of DMSO-H₂O, which clearly indicates that intermolecular interactions between perpendicular (DMSO) or planar (DMSO-water)

conformers of EDMAADCy molecule and γ -CD, causing host-guest type inclusion complex, are very different. Moreover, specific solute-solvent interactions (hydrogen bonds) occurring in the DMSO-water system have a significant influence on the stability of inclusion complexes.

- K_1 value is dependent on the excitation wavelength i.e., as the excitation wavelength is changed from 360 nm to 400 nm, the equilibrium constant increased about three and four times for DMSO-H₂O and DMSO system, respectively. This behavior is understandable in terms of the existence of distribution of spatial conformers in DMSO and DMSO-water systems. As mentioned earlier, the rotamers with different angles between their interatomic planes can be directly excited. Then, the excited conformers can differently interact with γ -CD.
- upon increasing the solute concentration from 10^{-4} to $5 \cdot 10^{-4}$ M, the K_1 value continuously increases, which is related to the fact that as the solute concentration increases the molecules become flattened and flattened conformers differently interact with γ -CD.

4. Conclusions

The steady-state and time-resolved spectroscopic measurements clearly show that investigated biphenyl derivative in DMSO and DMSO-H₂O forms a typically spectrally inhomogeneous system i.e., conformers having different geometry can absorb and emit at different energies. Noticed concentration-dependent spectral behaviors are understandable in terms of concentration-induced planarization model i.e., at high solute concentrations the distance between EDMAADCy molecules becomes closer enough to the significant solute-solute intermolecular interactions causing planarization of D-A dye. Performed analysis indicates that 1:1 inclusion complexes were formed between studied compounds and γ -cyclodextrins in DMSO and DMSO-H₂O. Moreover, the process of formation of inclusion complex in DMSO and DMSO-H₂O is different in terms of geometrical structure of EDMAADCy and specific (hydrogen bonds) interactions between solute and solvent molecules. In other words, the geometrical structure of EDMAADCy and the presence of hydrogen-bond complex have a significant influence on the stoichiometry and strength of binding interactions of the molecule with cyclodextrins.

Author statement

The work was created by the joint effort of all co-authors.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Table 5

Solute-concentration and excitation-wavelength dependent equilibrium constant K_1 (M⁻¹) of EDMAADCy in DMSO and DMSO-water (4:1) in the presence of γ -CD.

λ_{exc} (nm)	DMSO	DMSO-H ₂ O
	K_1 (M ⁻¹)	K_1 (M ⁻¹)
	$c = 5 \cdot 10^{-4}$ M	
360	646	54
370	757	171
380	1075	217
390	1798	155
400	2121	179
	$c = 2.5 \cdot 10^{-4}$ M	
360	362	60
370	493	156
380	521	164
390	587	179
400	1626	154
	$c = 10^{-4}$ M	
360	243	30
370	314	64
380	497	93
390	652	97
400	970	115

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Spectroscopic studies of the excited-state intramolecular proton and electron transfer processes of methyl benzoate derivatives in cucurbit[7]uril nanocage

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Supramolecular interactions

ABSTRACT

The encapsulation of four methyl benzoate derivatives (**I-IV**) in aqueous solution in the presence of the macrocyclic host molecule cucurbit[7]uril (CB[7]) was studied using steady-state, time-resolved and ¹H NMR spectroscopic techniques. The significant changes in the steady-state and time-resolved fluorescence spectra (and calculated from them fluorescence decay times) were attributed to the formation of a well-defined, stable 1:1 inclusion complex. To understand the mechanism of supramolecular solvation dynamics, the time-dependent fluorescence Stokes' shifts, represented by the normalized spectral response function *c*(*t*), were studied. Our studies have shown that solvation dynamics of water are significantly inhibited inside a cucurbit cavity. The experimental results were also used to calculate, according to Benesi-Hildebrand plot and nonlinear least-squares regression analysis, equilibrium constants of the formed inclusion complex. For all investigated dyes, the *K*₁ value is significantly larger than this previously calculated for **I-IV** inclusion into cyclodextrins (CDs), indicating the superior host abilities of CB[7] as compared to CDs. The position of the guest molecule inside the host cavity was investigated by ¹H NMR experiment. The nature of the ¹H NMR shifts for different protons is qualitatively different for methoxy derivative-CB[7] complex (**I**-CB[7] and **III**-CB[7] systems) and hydroxy derivative-CB[7] complex (**II**-CB[7] and **IV**-CB[7] systems), which indicates that the inclusion complexes of hydroxy and methoxy derivatives with CB[7] are of a difference in nature. The CB[7]-induced chemical shifts clearly demonstrate that for **II**-CB[7] and **IV**-CB[7] complexes, benzene ring is fully embedded in the interior of CB[7], while for **I**-CB[7] and **III**-CB[7] complexes, only the part of the benzene ring is located within the CB[7] cavity. Finally, ¹H NMR chemical shifts and evolution of the steady-state and time-resolved emission spectra clearly indicate that -NMe₂ group attached to the **III** and **IV** is located outside of the CB[7] portal.

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1. Introduction

One of the objectives of contemporary molecular spectroscopy is the research of the mechanisms and effects of the interaction of electromagnetic radiation with molecules, which may be present in various environments (solid, liquid and gas) [1–3]. During the last several years, one can observe a very intense increase in the development of research on supramolecular compounds, which due to their structure are able to form host-guest inclusion complexes with other organic and/or inorganic compounds. Among the main representatives of supramolecular compounds, there are the following: cyclodextrins (CDs), cucurbit[*n*]urils (CBs), calix[*n*]arenes, pillar[*n*]arenes, crown ethers and cyclophanes [4–6]. These fascinating macrocyclic compounds are of

particular interest mainly for two reasons: first, the geometrical structure of the abovementioned macrocyclic compounds gives rise to the fact that they have found numerous applications in pharmacy, medicine, agriculture, nanotechnology or the cosmetics industry [7–12]. On the other hand, systematic research into various photophysical and photochemical processes taking place in the organic molecule-macrocyclic compound system becomes an extremely interesting thing also from the purely theoretical point of view i.e., the understanding of the mechanisms of the processes leading to the formation of a stable inclusion complex [13–20].

Among different classes of macrocycles reported to date, cyclodextrins (cyclic oligosaccharides composed of six (α-CD), seven (β-CD) and eight (γ-CD) glucopyranose units) and cucurbit[*n*]urils (rigid cage consisting of *n*-glycoluril monomers joined by pairs of methylene bridges) are both important host molecules that have been extensively investigated [21,22]. Because of the fact, that cavity sizes of CBs (CB[6],

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CB[7] and CB[8]) are similar to those of CDs (α -CD, β -CD and γ -CD), cucurbiturils are often compared with cyclodextrins. However, these size similarities should not be confused with similarities in composition and binding properties. It is documented that structural differences between the constituent monomers and differences in the shape of the hosts (CBs exhibit an equatorial symmetry plane which does not exist in the CDs) can lead to different binding features of the studied dye with these two classes of macrocyclic compounds [4–6,21,22].

Several scientific groups, including ours, have presented many of interesting phenomena resulting from host-guest interactions, such as enhanced emission of the Twisted Intramolecular Charge Transfer State (TICT) and/or Excited-State Intramolecular Proton Transfer (ESIPT) [13–20], the effect of alcohols on the inclusion complexation, changes in acid-base behavior of encapsulated guests [23], guest relocation from host cavity to biomolecular binding pocket [24], biological catalysis [25]. In the last few years, our scientific attention has been paid to the description of the TICT and ESIPT processes of organic molecules in neat solvents of different polarity [26–29] and aqueous solutions with CDs [17–20] with particular focus on photophysical and photochemical properties of methyl benzoate derivatives. This group of molecules is interesting for two reasons: (i) molecules provide means of studying the general theory of ICT and ESIPT processes, (ii) they can be used as fluorescence probes [30,31], lasing medium in proton transfer lasers [32,33] and active materials in dry xerographic toners and metal ion sensors [34,35].

Intrigued by other researchers, who postulate some differences in the formation of inclusion complex dye-CD and dye-CB, our group has developed a great interest in the studies of the possible interactions between CBs and some methyl benzoate derivatives which are capable of exhibiting TICT- and/or ESIPT-type behavior. Having knowledge regarding the basic spectroscopic characteristics of the inclusion complexes of the investigated methyl benzoate derivatives with α -, β -, and γ -cyclodextrins [17–19], in the present paper, we describe the photophysical and photochemical properties of **I–IV** in CB[7] cavities. Moreover, this study focuses on the improved and complete knowledge of the effect of two different type of macrocycles of similar cavity size (β -CD and CB[7]) on the intramolecular electron and proton transfer processes in investigated dyes i.e., comparing whether or not the abovementioned organic molecules show similar photophysical and photochemical properties in the presence of CB[7] as in the case of cyclodextrins. Finally, the role of intermolecular hydrogen bonds in deactivation processes of excited inclusion complexes has been investigated using steady-state, time-resolved and ^1H NMR spectroscopic techniques. We believe that our experimental studies can give additional insights into the guest structure-host structure relationship of the inclusion complexes.

2. Experimental details

The investigated dyes have been synthesized and purified by Gormin [36–38]. The purity of dyes was controlled by thin layer chromatography. Cucurbit[7]urils were purchased from Sigma-Aldrich and used without further purification. D_2O was also purchased from Sigma-Aldrich.

Absorption, fluorescence excitation and fluorescence spectra were recorded using, respectively, a Shimadzu UV-2401 PC spectrophotometer and Shimadzu RF-5301 PC spectrofluorometer. The fluorescence emitted was observed perpendicular to the direction of the exciting beam. The emission spectra have been corrected for the spectral response of the photomultiplier (Hamamatsu R-928) and monochromator pass, but not for reabsorption which was negligible in these samples. Time-resolved emission spectra were measured applying streak camera (C4334-01 Hamamatsu) and 2501 S spectrograph (Bruker Optics). Solid state Nd: YAG laser (PL 2143A/SS EKSPILA) and optical parametric generator (PG 401/SH EKSPILA) were used as an excitation light pulses source [39].

Quantum yield of the molecules under study was determined by a relative method using Coumarin I in ethanol as standard [40]. The fluorescence quantum yield was calculated from the relationship:

$$\phi_F = \phi_s \frac{\int I(\nu) d\nu}{\int I_s(\nu) d\nu} \frac{1 - 10^{-D_s} n^2}{1 - 10^{-D} n_s^2}$$

where ϕ_F is the fluorescence quantum yield of studied fluorophore, the integral is the area under the corrected fluorescence spectrum, D is the optical density of the studied molecule, n is the refractive index of the solvent, and the subscript S refers to the standard fluorophore in all cases.

Solution pHs were measured by a pH meter Eutech Instruments PC 510. Before measurements, the pH meter was calibrated at pH 3 and 7 by using standard pH buffer solution from Merck.

All ^1H NMR spectra were recorded with Bruker Avance III 500 MHz spectrometer at 300 K. 5 mM solutions of **I–IV** and CB[7] were prepared in 99.95% $^2\text{H}_2\text{O}$. Chemical shifts were referenced to the internal standard of acetone (δ_{H} 2.225).

3. Result and discussion

3.1. Steady-state absorption and fluorescence spectra in aqueous solution with CB[7]

Before undertaking the investigation of the methyl benzoate derivatives in the presence of CB[7], it is necessary to remind the ground- and excited-state spectroscopic behaviors of these molecules in neat aprotic and protic solvents. On the basis of the experimental results and quantum-chemical calculations, it has been shown that the long-wavelength absorption band of the investigated dyes consists of two overlapping components (electronic transitions ($S_0 \rightarrow S_1$ and $S_0 \rightarrow S_2$) of different character: $\pi\pi^*$ and charge-transfer (CT)) (Figs. S1, S2). Also, it has been reported that the absorption spectrum in protic solvents (water, methanol, ethanol) differs significantly from this registered in aprotic medium [36–38,41–45]. The dominant differences (related to the shape, absorption maxima positions and molar absorption coefficient values of the respective absorption bands) are caused by the presence of solute-solvent H-bonded complexes in protic medium.

The excited-state spectroscopic characteristics of the molecules under study were also the subject of numerous investigations owing to their very interesting photophysical and photochemical properties in different homogeneous and heterogeneous media [36–38,41–45]. It is well established that some of the investigated molecules dissolved in a polar aprotic medium besides the normal (locally-excited (LE)) fluorescence exhibit the emission from the excited-state intramolecular proton transfer (ESIPT) (molecules **II** and **IV**) and/or excited-state intramolecular charge transfer (ICT) (molecules **III** and **IV**) states [41–45] (Fig. S1). Moreover, spectroscopic studies clearly demonstrate that intermolecular hydrogen bonding interaction between methyl benzoate derivative and protic medium has also significant influence on the excited-state spectroscopic behaviors i.e., ESIPT and ICT processes [36–38,41–45].

Fig. 1 shows the changes observed in the absorption (left panel) and normalized (by scaling the area under the spectrum to be equal to unity) absorption spectra (right panel) of **I–IV** upon the gradual addition of CB[7] in aqueous solution. It was found that pH of the investigated dyes in the presence of CB[7] solution was in the range of 6.20 to 6.52 (Table S1). These pH values indicate that neutral form of the **I–IV** dyes exists in the studied solutions. Upon addition of CB[7], the molar absorption coefficient of the two long-wavelength absorption bands gradually changes and is accompanied by a small (~ 2 nm) blue-shift of the absorption bands which is a clear indication of the reduction of the microenvironment polarity around the fluorophore. As can be seen in Fig. 1, the observed changes in the spectral behavior of two hydroxy

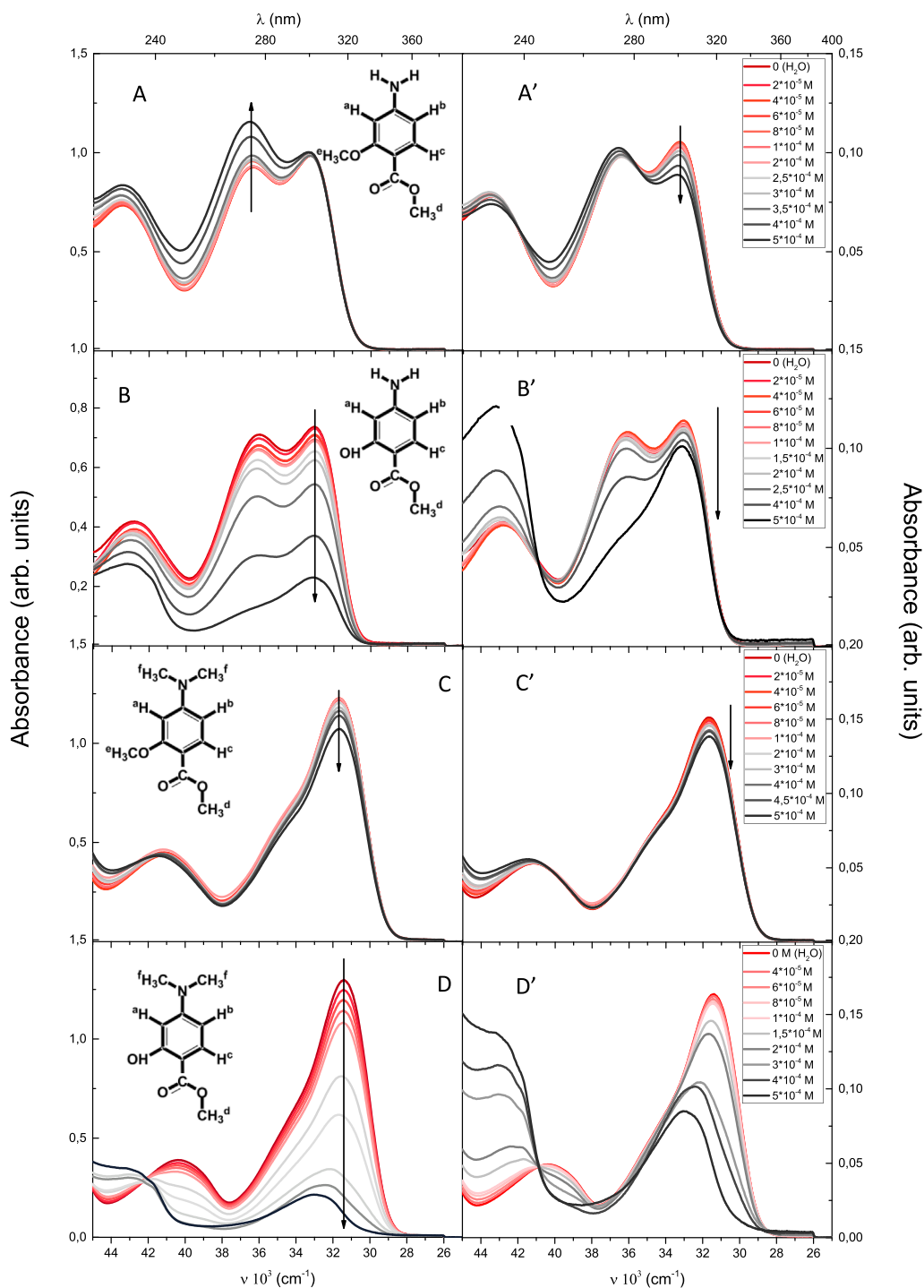


Fig. 1. Absorption (left panel) and normalized absorption (right panel) spectra of **I** (A, A'), **II** (B, B'), **III** (C, C') and **IV** (D, D') ($c = 5 \cdot 10^{-5}$ M) in aqueous solutions containing different concentration of CB[7].

derivatives (**II** and **IV**) are clearly greater than those observed for methoxy derivatives (**I** and **III**) suggesting that the inclusion complexes of hydroxy and methoxy derivatives with CB[7] are of a different nature. Nevertheless, the noticed changes definitely indicate some interaction between investigated dyes and CB[7] host. Moreover, the occurrence of an isosbestic point in the normalized absorption spectra (Fig. 1, right panel) suggests that an equilibrium is established between free molecule and molecule-CB[7] inclusion complex in the ground state.

The steady-state fluorescence (left panel) and normalized fluorescence (right panel) spectra of **I-IV** in aqueous solutions containing

different concentration of CB[7] are depicted in Fig. 2. It is clear from Fig. 2 that the fluorescence spectrum of **I-IV** in H_2O possesses besides the short-wavelength (locally-excited) fluorescence band, a much weaker, broad long-wavelength band. As previously reported, the long-wavelength fluorescence band of **I** in water results from the emission of intermolecular hydrogen-bonded complexes i.e., mono- and dihydrated forms of molecule **I**. Considering molecular structure of **II-IV** i.e., fact that studied molecules are capable of exhibiting TICT-type behavior (**III** and **IV**) and/or ESIPT-type behavior (**II** and **IV**), the long-wavelength fluorescence spectrum in H_2O consists of two or three

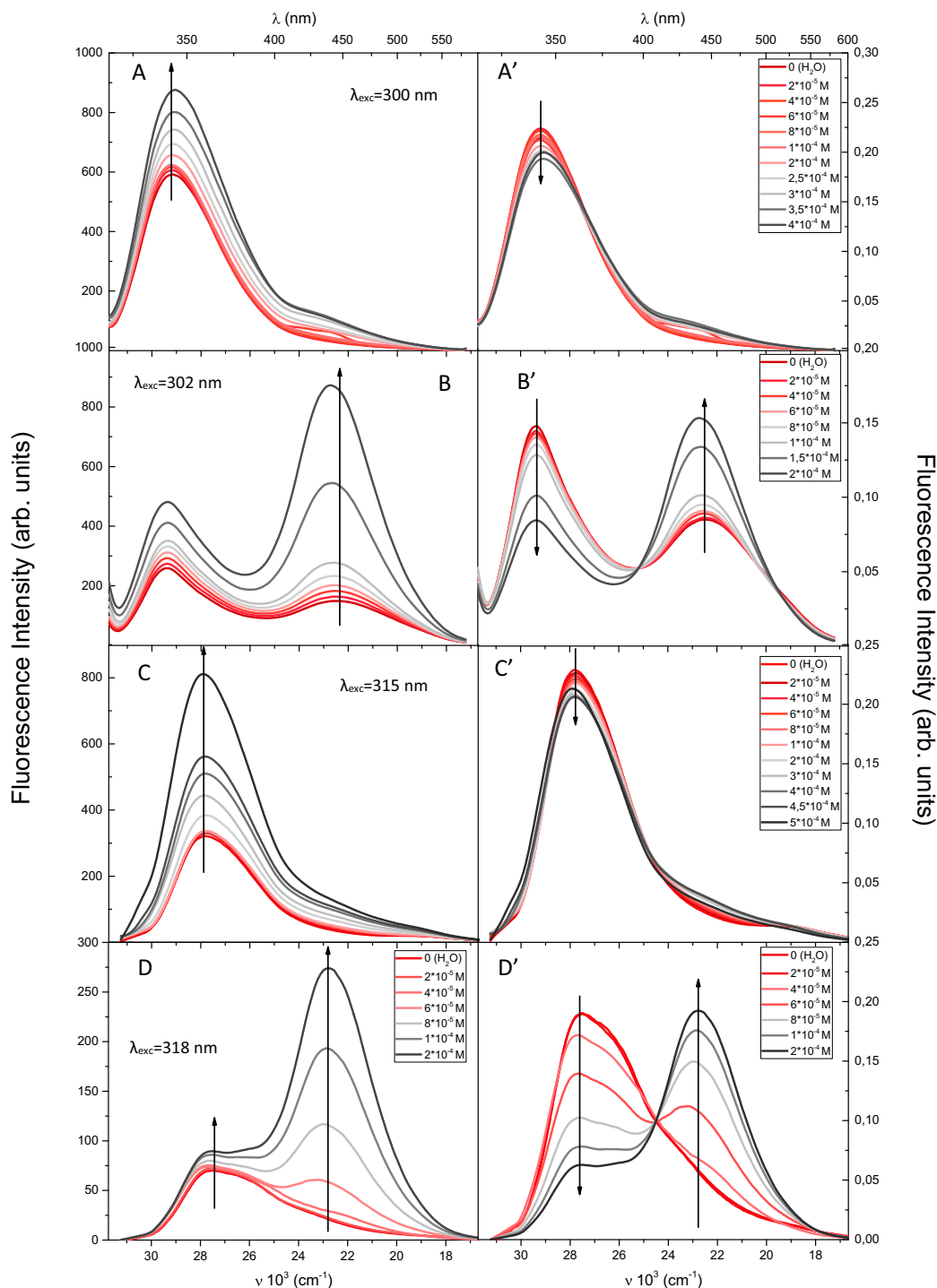


Fig. 2. Fluorescence (left panel) and normalized fluorescence (right panel) spectra of **I** (A, A'), **II** (B, B'), **III** (C, C') and **IV** (D, D') in aqueous solutions containing different concentration of CB[7].

partially overlapping bands: ESIPT and H-bonded complexes (molecule **II**), TICT and H-bonded complexes (molecule **III**), and ESIPT, TICT and H-bonded complexes (molecule **IV**).

As it is shown in Fig. 2, addition of CB[7] to the aqueous solution of **I** and **III** (molecules which are not capable of exhibiting ESIPT-type behavior) brings about a strong increase in the intensity of the LE fluorescence band with a slight change in the long-wavelength emission intensity. A small increase in the LW fluorescence intensity of **III** in water upon addition of the host CB[7] (absence of TICT fluorescence band) points toward strong solute-solvent interaction also in the presence of CB[7] i.e., the water molecules form a chemical bond with *n*

electrons of N atom of the -NMe₂ group which causes that the intramolecular charge transfer process in the excited state does not appear. Therefore, spectral changes observed for **III** (SW fluorescence enhancement, absence of TICT fluorescence band and presence of an isobestic point in the steady-state area normalized emission spectra) can be explained by encapsulation of **III** in the hydrophobic cage of CB[7] with the dimethylamino (-NMe₂) group exposed to the bulk phase.

Contrary to spectroscopic behavior of molecules **I** and **III**, upon addition of CB[7] to the aqueous solution of **II** and **IV** (molecules which are capable of exhibiting ESIPT-type behavior), the short-wavelength fluorescence band is rather weakly enhanced. However, distinct

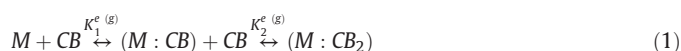
enhancement of the long-wavelength fluorescence band ($\lambda_{\max} \sim 450$ nm) can be observed, which is similar to the emission from the ESIPT state observed in neat nonpolar and polar aprotic solvents. Such increase in ESIPT fluorescence intensity can be interpreted in terms of incorporation of molecules **II** and **IV** within the hydrophobic micropockets of CB [7], resulting in a reduced polarity as compared to the aqueous phase and possibility of excited-state intramolecular proton transfer process. It is worth noting that, the absence of the long-wavelength TICT fluorescence band ($\lambda_{\max} \sim 520$ nm) of **IV** in aqueous solutions containing different concentrations of CB[7] (similar to the situation observed for molecule **III**) is related to the fact that molecule **IV** is also stabilized in protic water by hydrogen bonding involving the nitrogen electronic lone pair. The above behavior combined with the presence of an isosbestic point in the steady-state area normalized emission spectra of **IV** bear the evidence of formation of well-defined host-guest inclusion complex (**IV**-CB[7]), whose structural geometry is similar to that of **III**-CB[7] complex in terms of the orientation of the guest molecule (-OH group is included in the CB[7] cavity (possibility of ESIPT process), whereas -NMe₂ group is exposed to the bulk phase (impossibility of rotation of the dimethylamino group)).

Fig. S3 presents the fluorescence quantum yield (Φ_F) change with increasing host concentration. For all studied dyes, after a steep initial rise fluorescence quantum yield trends to level off when most of guest molecules are complexed. The relatively high increase of Φ_F upon increasing CB[7] concentration may be presumably due to decrease in nonradiative deactivation rates within the confined CB[7] cavities. Moreover, it is clearly seen that fluorescence quantum yield of complexes **I**-CB[7] and **II**-CB[7] is notably higher as compared to **III**-CB[7] and **IV**-CB[7] systems.

Fig. S1 shows a typical example of the effect of CB[7] on the fluorescence excitation spectra of **I-IV**. CB[7]-induced spectral behavior (small blue-shifts of the fluorescence excitation bands, presence of an isosbestic point) also indicate a structural change of the emitting species, brought out by the cucurbituril encapsulation.

3.2. Stoichiometry and equilibrium constants of inclusion complexes

Steady-state spectroscopic measurements can be used for the determination of the inclusion complex stoichiometry and equilibrium constants. A reliable determination of the inclusion complex stoichiometry in the excited (e) (or ground (g)) state



can be provided by analyzing the difference in fluorescence intensity (or absorbance) of the investigated molecule in the presence of CBs. Modified Benesi-Hildebrand (B-H) equation [46,47]:

$$\frac{1}{(I_{SW}/I_{LW}) - (I_{SW}/I_{LW})_0} = \frac{1}{K_n^e((I_{SW}/I_{LW})_1 - (I_{SW}/I_{LW})_0)} \cdot \frac{1}{[CB]^n} + \frac{1}{(I_{SW}/I_{LW})_1 - (I_{SW}/I_{LW})_0} \quad (2b)$$

$$\frac{1}{A - A_0} = \frac{1}{K_n^e(A_1 - A_0)} \cdot \frac{1}{[CB]^n} + \frac{1}{A_1 - A_0}$$

gives reliable information on the stoichiometry and equilibrium constants of the inclusion complex in the excited (or ground) state. In Eq. (2a), I_{SW} and I_{LW} are the intensity of the short-wavelength (SW) and the long-wavelength (LW) fluorescence bands, respectively; n represents the stoichiometry of the formed complex ($n = 1$ or 2) with respect to the CB[7], $(I_{SW}/I_{LW})_0$ (A_0), $(I_{SW}/I_{LW})_1$ (A_1) and (I_{SW}/I_{LW}) (A) are the fluorescence intensities (or absorbances) of the studied molecule in water (in the absence of CB[7]), in 1: n complex (when all of the molecules are complexed) and at the particular concentration of CB[7].

Thus, the $1/((I_{SW}/I_{LW}) - (I_{SW}/I_{LW})_0)$ should follow a linear dependence on $1/[CB]^n$ for the correct stoichiometry (n).

Here we would like to note that, graphical method (Benesi-Hildebrand plot) does not allow the determination of equilibrium constants properly and provides an estimation rather than accurate values. Nigam and Durocher [48–50] have shown that the changes in fluorescence intensity obtained at low macrocyclic compounds concentrations are widely spaced as compared to high-concentration values. Consequently, the slope of the line is very sensitive to the y value of the data points having the largest x value [48]. Therefore, when only 1:1 complex is formed, the nonlinear least-squares regression analysis (NL) is an excellent instrument to determine more reliable equilibrium constant value in the excited (or ground) state. The steady-state spectroscopic data should be fitted into the following equations [48–50]:

$$(I_{SW}/I_{LW}) = \frac{(I_{SW}/I_{LW})_0 + (I_{SW}/I_{LW})_1 K_1^e [CB]}{1 + K_1^e [CB]}$$

$$A = \frac{A_0 + A_1 K_1^e [CB]}{1 + K_1^e [CB]} \quad (3b)$$

Nonlinear regression requires preliminary parameters estimates, (K_1^e (K_1^g) and $(I_{SW}/I_{LW})_1$ (A_1)) which can be determined from the linear regression approach i.e., Benesi-Hildebrand plot, (Eqs. (2a) and (2b)).

As can be seen in Fig. 3, for all studied fluorophores, the plots of $1/((I_{SW}/I_{LW}) - (I_{SW}/I_{LW})_0)$ versus $1/[CB]$, as well as $1/(A - A_0)$ versus $1/[CB]$ ($n = 1$) indeed demonstrate linear dependence giving the linear correlation coefficient higher than 0.95. The linearity of the double reciprocal plot clearly indicates the formation of well-defined 1:1 inclusion complex between methyl benzoate derivatives (**I-IV**) and CB[7] both in the ground and excited states. From the intercept and slope of the obtained straight lines, the equilibrium constants (K_1^g , K_1^e) were determined and collected in Table 1. The equilibrium constants were also determined using nonlinear regression procedure (see Eqs. (3a) and (3b)). On the basis of the drawings presented in Figs. S4 and S5 and using initial values of K_1^e (K_1^g) and $(I_{SW}/I_{LW})_1$ (A_1) experimentally determined from Eqs. (2a) and (2b) (Benesi-Hildebrand plot) the final values of equilibrium constants have been calculated and collected in Table 1. It should be noted that equilibrium constants determined by two methods (B-H, NL) differ significantly, which confirms Durocher's observation that B-H method gives an estimation rather than proper K_1^e and K_1^g values. In order to avoid complexity, an average equilibrium constant value ($\langle K_1^e \rangle = (K_1^e(B-H) + K_1^e(NL))/2$) has been considered for further analysis (Table 1).

One of the best method used to recognize the inclusion complex stoichiometry is Job's method (continuous variation method) [51,52]. Job plots were generated by plotting $\Delta A \cdot R$ (or $\Delta I \cdot R$) against R , where ΔA (ΔI) is the difference in absorbance (fluorescence intensity) of the investigated organic molecule **M** (**I-IV**) without and with CB[7] and $R = [M]/([M] + [CB[7]])$. As can be seen in Figs. 4, S6 and Tables S2 and S3 for all investigated systems, the value of $R = 0.5$ clearly indicates the 1:1 stoichiometry of the formed inclusion complexes in the ground and excited states.

To understand how methyl benzoate derivatives interact with a different type of macrocycles the values of the ground- and excited-state equilibrium constants estimated for **I-IV** in α -, β - and γ -CD are also listed in Table 1. The results demonstrate that all the studied dyes have a high binding affinity to CB[7] in both ground and excited states (significantly higher than in α -, β - and γ -CD systems). The equilibrium constants K_1^g and K_1^e are 1–2 or even 3 orders of magnitude higher than that in α -, β - and γ -CD supramolecular assemblies. These data indicate the difference in the photophysical and photochemical properties of the studied dyes when they form inclusion complexes with CB[7] and cyclodextrins. The more stable inclusion complexes in CB[7] (compared to cyclodextrins) can be explained in terms of optimal size and shape of hydrophobic nanocage of CB[7] internal cavity to encapsulation the

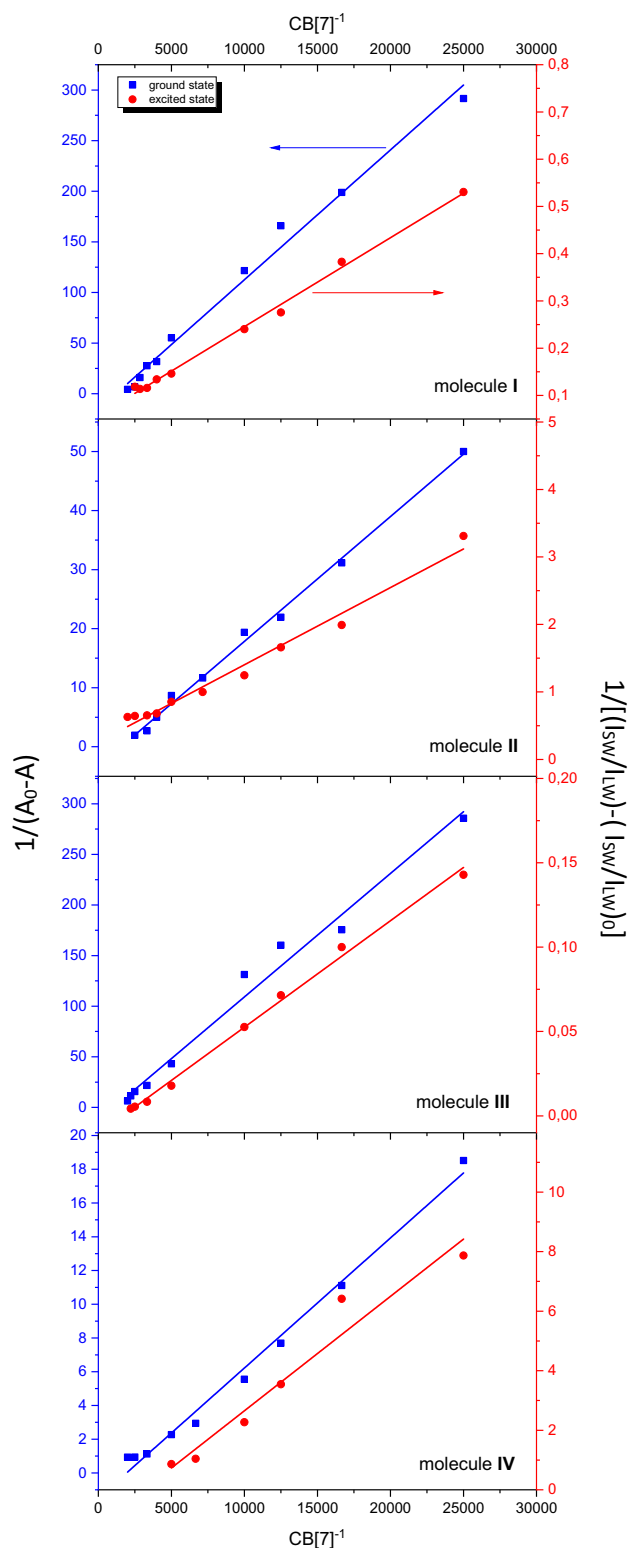


Fig. 3. The plots of $1/(I_{sw}/I_{LW}) - (I_{sw}/I_{LW})_0$ versus $1/[CB]$ and $1/(A - A_0)$ versus $1/[CB]$ for investigated dyes.

methyl benzoate derivatives. Taking into account the fact that β -CD host has a comparable cavity volume to CB[7], it can be argued that the shape of the cavity plays a very important role in the stability of the complexes. CBs exhibit an equatorial symmetry plane (which does not exist in the CDs) which lead to different binding features of the studied dye with these two classes of macrocyclic compound.

From the analysis of K_1^e and K_1^e values, it is evident that: (i) all investigated methyl benzoate derivatives have much higher affinity to bind guest molecule in the excited state than in the ground state. This behavior is understandable, if we consider that the value of the electric dipole moment of the investigated dyes distinctly increases upon excitation i.e., $\mu_e > \mu_g$; (ii) the investigated molecules (excluding IV) show similar ability to formation of the inclusion complex in the ground state ($\langle K_1^e(I) \rangle \approx \langle K_1^e(II) \rangle \approx \langle K_1^e(III) \rangle$); (iii) the molecules which are capable of exhibiting ESIPT-type behavior (II and IV) form more stable inclusion complexes with CB[7] in the excited state than two methoxy derivatives (I and III) ($\langle K_1^e(II) \rangle \approx \langle K_1^e(IV) \rangle \approx 2\langle K_1^e(I) \rangle \approx 2\langle K_1^e(III) \rangle$), which may be due to the fact that hydroxy derivatives (II and IV) are more deeply embedded in the CB[7] cavity.

3.3. Time-resolved studies in aqueous solution with CB[7]

To get information on the excited-state dynamics of I-IV in H₂O and aqueous solutions containing different concentration of CB[7], time-resolved emission spectra were recorded. Fig. 5 presents a typical streak camera image taken directly from the apparatus [39]. From a presented image both the fluorescence spectra at various times after excitation as well as the fluorescence decay times can be derived. The fluorescence lifetimes and preexponential coefficients describing the contribution of the i -th fluorescence decay component to the total emission are summarized in Table 2 and Table S4.

In our previous works [19], we have shown that fluorescence kinetics of I-IV in H₂O were reasonably well fitted by biexponential decays with shorter component ~ 10 – 40 ps and longer one ~ 200 – 400 ps. The fast component originates from the emission of the excited hydrogen-bonded complex (strong intermolecular hydrogen bonding between methyl benzoate derivative and water molecules results in an increase in the nonradiative decay rate in the excited state), whereas the longer component corresponds to the emission of unbound (free) fluorophore. In our current investigation, for all the studied dyes, the fluorescence decay curve in the absence of CB[7] can be satisfactorily fitted to the monoexponential function with the fluorescence decay time $\tau_1 \sim 250$ – 400 ps (Table 2). It should be clearly stated that this difference results from the fact that the decay time of the short component ($\tau_{TR} \sim 10$ – 40 ps) cannot be precisely determined in the streak camera experiment because it is much shorter than the temporal resolution (TR) of the setup (Table S4). Therefore, the designated fluorescence decay component τ_1 of methyl benzoate derivatives in water should be interpreted as average lifetime of two existing species (unbound (free) molecule and hydrogen-bonded complex).

The fluorescence decay curves in the presence of CB[7] can be satisfactorily deconvoluted by the monoexponential (molecule I), biexponential (II, III) and three-exponential (IV) fitting function. For all the investigated dyes, the value of fast decay component τ_1 slightly increases with increasing CB[7] concentration, which can be interpreted by considering inclusion of the fluorophore in a host cavity. CB[7] provides superior protection to the encapsulated fluorophore from the neighbouring water molecules (hydrogen bonding is reduced upon encapsulation) which leads to a suppression of the nonradiative decay channels and enhancement of the fluorescence lifetime. This is consistent with the CB[7]-induced enhancement of the LE fluorescence.

In the case of II, analysis of the fluorescence kinetics in CB[7] confirms conclusions drawn from the evolution of the steady-state emission spectra i.e., the existence of the two different emitting species. In the presence of low concentration of CB[7] ($c = 10^{-4}$ M), fluorescence decay exhibits a fast decay component (τ_1), as a major component (90%), with a small contribution of slow decay component (~ 1 ns), which is close to the lifetime of ESIPT tautomer reported for this molecule in polar aprotic solvents [36–38]. Moreover, the preexponential factor A_2 corresponding to decay of ESIPT tautomer increases with increasing CB[7] concentration, whereas A_1 factor presents opposite behavior. These results support that molecule II is encapsulated in the

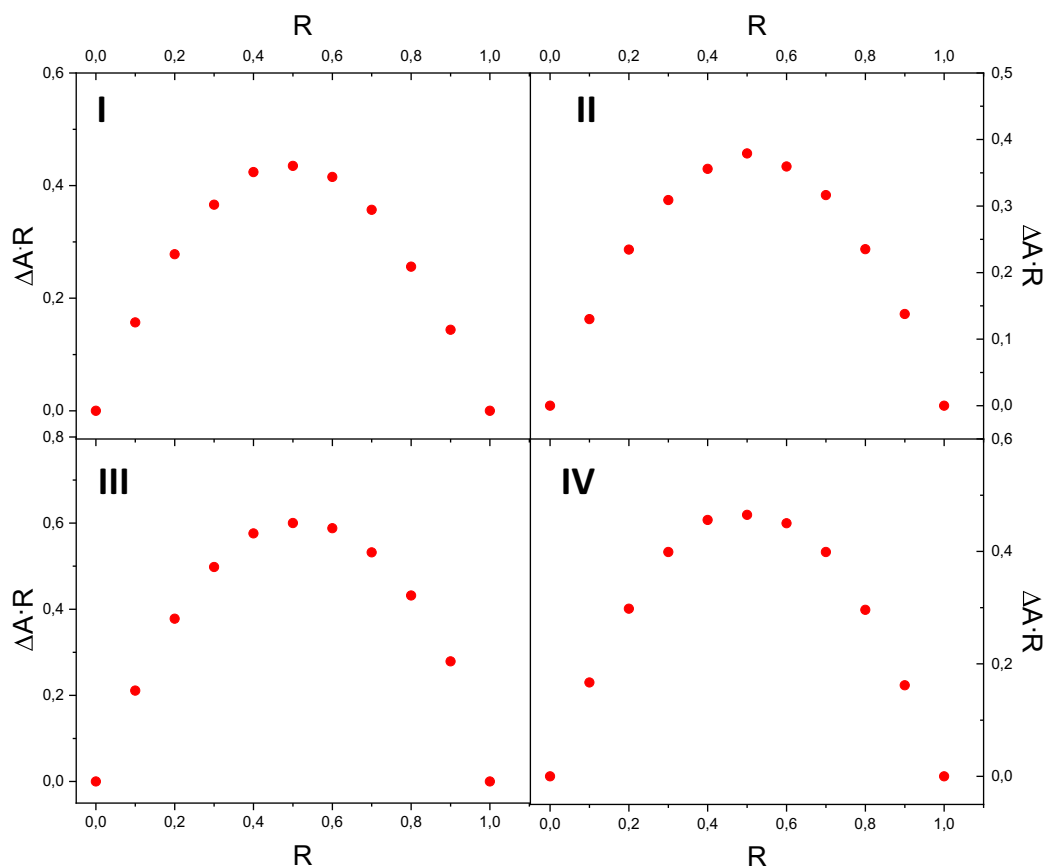


Fig. 4. Job plots of the investigated dyes-CB[7] systems prepared by using steady-state absorption data.

host cavity and the protection of fluorophore by CB[7] leads to excited-state intramolecular proton transfer process.

For molecule **III**, at the lowest concentration of CB[7], only one lifetime is exhibited, whereas at the highest CB[7] concentration two emissive species contribute to the observed decay curve (see τ_1 and τ_3 in Table 2). The preexponential factor associated with the slower lifetime component (A_3) shows progressive increase with the concentration of hosts. Taking into account that (i) upon addition of CB[7] to the aqueous solution of **III**, the locally-excited fluorescence band shows a pronounced enhancement, whereas the TICT emission band is rather weakly enhanced (ii) the value of τ_3 decay component does not correlate very well with the lifetime of TICT conformer of molecule **III**

registered in aprotic polar solvents (a few nanoseconds), led us to conclude that in 1:1 inclusion complex (**III**-CB[7]) dimethylamino group remains outside the cavity in aqueous medium and τ_3 decay component can be attributed to the emission from the HICT (Hydrogen-bonded Intramolecular Charge Transfer) state [27] (possibility of photoinduced rotation of dimethylamino group is limited due to the presence of hydrogen bonds).

As can be seen in Table 2, the fluorescence kinetics of **IV** in the presence of CB[7] is of the most complex nature. In CB[7]-free aqueous solution fluorescence decay curve was fitted by a monoexponential function ($\tau_1 \sim 250$ ps), in the presence of CB[7] at low concentration the decay fits to biexponential function with time constants of $\tau_1 \sim 300$ ps (96%) and $\tau_2 \sim 1.5$ ns (4%), whereas at the highest concentration, three discrete emissive centers with time constants of $\tau_1 \sim 360$ ps (8%), $\tau_2 \sim 1.5$ ns (26%) and $\tau_3 \sim 3.8$ ns (66%) contribute to the observed total emission. These results in connection with CB[7]-dependent steady-state emission spectra as well as fluorescence lifetimes for methyl benzoate derivatives in different media available in the literature, indicate that τ_2 component is associated with the fluorescence from ES IPT state whereas the longest time component (τ_3) can be assigned to the emission from excited ICT state.

To understand the mechanism of supramolecular solvation dynamics in an even more quantitative sense, we have studied the time-dependent fluorescence Stokes' shift represented by the normalized spectral response function $c(t)$, defined by Maroncelli and Fleming as [53]:

$$c(t) = \frac{\nu(t) - \nu(\infty)}{\nu(0) - \nu(\infty)}$$

where $\nu(t)$, $\nu(0)$, and $\nu(\infty)$ are the wavenumbers of the fluorescence band maximum at times t , 0, and ∞ . Fig. 6 presents the emission spectra

Table 1
Equilibrium constants of investigated molecules in CB[7] and cyclodextrins.

Molecule	Medium	K_f^i (M^{-1})	$\langle K_f^i \rangle$ (M^{-1})	$K_f^i(M^{-1})/K_f^c$ (M^{-1})	$\langle K_f^i \rangle$ (M^{-1})
I	CB[7]	1301 ($\pm 3\%$) ^a 1531 ($\pm 7\%$) ^b	1416	1642 ($\pm 9\%$) ^a 1626 ($\pm 3\%$) ^b 14 ^c	1634
	α -CD			4.5/12 ^c	
	γ -CD			2153 ($\pm 12\%$) ^a 4572 ($\pm 21\%$) ^b 210/5 ^c	3362
II	CB[7]	1176 ($\pm 17\%$) ^a 840 ($\pm 17\%$) ^b	1008	2153 ($\pm 12\%$) ^a 4572 ($\pm 21\%$) ^b 210/5 ^c	3362
	α -CD			379/15 ^c	
	γ -CD			1677 ($\pm 15\%$) ^a 1731 ($\pm 9\%$) ^b 106 ^c	1704
III	CB[7]	1053 ($\pm 3\%$) ^a 1647 ($\pm 3\%$) ^b	1350	1677 ($\pm 15\%$) ^a 1731 ($\pm 9\%$) ^b 106 ^c	1704
	β -CD				
	γ -CD				
IV	CB[7]	2250 ($\pm 12\%$) ^a 1925 ($\pm 6\%$) ^b	2087	3096 ($\pm 9\%$) ^a 4231 ($\pm 16\%$) ^b 38/52 ^c	3663
	α -CD				
	γ -CD				

^a Determined by Benesi-Hildebrand method.

^b Determined by the nonlinear least-squares regression.

^c From [17,19].

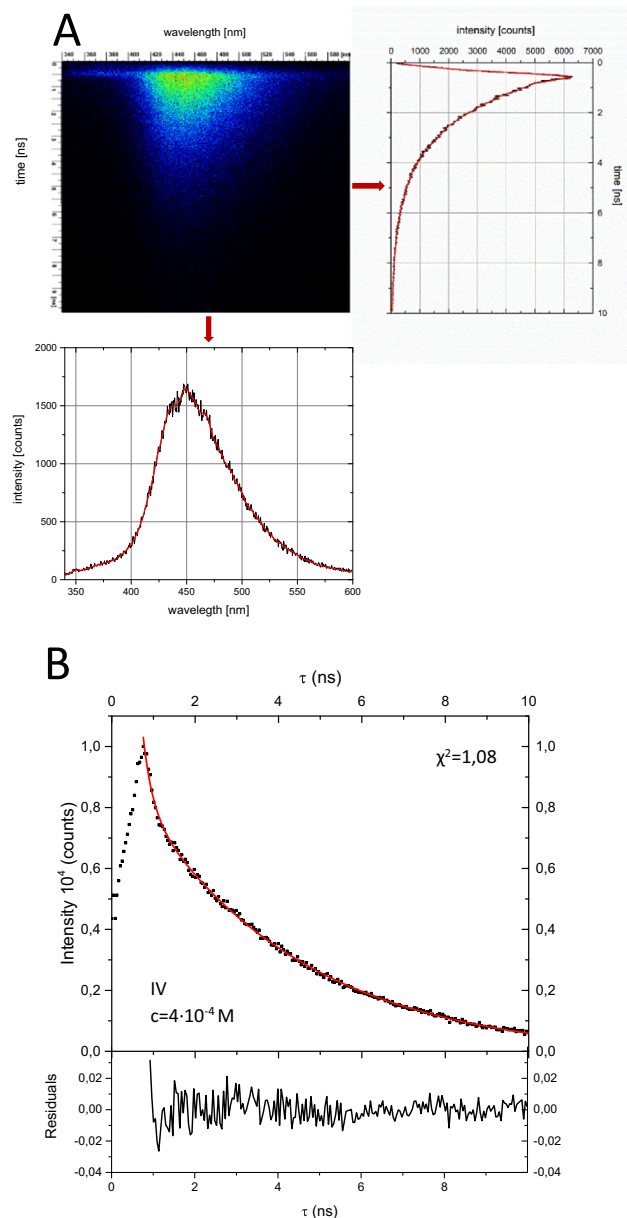


Fig. 5. (A) The original streak camera image for **IV** in aqueous solution with CB[7] and fluorescence spectrum at 100 ps after excitation as well as the fluorescence decay curve. (B) Representative fluorescence decay profile of **IV** in aqueous solution of CB[7] and distributions of the weighted residuals.

of **II** (A, A') and **IV** (B, B') in aqueous solution with CB[7] at various times after excitation. Because of the limited time resolution of a streak camera set up (~60 ps) an analysis of the dynamic Stokes shift for **I** and **III** was not carried out. Furthermore, we are also missing a part of the solvation dynamics of the molecules **II** and **IV** being in the locally-excited state which also occurs in a time scale faster than the time resolution of the set up used.

As can be seen in Fig. 6, upon increasing the time after excitation the position of the long-wavelength fluorescence band maximum ($\nu^{max}(ESIPT)$ for **II** and $\nu^{max}(ESIPT + ICT)$ for **IV**) is redshifted. Fig. 7 illustrates the temporal behavior of $c(t)$ function for the long-wavelength band of **II** (Fig. 7A) and **IV** (Fig. 7B) in aqueous solution with CB[7]. For molecule **II**, $c(t)$ versus time after excitation is very well fitted by a monoexponential function with the decay component of $\tau_{solv.} = 254$ ps, whereas for **IV**, the data could be well reproduced with the sum of two exponential functions with a fast ($\tau_{solv. (1)} = 50$ ps) component ($A_{solv. (1)} =$

Table 2

Fluorescence lifetimes of **I-IV** in aqueous solutions containing different concentrations of CB[7].

Molecule	Concentration (M)	τ_1 [ns]	A_1 [%]	τ_2 [ns]	A_2 [%]	τ_3 [ns]	A_3 [%]	χ^2
I	0	0.29	100	–	–	–	–	1.02
	0.0001	0.26	100	–	–	–	–	1.18
	0.0002	0.35	100	–	–	–	–	1.10
	0.0004	0.33	100	–	–	–	–	1.12
II	0	0.37	100	–	–	–	–	1.20
	0.0001	0.38	90	0.99	10	–	–	1.05
	0.0002	0.36	86	0.63	14	–	–	1.10
	0.0004	0.32	71	0.80	29	–	–	1.12
III	0	0.29	100	–	–	–	–	1.18
	0.0001	0.27	100	–	–	–	–	1.21
	0.0002	0.36	87	–	–	0.66	13	1.13
	0.0005	0.66	71	–	–	0.86	29	1.19
IV	0	0.25	100	–	–	–	–	1.16
	0.0001	0.30	96	1.52	4	–	–	1.06
	0.0002	0.23	34	1.64	24	3.85	42	1.19
	0.0005	0.36	8	1.48	26	3.73	66	1.17

12%) and a slow component of $\tau_{solv. (2)} = 337$ ps ($A_{solv. (2)} = 88\%$), with an average solvation time ($\langle \tau_{solv.} \rangle = (A_{solv. (1)} \tau_{solv. (1)}^2 + A_{solv. (2)} \tau_{solv. (2)}^2) / (A_{solv. (1)} \tau_{solv. (1)} + A_{solv. (2)} \tau_{solv. (2)})$) of 304 ps.

It is evident that the obtained solvation component (~300 ps) of **II** and **IV** in aqueous solution with CB[7] is significantly slower (at least 3 orders of magnitude) than the solvation dynamics of some organic molecule e.g., Coumarin 480 (310 fs) reported by Fleming et al. in bulk water [54]. These results indicate that the water molecules encapsulated in a supramolecular assembly are fundamentally different from bulk water. Several experimental and theoretical studies have shown [54,55] that solvation dynamics of water and DMF are significantly inhibited inside a cyclodextrin cavity, micelles, lipids, proteins and DNA [56]. Nandi and Bagchi ascribed the slow dynamics inside cyclodextrin cavity to almost complete suppression of the translational modes of the trapped water molecules in the γ -CD cavity [56,57]. According to the above mentioned investigations, we can state that the observed dramatic retardation of the solvation dynamics inside the studied CB[7] cavity can be attributed to the loss of translational modes in analogy to a similar model proposed for slow solvation dynamics of water in different supramolecular assemblies. Summarising, the received component arises mainly from the disruption of the hydrogen-bond network of water and the binding of water molecules to CB[7].

3.4. ^1H NMR studies on the host-guest complex

The interaction of methyl benzoate derivatives with CB[7] was also studied using ^1H NMR spectroscopic technique. It is accepted that the structure of the formed inclusion complex can be recognized by observing the chemical shift changes of the guest resonances upon addition of a macrocyclic host [58,59]. The upfield shifts of the proton resonances are characteristic of dyes encapsulated in the cucurbit cavity, whereas downfield shifts are observed for resonances from guest protons located outside of the cavity [58,59]. Fig. 7 shows the ^1H NMR spectra of CB[7], **I-IV** in D_2O in the absence and presence of 1.0 equiv. of CB[7]. As can be seen, the observed changes in the NMR signals possess very complex nature (Table 3). Interestingly, the nature of the NMR shifts for different protons are qualitatively different for methoxy (**I**-CB[7] and **III**-CB[7] systems) and hydroxy (**II**-CB[7] and **IV**-CB[7] systems) derivatives with CB[7], which indicates that the inclusion complexes of hydroxy and methoxy derivatives with CB[7] are of a different nature.

Upon addition of 1.0 equiv. of CB[7] to aqueous solution of **II** and **IV**, all aromatic protons of the benzene skeleton (H_a , H_b and H_c) experience an upfield shift, while the H_d proton of the methoxy group

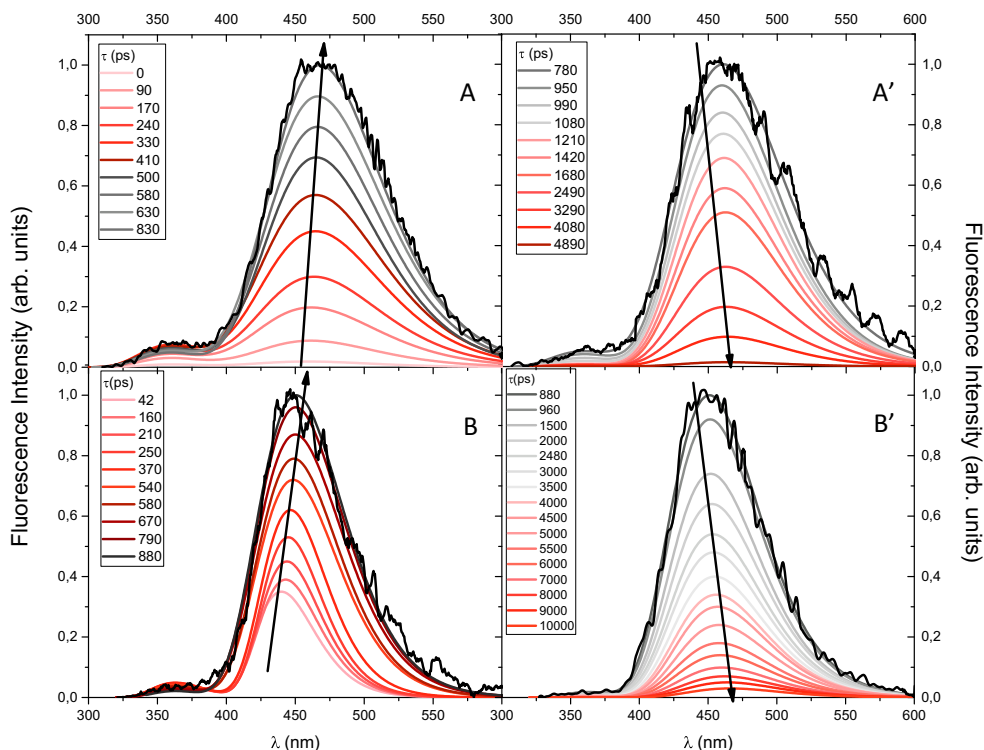


Fig. 6. Time-resolved emission spectra of **II** (A, A') and **IV** (B, B') ($c = 5 \cdot 10^{-5}$ M) in aqueous solution with CB[7] ($5 \cdot 10^{-4}$ M) at various times after excitation.

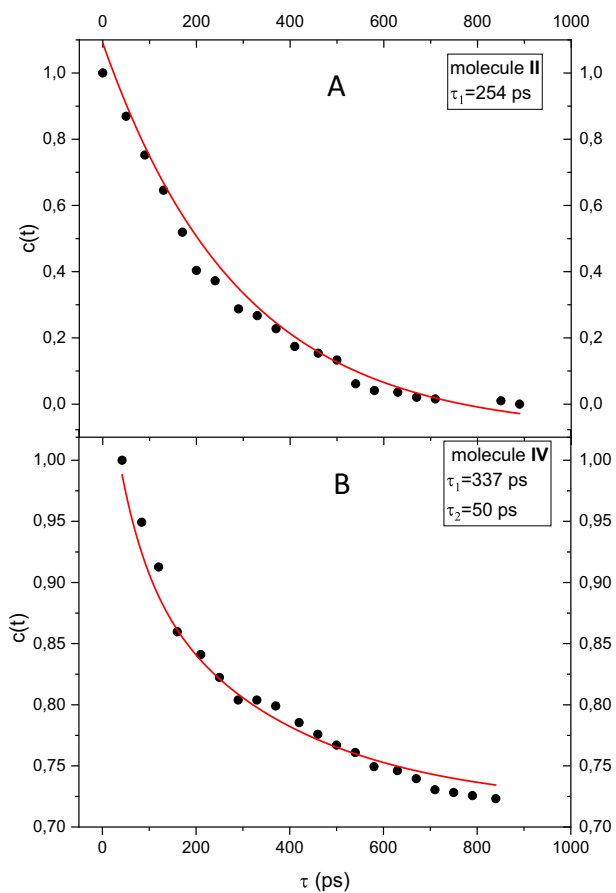


Fig. 7. Temporal behavior of $c(t)$ function for the long-wavelength band of **II** (A) and **IV** (B) in aqueous solution with CB[7] ($c_{\text{CB}[7]} = 5 \cdot 10^{-4}$ M).

undergoes marginal downfield shift (**II**-CB[7] system) or remains almost unchanged (**IV**-CB[7] system) (Fig. 8). These CB[7]-induced chemical shifts clearly demonstrate that the benzene ring is included inside the host cavity, while the methyl part of the $-\text{COOCH}_3$ group interacts with the carbonyl oxygens on the host portal. Moreover, the fact that the signals of the aromatic protons (H_a , H_b and H_c) are remarkably broadened indicates that the benzene ring is fully embedded in the interior of CB[7]. A similar broadening caused by a dynamic distribution of host-guest species during the time scale of the NMR experiment has been reported previously [60–62]. It is important to note that the chemical shift of the dimethylamino proton (H_f) of **IV**-CB[7] complex is practically unchanged, which also confirms the conclusions drawn from the evolution of the steady-state and time-resolved emission spectra described above i.e., $-\text{NMe}_2$ group is located outside of the CB[7] portal (negligible interaction of $-\text{NMe}_2$ group with CB[7]).

As can be seen in Figs. 8 and S7, in contrast to **II**-CB[7] and **IV**-CB[7] systems, the ^1H NMR signals corresponding to the H_a and H_b protons of the benzene ring of **I** and **III** are displaced downfield, while protons H_c and H_e show upfield shifts. These behaviors imply that only the part of the benzene ring is located within the CB[7] cavity. On the other hand,

Table 3
 ^1H NMR chemical shifts for **I**-**IV** protons in the absence and presence of CB[7].

Compound	CH_2	CH	CH_2	a	b	c	d	e	f
CB[7]	4.245	5.544	5.811	–	–	–	–	–	–
I	–	–	–	6.506	6.440	7.763	3.844	3.874	–
I -CB[7]	4.239	5.537	5.807	6.536	6.495	7.751	3.841	3.841	–
II	–	–	–	6.300	6.393	7.724	3.911	–	–
II -CB[7]	4.187	5.475	5.785	6.128	6.128	7.469	3.911	–	–
III	–	–	–	6.366	6.490	7.845	3.848	3.933	3.059
III -CB[7]	4.241	5.539	5.804	6.659	6.659	7.821	3.837	3.837	3.053
IV	–	–	–	6.269	6.477	7.783	3.914	–	3.032
IV -CB[7]	4.236	5.533	5.804	6.216	6.388	7.691	3.914	–	3.042

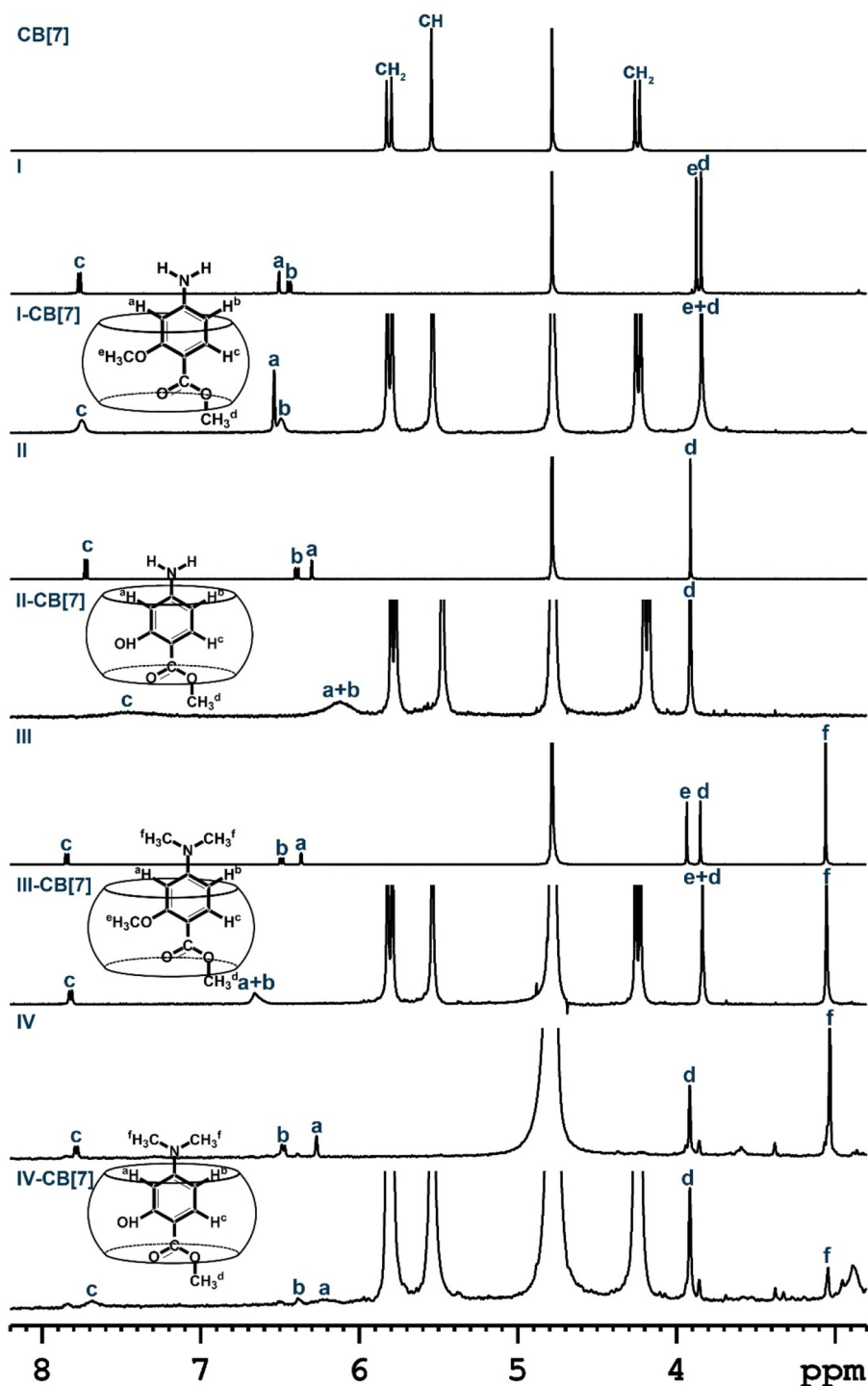


Fig. 8. ^1H NMR spectra of CB[7], I-IV in D_2O in the absence and presence of 1.0 equiv. of CB[7].

the chemical shifts of the H_a and H_f protons of I-CB[7] and III-CB[7] systems are very similar to those in II-CB[7] and IV-CB[7] systems suggesting that H_a and H_f protons interact with the carbonyl oxygens of the host portal.

4. Conclusions

In the present work the supramolecular interactions of four methyl benzoate derivatives with CB[7] were studied in aqueous solution using steady-state, time-resolved and ^1H NMR spectroscopic

techniques. We found that investigated dyes form stable 1:1 inclusion complex in the ground and excited states. Our experimental data clearly indicate that the molecules which are capable of exhibiting ESIPT-type behavior (II and IV) form more stable inclusion complexes with CB[7] in the excited state than two methoxy derivatives (I and III). The observed changes in the ^1H NMR signals point toward the fact that hydroxy derivatives (II and IV) are more deeply embedded in the CB[7] cavity. Finally, ^1H NMR chemical shifts and evolution of the steady-state and time-resolved emission spectra clearly indicate that -NMe₂ group attached to the III and IV is located outside of the CB[7] portal

(water molecules form a chemical bond with n electrons of N atom of the $-NMe_2$ group and twisted intramolecular charge transfer process in the excited state does not appear).

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.molliq.2020.113921>.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author statement

This paper is dedicated to Professor Janina Heldt on the occasion of her 75th birthday. The work was created by the joint effort of all co-authors.

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Article

Insight into Molecular Interactions of Two Methyl Benzoate Derivatives with Bovine Serum Albumin

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Abstract: The nature and mechanisms of interaction between two selected methyl benzoate derivatives (methyl *o*-methoxy *p*-methylaminobenzoate–I and methyl *o*-hydroxy *p*-methylaminobenzoate–II) and model transport protein bovine serum albumin (BSA) was studied using steady-state and time-resolved spectroscopic techniques. In order to understand the role of Trp residue of BSA in the I-BSA and II-BSA interaction, the effect of free Trp amino acid on the both emission modes (LE–locally excited (I and II) and ESIPT–excited state intramolecular proton transfer (II)) was investigated as well. Experimental results show that the investigated interactions (with both BSA and Trp) are mostly conditioned by the ground and excited state complex formation processes. Both molecules form stable complexes with BSA and Trp (with 1:1 stoichiometry) in the ground and excited states. The binding constants were in the order of 10^4 M^{-1} . The absorption- and fluorescence-titration experiments along with the time-resolved fluorescence measurements show that the binding of the I and II causes fluorescence quenching of BSA through the static mechanism, revealing a 1:1 interaction. The magnitude and the sign of the thermodynamic parameters, ΔH , ΔS , and ΔG , determined from van't Hoff relationship, confirm the predominance of the hydrogen-bonding interactions for the binding phenomenon. To improve and complete knowledge of methyl benzoate derivative-protein interactions in relation to supramolecular solvation dynamics, the time-dependent fluorescence Stokes' shifts, represented by the normalized spectral response function $c(t)$, was studied. Our studies reveal that the solvation dynamics that occurs in subpicosecond time scale in neat solvents of different polarities is slowed down significantly when the organic molecule is transferred to BSA cavity.

Keywords: inclusion complex; bovine serum albumin; tryptophan; excited-state intramolecular proton transfer; supramolecular interactions



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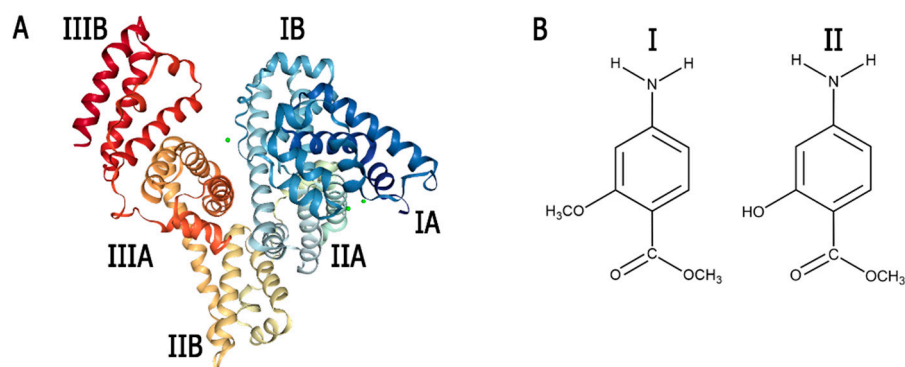
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1. Introduction

During the past decade, considerable attention has been focused on characterization of noncovalent interactions between a macrocyclic host (chemical and/or biological) and different guest molecules (especially drugs and/or small bioactive molecules) [1–3]. Cyclodextrins (α , β and γ), calixarenes, cucurbiturils, pillararenes, crown ethers and cyclophanes are one of such chemical organized assemblies possessing a hydrophobic nanocavity, which can accommodate guest molecules [4–6]. The understanding of the host–guest interactions is highly desirable for at least two main reasons. First, the explanation of the photochemical and photophysical processes leading to the formation of host–guest inclusion complex is interesting from a spectroscopic point of view, because the incorporation of the molecule into the chemical or biological nanocavity creates a new chemical system with significantly different spectroscopic properties [7,8]. Conversely, owing to complexation ability, chemical and biological cavities have received widespread attention for applications in medicine, agriculture, nanotechnology, cosmetics, food and pharmaceutical industries [9–14]. Among various representatives of chemical and biological macromolecules, nontoxic cyclodextrins (chemical) and human as well as bovine serum

albumin (biological) are the most investigated ones because of their use in medicine as drug delivery carriers (cyclodextrins) and transport proteins (serum albumins) [15–19].

Serum albumins, the most abundant proteins in the blood plasma, play a significant role in the transport of both exogenous and endogenous compounds. Several experimental and theoretical studies have shown that human serum albumin (HSA) and bovine serum albumin (BSA) present an ideal model of serum carrier protein, and therefore they are most widely used as model proteins in evaluating small organic molecules (drug)–protein interactions [15,20–23]. BSA finds a wider range of applications because of its (i) low cost, (ii) high water solubility (iii) almost 80% sequence homology with HSA and (iv) widespread availability in a pure form. The crystal structure of BSA shows three domains (I, II, III), which are divided into two subdomains, named A and B [24,25] (Scheme 1A). The drug-binding sites are located in hydrophobic cavities in subdomains IIA and IIIA. Although HSA and BSA exhibit similar conformation properties, their spectroscopic properties are different. This is because BSA has two tryptophan residues that possess intrinsic fluorescence (Trp 134 and Trp 212), whereas HSA has only one residue (Trp 214) [26,27]. Trp 212 is located within a hydrophobic binding pocket of the protein and Trp 134 is located on the surface of the molecule [24].



Scheme 1. Structure of bovine serum albumin (BSA) (A) and methyl benzoate derivatives (methyl *o*-methoxy *p*-methylaminobenzoate-I, methyl *o*-hydroxy *p*-methylaminobenzoate-II) (B).

We have recently undertaken spectroscopic investigations of the some methyl benzoate derivatives which, on the one hand, are interesting from the spectroscopic point of view (they are capable of exhibiting Twisted Intramolecular Charge Transfer- and/or Excited State Intramolecular Proton Transfer-type behaviour) and, conversely, they have potential in applications e.g., TICT and ESIPT fluorescence probes, metal ion sensors, active materials in dry xerographic toners or lasing medium in proton transfer lasers [28–32]. Our scientific attention has been focused not only on the description of the TICT and ESIPT processes of organic molecules in different organic solvents and heterogeneous systems, but also in aqueous solution in the presence of the supramolecular compounds (α -, β - and γ -cyclodextrins, cucurbit[7]uril) [33–37]. We have shown that photochemical and photophysical properties of investigated molecules encapsulated in macrocyclic host macrocyclic host are significantly different from those observed in bulk solution. These significant changes in spectroscopic behaviours were attributed to the formation of stable 1:1 and/or 1:2 inclusion complexes. The effect of macrocyclic host complexation on TICT and ESIPT photochemistry has been interpreted in terms of the protection of the embedded organic molecule in host cavity from the neighbouring water molecules (reduction of hydrogen bonds upon encapsulation), size and shape of macrocyclic host, orientation of the guest molecule in micropockets, restriction on molecular motion and the reduced polarity effect introduced by cavities as compared to the aqueous phase [33–37].

However, important issues on the nature and mechanisms of host guest interactions remain open and require clarification. Herein, we focus on the deepening the knowledge of the nature and mechanisms of interactions between two selected methyl

benzoate derivatives (methyl *o*-methoxy *p*-methylaminobenzoate–I, methyl *o*-hydroxy *p*-methylaminobenzoate–II (Scheme 1B))—showing locally-excited (I and II) and ESIPT fluorescence (II)—and biological cavity (BSA). In particular, it is compared whether or not the investigated fluorophores show similar spectroscopic and photochemical properties in the presence of biological cavities (BSA) as in the case of chemical nanocages (cyclodextrins (α , β and γ) and cucurbit[7]urils). The interaction of I and II with the model transport protein BSA is studied using steady-state and state-of-the-art time-resolved spectroscopic techniques, which proved to be helpful in understanding the mechanisms of the processes leading to the formation of a stable small organic molecule–protein complexes (quenching, binding and thermodynamic parameters). Overall, our current (in combination with our previous) studies offer a detailed outlook into the dynamic and structural aspects of chemical (CDs, cucurbit[7]urils) [33–37] and biological (BSA) host–guest interactions. Especially, we investigate the effects exerted by biological and chemical nanocavity on the excited-state intramolecular proton and electron transfer processes that may occur in supramolecular systems.

2. Results and Discussion

2.1. Steady-State Absorption and Fluorescence Spectra of I and II in the Phosphate Buffer in the Presence of BSA

As mentioned earlier, UV–visible absorption and fluorescence spectroscopy belongs to the most important techniques used to explore small organic molecule–protein interactions. This is due to the fact that the spectroscopic properties of HSA and/or BSA (in fact protein’s fluorophores) are sensitive to the immediate neighbourhood i.e., polarity of their surroundings, as well as composition of the solvation shell. Thus, before undertaking the investigation of the interactions between BSA and two methyl benzoate derivatives (I and II), it is necessary to recall the spectroscopic behaviour of these two organic fluorophores, BSA, as well as three crucial amino acid residues: tryptophan (Trp), tyrosine (Tyr), and phenylalanine (Phe) (because, as mentioned, spectroscopic properties of the BSA are mainly governed by the absorption/fluorescence of above mentioned residues) [38].

Several spectroscopic investigations were carried out on the I-BSA and II-BSA systems (and in parallel on the I-Trp, II-Trp systems) to recognize whether some interactions occurred and to obtain insight into the nature of such interactions. The spectral characteristics of the two investigated methyl benzoate derivatives, BSA and three crucial amino acid residues (Trp, Tyr, Phe) in the phosphate buffer are presented in Figure 1. As can be seen, these residues have distinct absorption and emission wavelengths and differ in the quantum yield (in aqueous medium quantum yields of Trp, Tyr and Phe are near 0.2, 0.14 and 0.03, respectively) [38]. Several experimental and theoretical studies have shown that, proteins emission is dominated by Trp, which absorbs at the longest wavelength ($\lambda_{max}^{abs}(Trp)$ in phosphate buffer occurs at 280 nm) and displays the largest molar absorption coefficient [38]. Although Tyr quantum yield is similar to that of Trp, emission is often quenched, mostly due to its interactions with the peptide chain or as a result of energy transfer to Trp [38]. As can be seen, Tyr can be excited at wavelengths similar to that of Trp ($\lambda_{max}^{abs}(Tyr)$ in phosphate buffer occurs at 275 nm) but emits at a distinctly different wavelength. The wavelength of maximum intensity of the fluorescence band of Tyr is distinctly blue-shifted in comparison to the fluorescence band of Trp ($\lambda_{max}^{fl}(Tyr) = 303$ nm, $\lambda_{max}^{fl}(Trp) = 351$ nm). It is also evident that among the three crucial amino acids, Phe displays the shortest absorption and emission wavelengths. Considering that proteins fluorescence is generally excited at 280 nm or at longer wavelengths, Phe is not excited in the majority of experiments (this holds true also in our case).

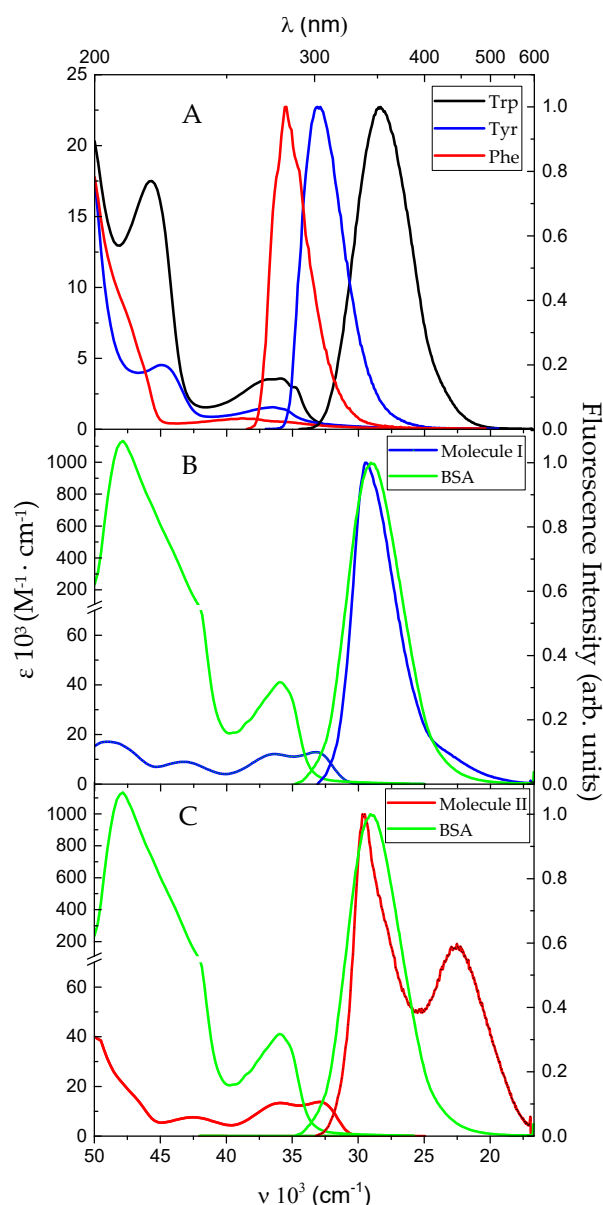


Figure 1. Absorption and emission spectra of the three crucial aromatic amino acids (A), BSA (B,C) and investigated dyes (B,C) in phosphate buffer solution (pH 7.4). In order to better visualize the absorbance scale (representing the molar absorption coefficient) for BSA and investigated molecules, the ordinate axis has been truncated.

In previous reports [28–32], we have shown that the long-wavelength absorption band of the studied fluorophores in neat solvents consists of two electronic transitions $S_0 \rightarrow S_1$ and $S_0 \rightarrow S_2$ of different character: $\pi\pi^*$ and charge-transfer (CT). Moreover, it is well established that molecule II dissolved in some media besides the locally-excited (LE) fluorescence exhibits the long-wavelength emission from the excited-state intramolecular proton transfer (ESIPT) state [28–32]. In Figure 1B,C, the long-wavelength absorption and fluorescence spectra of BSA in the phosphate buffer are compared with those of I and II. As the spectral behaviours of BSA mainly arise due to the presence of two tryptophan residues (Trp-212 (located in hydrophobic subdomain IB) and Trp-134 (located near the surface of the albumin molecule in the second helix of the first domain)), the steady-state absorption and fluorescence spectra of BSA and Trp show similar spectral features ($\lambda_{max}^{abs}(\text{Trp}) = 280 \text{ nm}$ versus $\lambda_{max}^{abs}(\text{BSA}) = 278 \text{ nm}$, $\lambda_{max}^{fl}(\text{Trp}) = 351 \text{ nm}$ versus

λ_{max}^{fl} (BSA) = 344 nm). The long-wavelength absorption spectrum of BSA overlaps with the broad, long-wavelength absorption band (consisting of two different electronic transitions) of the investigated methyl benzoate derivatives, as well as the short-wavelength, locally-excited fluorescence band of **I** and **II** considerably overlap with that of serum protein.

In order to obtain more insight into **I**-BSA and **II**-BSA interaction process, the absorption- and fluorescence-titration experiments were performed with varying concentrations of BSA. Figure 2 shows the LW absorption spectra of **I** and **II** in the phosphate buffer solutions containing different concentrations of BSA. Upon addition of BSA, the molar absorption coefficient of the first long-wavelength absorption band centred at about 275 nm (molecule **I**) and 279 nm (molecule **II**) gradually increases, whereas no significant changes in the second long-wavelength absorption band (centred at 300 nm (**I**) and 305 nm (**II**)) were observed in the presence of BSA. Considering the absorption characteristics of BSA in phosphate buffer solution, this spectral behaviour probably comes from the absorption of BSA molecule. Moreover, an isosbestic point can be clearly seen between these two bands, which indicates the presence of equilibrium between two different species (**I** (or **II**) and the ground-state complex **I**-BSA (or **II**-BSA)).

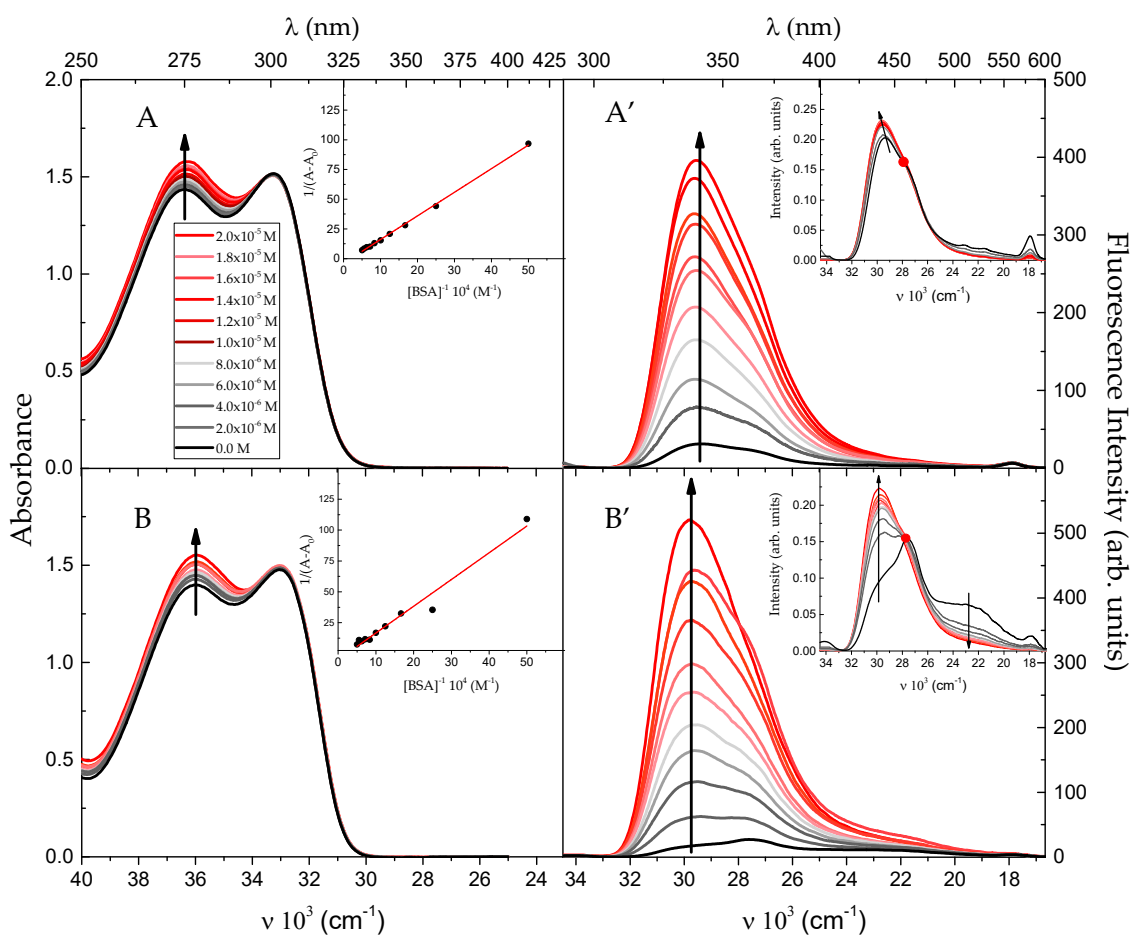


Figure 2. Absorption and fluorescence spectra ($\lambda_{exc.} = 300$ nm) of **I** (**A,A'**), **II** (**B,B'**) ($c = 5 \times 10^{-5}$ M) in phosphate buffer solutions containing different concentration of BSA (0 M, 2.0×10^{-6} M, 4.0×10^{-6} M, 6.0×10^{-6} M, 8.0×10^{-6} M, 1.0×10^{-5} M, 1.2×10^{-5} M, 1.4×10^{-5} M, 1.6×10^{-5} M, 1.8×10^{-5} M, 2.0×10^{-5} M). Insert the ground-state Benesi–Hildebrand dependence for **I** (**A**), **II** (**B**) and the normalized fluorescence spectra of **I** (**A'**) and **II** (**B'**) in phosphate buffer solutions containing different concentration of BSA.

Addition of BSA to the phosphate buffer solutions of **I** and **II** brings about a strong increase in the fluorescence intensity with a slight shift in the position of the short-wavelength fluorescence band maximum (~ 4 nm). Moreover, when the protein concentration is in-

creased, an isosbestic point can be clearly seen in normalized fluorescence spectra (we normalized each of the area under the fluorescence curve to unity, in this case, fluorescence spectrum corresponds to the emission of the same number of emitting molecules) points that equilibrium is established also in the excited state for both studied systems (see insert of Figure 2). The appearance of an isosbestic point-like feature in the normalized fluorescence spectra of molecule which is capable of exhibiting ESIPT-type behaviour (molecule II) and gradual decrease in the intensity of the ESIPT fluorescence band indicate that the binding between II and BSA is specific and involves the hydroxy (-OH) and/or ester (-COOCH₃) groups, which causes that ESIPT does not appear.

The changes in the absorbance and fluorescence intensity of I and II in the presence of BSA have been used to create the Benesi–Hildebrand (B–H) plot (see Equation (2a,b)). As can be seen in the insert of Figure 2 and Figure S1 (Supplementary Materials), for both investigated systems, the plots of $1/(A - A_0)$ versus $1/[BSA]$, as well as $1/(I - I_0)$ versus $1/[BSA]$ demonstrate linear dependence, which indicates the formation of well-defined 1:1 complexes with BSA, both in the ground and excited states. The binding constants were found to be $(1.9 \pm 0.1) \times 10^4 \text{ M}^{-1}$ and $(2.3 \pm 0.1) \times 10^4 \text{ M}^{-1}$ in the ground state and $(2.1 \pm 0.1) \times 10^4 \text{ M}^{-1}$ and $(2.0 \pm 0.1) \times 10^4 \text{ M}^{-1}$ in the excited state for I and II, respectively. These results indicate the strong binding affinity of the investigated dyes to BSA in the ground and excited states.

2.2. Time-Resolved Studies of I and II in the Phosphate Buffer in the Presence of BSA

To obtain information on the excited-state dynamics of the methyl benzoate derivative-BSA system in the phosphate buffer, the time-resolved emission spectra (TRES) of the investigated dyes upon the gradual addition of BSA were recorded. Table 1 gives the fluorescence lifetimes and preexponential factors describing the contribution of the *i*-th fluorescence decay component to the total emission.

Table 1. Fluorescence lifetimes of I and II in phosphate buffer solutions containing different concentrations of BSA.

Molecule	BSA Concentration 10 ⁻⁵ (M)	τ_1 (ns)	A_1 (%)	τ_2 (ns)	A_2 (%)	τ_3 (ns)	A_3 (%)	r^2
I	0	0.19	100					0.98
	0.1	0.20	7			4.49	93	0.99
	0.2	0.30	3			5.36	97	0.99
	0.4	0.30	2			5.18	98	0.99
	0.6	0.32	1			5.57	99	0.99
	0.8					5.51	100	0.99
	1.4					5.69	100	0.99
	2.0					5.78	100	0.99
II	0	0.20	24	0.80	76			0.95
	0.1	0.21	14	1.15	15	5.79	71	0.99
	0.2	0.19	5	1.19	17	5.56	78	0.99
	0.4	0.19	2	1.00	9	5.71	89	0.99
	0.6	0.18	1	1.00	6	5.23	93	0.99
	0.8					5.58	100	0.99
	1.4					5.52	100	0.99
	2.0					5.84	100	0.99

We will first discuss the changes in the fluorescence lifetimes of molecule II, which is capable of exhibiting ESIPT-type behaviour, in the presence of BSA in the phosphate buffer medium. As expected, fluorescence decay profile of II recorded in the absence of BSA

consists of a slow decay $\tau_2 = 0.8$ ns as a major component (76%) with a small contribution of a fast decay component ($\tau_1 = 200$ ps, $A_1 = 24\%$). According to our previous works [35,37], we can state that the slow component can be assigned to the emission from ESIPT state, whereas the decay time of the fast component originates from the emission of LE state.

The fluorescence kinetics of **II** in the presence of BSA are of the most complex nature (see Table 1 and Figure 3). In the presence of BSA at low concentration (10^{-6} – 6×10^{-6} M), the decay fits to three-exponential function with time constants of $\tau_1 \sim 200$ ps, $\tau_2 \sim 1$ ns and $\tau_3 \sim 5.7$ ns, whereas at the highest concentration (8×10^{-6} – 2×10^{-5} M), fluorescence decays can be reasonably well fitted by monoexponential function ($\tau_3 \sim 5.7$ ns) (see Table 1 and Figure 3). Through the individual form of the time constants of **II** (LE and ESIPT) remain almost unchanged, their relative preexponential factors are distinctly modified upon interactions with BSA. The preexponential factors associated with the LE and ESIPT emission (A_1 and A_2) are found to be decreased from 24% and 76% to 1% and 6%, respectively, whereas A_3 factor presents opposite behaviour. These results clearly indicate that the short components (τ_1 and τ_2) correspond to the unbound forms (LE and ESIPT), while the long component τ_3 corresponds to the bound form (**II**-BSA). Moreover, this binding results in the absence of the intramolecular proton transfer process in the excited state, confirming the involvement of the hydroxy (-OH) and/or ester (-COOCH₃) groups in specific interactions.

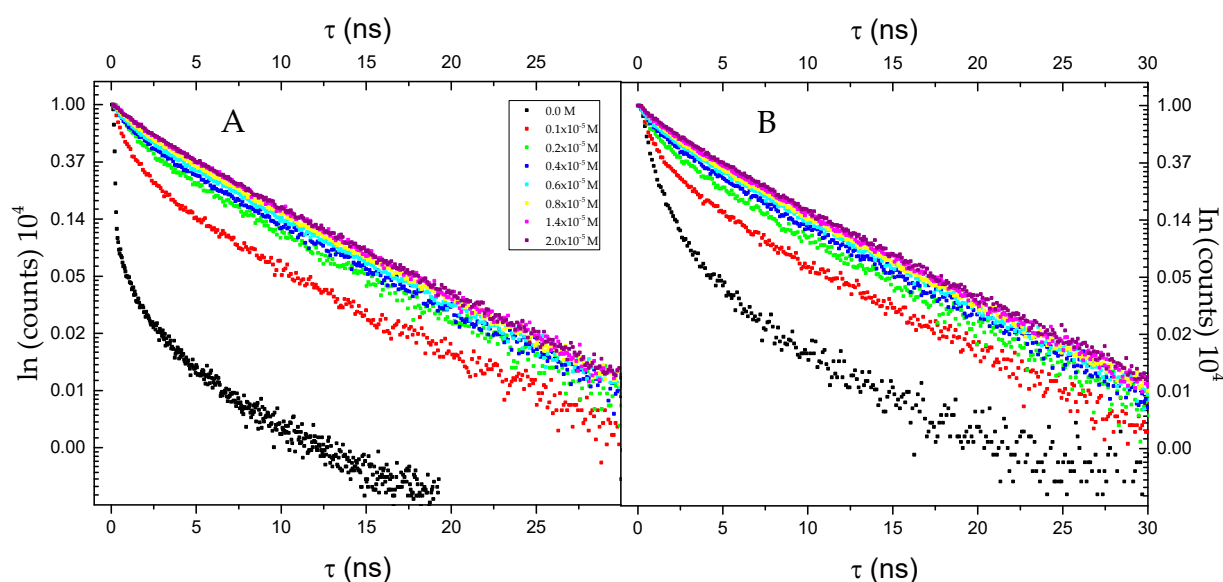


Figure 3. Fluorescence decay profiles of **I** (A) and **II** (B) in absence and different concentration of BSA (0.0 M, 0.1×10^{-5} M, 0.2×10^{-5} M, 0.4×10^{-5} M, 0.6×10^{-5} M, 0.8×10^{-5} M, 1.4×10^{-5} M, 2.0×10^{-5} M).

For the molecule **I** in the phosphate buffer medium, the fluorescence decay profile can be satisfactorily fitted by monoexponential function with the fluorescence decay time $\tau_1 \sim 200$ ps (Table 1), in the presence of BSA at low concentration (10^{-6} – 6×10^{-6} M), the sum of two exponential functions was required, whereas at the highest concentration (8×10^{-6} – 2×10^{-5} M), again only one emissive centre was observed ($\tau_3 \sim 5.7$ ns) (Figure 3). In the presence of low concentration of BSA (10^{-6} M), fluorescence kinetics exhibits a slow decay component τ_3 as a major component (93%), which is close to the average lifetime of two Trp moieties [21], with a small contribution of fast decay component ($\tau_1 \sim 200$ ps). It should be also clearly state that, the values of fast and slow decay components (τ_1 and τ_3) remain almost unchanged (τ_1 slightly increases) with increasing BSA concentration. Conversely, the preexponential factor A_1 corresponding to the decay of LE form of the molecule **I** decreases with increasing BSA concentration, whereas A_3 factor presents opposite behaviour. These findings indicate that the possibility of the emission from LE state

is limited due to the presence of specific interactions between **I** and BSA (formation of **I**-BSA complex).

In our previous paper, the mechanism of supramolecular solvation dynamics of the methyl benzoate derivatives in aqueous solution with CB[7] has been investigated according to the procedure described by Maroncelli and Fleming [37,39,40]. We have shown that solvation dynamics which occurs in subpicosecond time scale in neat solvents of different polarities is slowed down significantly when organic molecule is relocated to CB[7] cavity. In order to determine and compare solvation dynamics in selected chemical and biological nanocavities (i.e., CB[7]) versus BSA), the time-dependent fluorescence Stokes' shifts (determined from the emission spectra registered at various times after excitation (see Figure 4), were used to create the normalized solvation correlation function $c(t)$ (see Equation (7)). Figure 5 presents the temporal behaviour of $c(t)$ function for **I** and **II** bound to BSA. For both molecules, decay of $c(t)$ is well fitted by the sum of two exponential functions (see Equation (8)) with a fast component $\tau_{1(\text{solv.})}$ (**I**) = 430 ps, $\tau_{1(\text{solv.})}$ (**II**) = 520 ps ($A_{1(\text{solv.})}$ (**I**) = 2%, $A_{1(\text{solv.})}$ (**II**) = 3%) and a slow component of $\tau_{2(\text{solv.})}$ (**I**) = 4.07 ns, $\tau_{2(\text{solv.})}$ (**II**) = 5.25 ns ($A_{1(\text{solv.})}$ (**I**) = 98%, $A_{1(\text{solv.})}$ (**II**) = 97%), with an average solvation time $\langle \tau_{(\text{solv.})} \rangle$ (**I**) = 4.06 ns, and $\langle \tau_{(\text{solv.})} \rangle$ (**II**) = 5.24 ns.

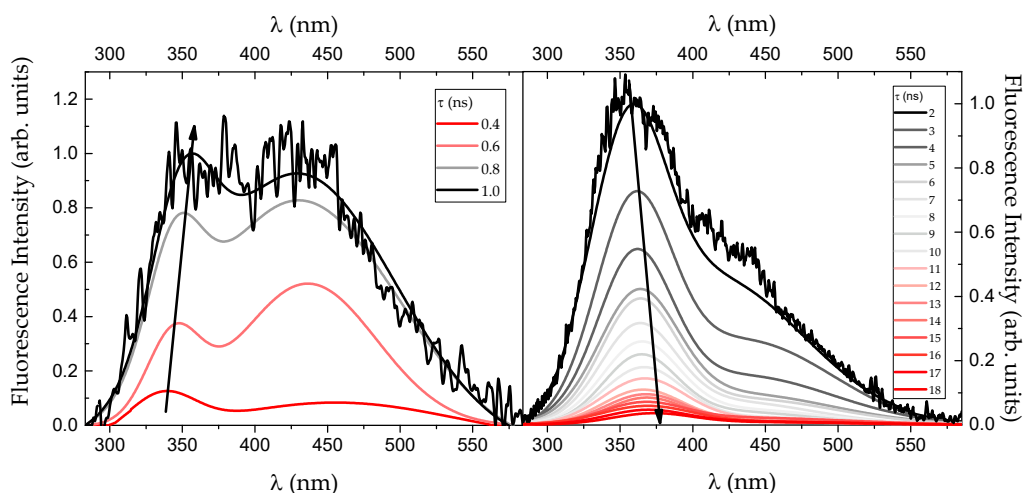


Figure 4. Time-resolved emission spectra ($\lambda_{\text{exc.}} = 300$ nm) of **II** in phosphate buffer solution with BSA ($c_{\text{BSA}} = 10^{-6}$ M) at various times after excitation.

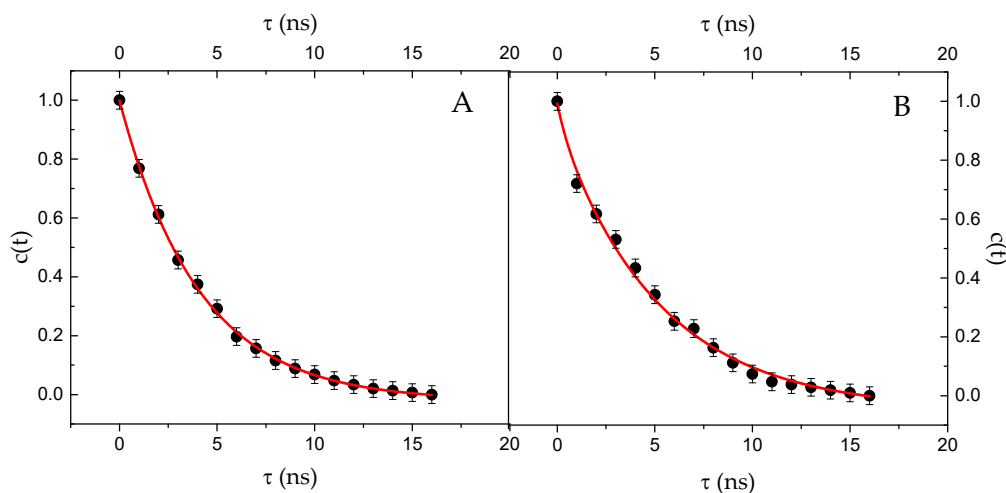


Figure 5. Temporal behaviour of $c(t)$ function for the short-wavelength fluorescence band of **I** (**A**) and **II** (**B**) in phosphate buffer solution with BSA ($c_{\text{BSA}} = 10^{-6}$ M).

In order to explain the presence of the nanosecond component of the solvation dynamics observed in our studies, it is necessary to assume the restriction on the movement of the biological water molecules in the vicinity of the protein. Similar behaviour has been reported previously and explained as a result of three main causes: (i) in bulk, the movement of water is cooperative and involves an extensive network of hydrogen bonds, (ii) in the case of protein-bound water, the hydrogen bond network is significantly disturbed, (iii) the different solvation times of biological water molecules may correspond to different parts of albumin [41,42]. Thus, the present investigation clearly shows that the solvation dynamics of **I** and **II** in aqueous solution with CB[7] is different from that in biological nanocavities (BSA). The average solvation time in methyl benzoate derivative-BSA system is found to be about one order of magnitude longer than that in **I**-CB[7] (or **II**-CB[7]) system. This indicates that at BSA hydrophobic interior, where investigated organic molecule is buried, the water molecules are more strongly confined than in the CB[7] cavity and therefore show slower solvation dynamics.

2.3. Effect of Trp on the Steady-State and Time-Resolved Spectroscopic Behaviour of **I** and **II**

In order to understand the role of Trp residue of BSA in the **I**-BSA and **II**-BSA interaction, it is useful to also study the interactions between molecules under study and the free Trp amino acid. As seen in Figure 6, the steady-state absorption and fluorescence spectra of buffered solutions of **I** and **II** are also affected by the presence of tryptophan molecules. For both fluorophores, with the gradual addition of Trp, the molar absorption coefficient of the first long-wavelength absorption band increases without change in spectral distribution of the second LW absorption band. Moreover, the occurrence of an isosbestic point in the titration experiment suggests that the equilibrium is established between methyl benzoate derivative (**I** or **II**) and the methyl benzoate derivative-Trp complex in the ground state. The excited-state spectral behaviours of **I**-Trp and **II**-Trp systems (e.g., increase in the fluorescence intensity and presence of an isosbestic point in the steady-state area normalized emission spectra) are similar to those in **I**-BSA and **II**-BSA systems suggesting that methyl benzoate derivative-Trp complex is also formed in the excited state. It is also evident from the normalized fluorescence spectra that for fluorophore **II**, upon increasing the Trp concentration, the intensity of the ESIPT fluorescence band decreases and simultaneously the short-wavelength (LE) emission band increases. The described experiment indicates (as was the case with **II**-BSA system) the involvement of the -OH and/or -COOCH₃ substituents in specific interactions between **II** and Trp. Here, the same absorption- and fluorescence-titration experiments were performed for **I**-Tyr, **II**-Tyr, **I**-Phe and **II**-Phe systems. For these systems, the absorption and fluorescence spectra of **I** and **II** in phosphate buffer solution containing different concentration of two amino acid residues (Tyr and Phe) were practically unchanged, which indicates that probably no specific interaction with Tyr and Phe occurs.

As can be seen in Figure S2, for both studied systems, the plots of $1/(A - A_0)$ versus $1/[\text{Trp}]$ and $1/(I - I_0)$ versus $1/[\text{Tryp}]$ show linear dependence over the whole range of studied Trp concentration, confirming the formation of complexes with 1:1 stoichiometry between methyl benzoate derivatives (**I** and **II**) and Trp in the ground and excited states. The binding constants were found to be $(2.1 \pm 0.1) \times 10^4 \text{ M}^{-1}$ and $(2.6 \pm 0.1) \times 10^4 \text{ M}^{-1}$ in the ground state and $(1.9 \pm 0.1) \times 10^4 \text{ M}^{-1}$ and $(2.6 \pm 0.1) \times 10^4 \text{ M}^{-1}$ in the excited state for **I** and **II**, respectively, which is similar to the previously calculated values for **I**-BSA and **II**-BSA complexes. This behaviour may indicate that mainly the Trp residue of BSA is involved in process of **I**-BSA and **II**-BSA specific interactions.

As can be seen in Table 2, the fluorescence kinetics of the investigated molecules in the presence of Trp is similar to that in **I**-BSA and **II**-BSA systems ((i) decay components (τ_1 , τ_2 and τ_3) remain almost unchanged; (ii) the preexponential factors A_1 and A_2 corresponding to decay of LE and ESIPT (molecule **II**) emission decrease with increasing Trp concentration, whereas A_3 factor (corresponding to the decay of bound form (**I**-Trp and **II**-Trp)) presents opposite behaviour) confirming the formation of complex between methyl benzoate derivatives (**I** and **II**) and Trp.

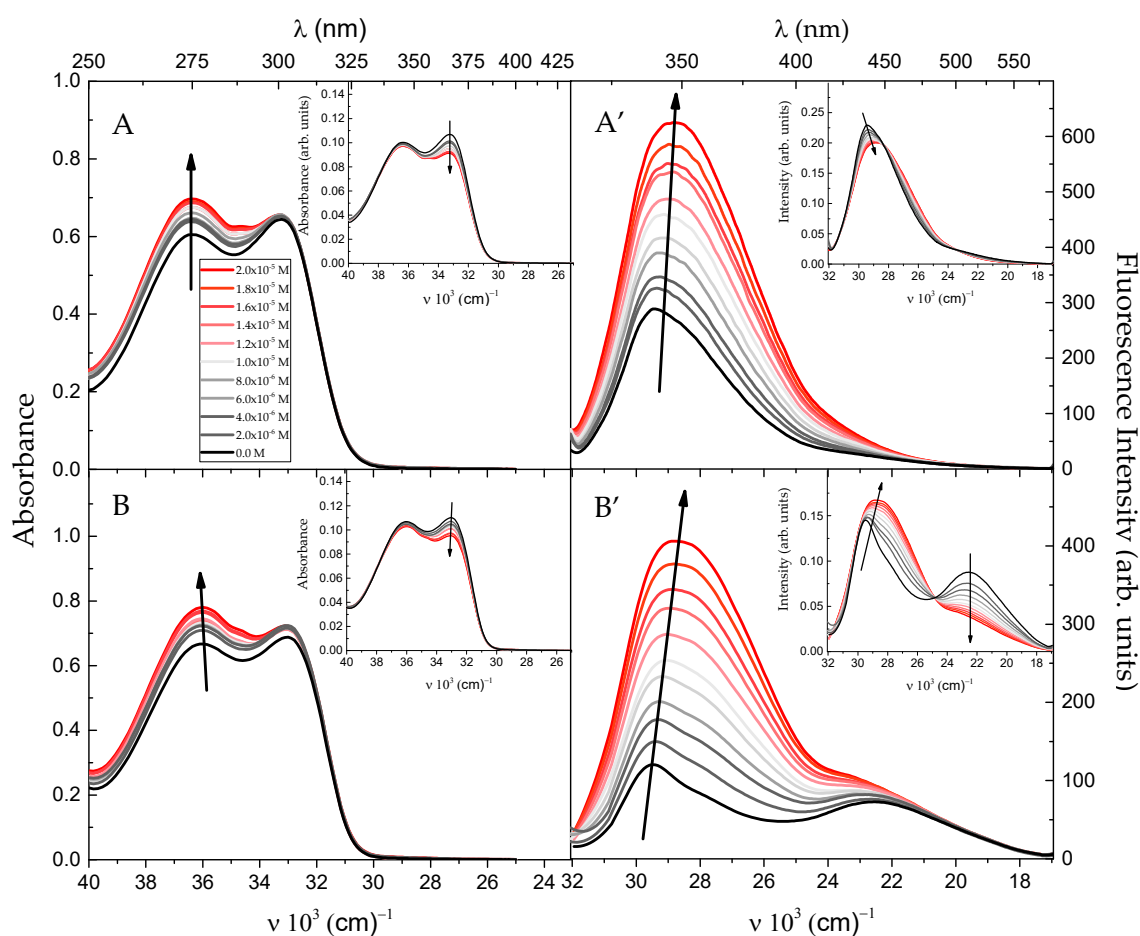
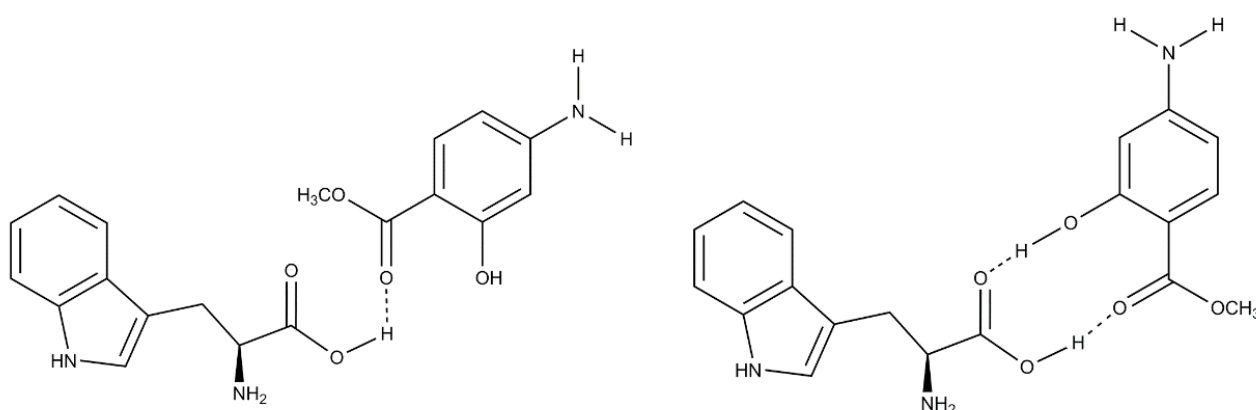


Figure 6. The long-wavelength absorption and fluorescence spectra ($\lambda_{\text{exc.}} = 300 \text{ nm}$) of **I** (**A,A'**) and **II** (**B,B'**) ($c = 5 \times 10^{-5} \text{ M}$) in phosphate buffer solutions containing different concentration of Trp (0 M, $2.0 \times 10^{-6} \text{ M}$, $4.0 \times 10^{-6} \text{ M}$, $6.0 \times 10^{-6} \text{ M}$, $8.0 \times 10^{-6} \text{ M}$, $1.0 \times 10^{-5} \text{ M}$, $1.2 \times 10^{-5} \text{ M}$, $1.4 \times 10^{-5} \text{ M}$, $1.6 \times 10^{-5} \text{ M}$, $1.8 \times 10^{-5} \text{ M}$, $2.0 \times 10^{-5} \text{ M}$). In order to better visualize the changes in the absorption (in the long-wavelength (LW) region) and emission spectra, the normalized spectra (by scaling the area under the spectrum to be equal to unity) are presented in insert.

Table 2. Fluorescence lifetimes of **I** and **II** in phosphate buffer solutions containing different concentration of Trp.

Molecule	Trp Concentration 10^{-5} (M)	$\tau_1 \text{ (ns)}$	$A_1 \text{ (%)}$	$\tau_2 \text{ (ns)}$	$A_2 \text{ (%)}$	$\tau_3 \text{ (ns)}$	$A_3 \text{ (%)}$	r^2
I	0	0.19	100					0.97
	0.4	0.14	12			3.45	88	0.99
	0.8	0.12	6			3.34	94	0.99
	1.2	0.12	5			3.5	95	0.99
	1.6	0.13	3			3.5	97	0.99
	2.0	0.14	2			3.5	98	0.99
II	0	0.20	24	0.80	76			0.95
	0.4	0.13	4	0.65	15	3.46	81	0.98
	0.8	0.11	3	0.87	7	3.71	90	0.99
	1.2	0.16	1	0.61	3	3.49	96	0.96
	1.6					2.90	100	0.98
	2.0					2.98	100	0.98

The presented above findings suggest that mainly Trp residue of BSA is involved in the process of specific interaction between **I** (or **II**) and BSA. Considering possible location of Trp in BSA, it can be concluded that the molecules **I** and **II** enter the hydrophobic cleft of the IB subdomain (location of the Trp-212 molecule) or near the surface of the albumin molecule in the second helix of the first domain (location of the Trp-134 molecule) and form specific hydrogen bonds with Trp-212 and/or Trp-134, thereby causing static fluorescence quenching of BSA (Trp). Moreover, as the investigated fluorophores have the chemically active substituents which are likely sites for intermolecular hydrogen bonding with Trp, Scheme 2 presents the most probable structures of complexes formed between **II** and Trp molecule. As can be seen, the Trp residue was attached to the investigated molecules only at the sites considered important for hydrogen bond formation. It is obvious that actual situation in **I**-BSA, **II**-BSA, **I**-Trp and **II**-Trp systems is not simple, but the considered model can give a first insight into the specific Trp-methyl benzoate derivative interactions. In future works, separate molecular docking studies will be used to confirm the most probable binding site with BSA.



Scheme 2. Two of the possible structures of complexes formed between **II** and Trp molecule.

2.4. Effect of **I** and **II** on the Steady-State and Time-Resolved Spectroscopic Behaviour of BSA and Trp

In order to clarify the interaction process between two methyl benzoate derivatives and BSA and Trp, we have also carried out a series of the steady-state absorption and fluorescence studies of BSA and Trp in the presence of **I** and **II**. To begin with, Figures 7 and S3 show the changes observed in the absorption (A,B) and emission (A',B') spectra of BSA (Figure 7) and Trp (Figure S3) in the phosphate buffer upon the gradual addition of **I** (A,A') and **II** (B,B'). In order to better visualize the changes in the absorption spectra in the long-wavelength (LW) region (250–340 nm), the normalized LW absorption bands (by scaling the area under the spectrum to be equal to unity) are presented in insert of Figures 7 and S3. For both investigated systems (BSA-investigated molecules and Trp-investigated molecules), upon the gradual addition of the methyl benzoate derivative to the aqueous buffer solution of BSA (or Trp), the absorbance of the short-wavelength (centred at about 210 nm (BSA-I and BSA-II) and 218 nm (Trp-I and Trp-II)) and long-wavelength (centred at about 280 nm (BSA-I and BSA-II) and 275 nm (Trp-I and Trp-II)) bands gradually increases and simultaneously a new absorption band centred at around 310 nm appears. An isosbestic point in normalized LW absorption bands can be clearly seen between these two bands (see insert of Figures 7 and S3), which confirms formation of the ground-state complex between BSA or Trp and both investigated dyes.

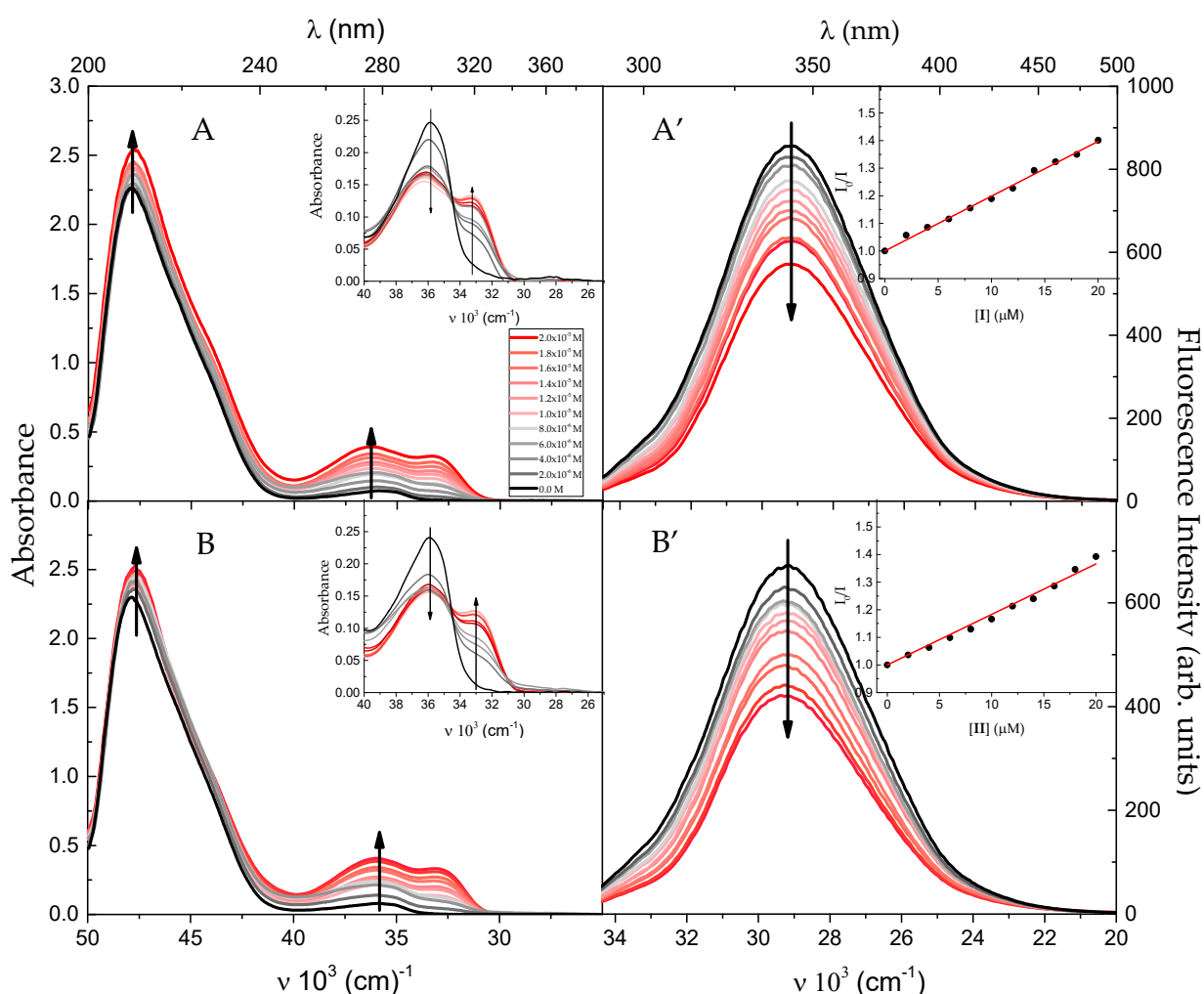


Figure 7. Absorption and fluorescence spectra ($\lambda_{exc.} = 280$ nm) of BSA ($c = 2 \times 10^{-6}$ M) in phosphate buffer solutions containing different concentration of **I** (A,A') and **II** (B,B') (0 M, 2.0×10^{-6} M, 4.0×10^{-6} M, 6.0×10^{-6} M, 8.0×10^{-6} M, 1.0×10^{-5} M, 1.2×10^{-5} M, 1.4×10^{-5} M, 1.6×10^{-5} M, 1.8×10^{-5} M, 2.0×10^{-5} M). The normalized LW absorption bands (by scaling the area under the spectrum to be equal to unity) are presented in insert of (A,B). The insert in (A',B') shows a graphical presentation of the relationship between I_0/I versus (I) (A') and (II) (B').

The interactions of BSA and Trp with two studied dyes were also investigated by the fluorescence quenching spectroscopy. In both cases (BSA and Trp), the addition of methyl benzoate derivative to a solution of BSA (or Trp) quenches significantly the single, broad fluorescence band centred at about 340 nm (BSA) and 352 nm (Trp) (registered upon excitation at 280 nm) without significant changes in the shape of the fluorescence spectrum (see Figures 7 and S3). There is no shift in the wavelength of maximum intensity of BSA and Trp when it was quenched by **I** and **II**.

Using data obtained from the absorption and fluorescence titration experiments (BSA-investigated molecules and Trp-investigated molecules), the Benesi–Hildebrand plots were constructed (Figure S4). Since the plots of $1/(A - A_0)$ versus $1/[I]$ ($1/[II]$) and $1/(I - I_0)$ versus $1/[I]$ ($1/[II]$) produce a straight line for both systems with a good correlation coefficient ($r > 0.95$), the stoichiometry of the formed complexes between BSA (Trp) and methyl benzoate derivatives in the ground and excited states is 1:1. The binding constants were found to be $(2.1 \pm 0.1) \times 10^4$ M $^{-1}$ and $(2.4 \pm 0.1) \times 10^4$ M $^{-1}$ in the ground state and $(2.0 \pm 0.1) \times 10^4$ M $^{-1}$ and $(2.7 \pm 0.1) \times 10^4$ M $^{-1}$ in the excited state, for BSA-I and BSA-II, respectively and $(2.0 \pm 0.1) \times 10^4$ M $^{-1}$ and $(2.2 \pm 0.1) \times 10^4$ M $^{-1}$ in the ground state and $(1.8 \pm 0.1) \times 10^4$ M $^{-1}$ and $(2.4 \pm 0.1) \times 10^4$ M $^{-1}$ in the excited state, for Trp-I and Trp-II,

respectively. It is clearly seen that they are in a good agreement with data presented in previous section (Section 2.1). The binding constant values determined on the basis of the two independent titration experiments differ by about 10%.

In order to confirm the existence of the ground and excited states complexes (BSA-I and BSA-II, as well as Trp-I and Trp-II) and the 1:1 stoichiometry of the complexes, the results of the steady-state spectroscopic measurements are analysed using the Job's plot technique (continuous variation method) [43]. Job plots were generated by plotting the difference in absorbance (ground state complex) or fluorescence intensity (excited state complex) versus the mole fraction of the investigated dye (R) in the used solutions (Figure S5). The curve maximum at about 0.5 supports the previously mentioned 1:1 stoichiometry in the ground and excited states i.e., the number of I (or II) molecules binding to BSA and Trp is close to unity.

2.4.1. Mechanism of the Observed Fluorescence Quenching

It is well known that the progressive fluorescence quenching can be explained by three main reasons: static quenching (nonfluorescent complex is formed between fluorophore and quencher), dynamic (resulting from collisional encounters between fluorophore and quencher) and quenching both by collisions and complex formation [38]. In order to determine the extent and mechanism of the fluorescence quenching of BSA by I and II molecules, the results of spectroscopic measurements are analysed using Stern-Volmer equation (see Equation (3)). As can be seen in insert of Figure 7, the Stern-Volmer plot (I_0/I versus [I] and [II]) at 298 K is linear for both systems, which indicates that only one type of quenching occurs (static or dynamic). The K_{SV} values obtained from linear plots are listed in Table 3. From the K_{SV} values obtained for both of the systems at room temperature (298 K), it is evident that the molecule II has only a slightly stronger binding affinity for BSA in comparison to I (the ratio of the corresponding Stern–Volmer quenching constant, $K_{SV}(\text{I})/K_{SV}(\text{II})$, is about 1.05).

Table 3. Stern–Volmer quenching constant (K_{SV}), binding constant determined using Benesi–Hildebrand (K_{BH}) and Scatchard (K_b) methods and thermodynamic parameters (binding number (n), changes in enthalpy (ΔH), entropy (ΔS), and Gibb's free energy (ΔG) of the binding process) at different temperatures.

Molecule	Temp. (K)	$K_{SV} 10^4$ (M^{-1})	$K_{BH} 10^4$ (M^{-1})	$K_b 10^4$ (M^{-1})	n	ΔH (kJ)	ΔS (J)	ΔG (kJ)
I	278	2.4	2.6	3.5	1.07	−25.66	−4.86	−24.308
	288	2.1	2.4	2.7	1.04			−24.260
	298	1.9	2.0	2.1	1.00			−24.211
	303	1.5	1.3	1.3	0.93			−24.187
II	278	2.6	3.6	3.5	1.07	−25.12	−2.17	−24.512
	288	2.2	3.0	3.0	1.05			−24.491
	298	1.8	2.7	2.7	1.02			−25.469
	303	1.5	1.8	1.3	0.99			−25.458

Because character of fluorescence quenching (static versus dynamic) can be differentiated by their different dependence on temperature (higher temperatures result in faster diffusion and dissociation of weakly bound complexes), temperature-dependent fluorescence spectral measurements were conducted at four different temperatures, and data are analysed using Stern–Volmer relation. Plots of I_0/I versus [I] and [II] at four temperatures are shown in Figure 8. Analysing the K_{SV} values (obtained at different temperatures) assembled in Table 3, in connection with the graphical presentation in Figure 8, it is evident that the quenching mechanism of the investigated systems is sensitive to temperature. As

can be seen, upon increasing the temperature, K_{SV} value decreases, which indicates that the quenching process is static i.e., complex is formed between I (or II) and BSA.

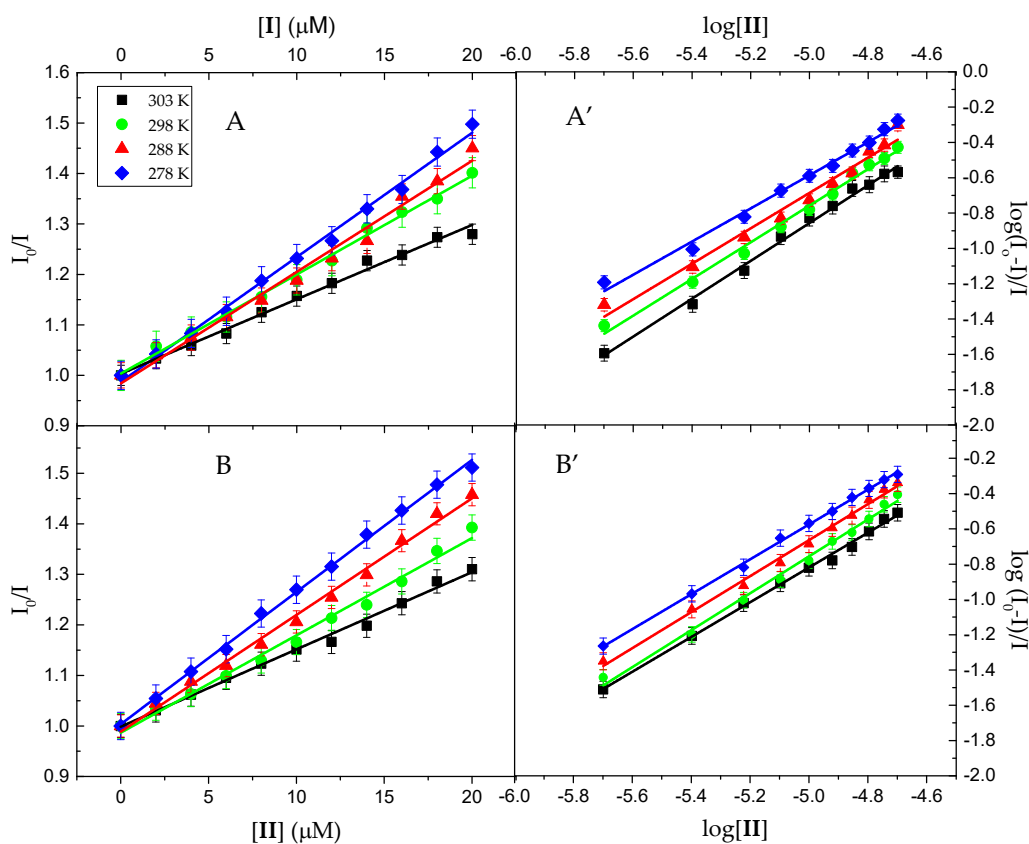


Figure 8. Stern-Volmer (A,B) and $\log(I_0-I)/I$ versus $\log(\text{molecule})$ (A',B') plots for quenching of BSA by I (A,A') and II (B,B') at different temperatures.

Static character of fluorescence quenching is also confirmed by temperature-dependent Benesi–Hildebrand plot experiment. The plots of $1/(I - I_0)$ follow a linear dependence on $1/[I]$ ($1/[II]$) for four investigated temperatures (278–303 K). This finding indicates 1:1 complex formation between studied compounds and BSA at all temperatures. As seen in Table 3, the increase of the solute temperature from 278 to 303 K results also in the decrease of binding constant (K_{BH}) value by a factor of 2 (for BSA-I system) and 1.5 (for BSA-II), which confirms the occurrence of static quenching mechanism - higher temperatures results in dissociation of weakly bound complexes.

It is well-known that the analysis of fluorescence kinetics is the most definite method to distinguish the character of fluorescence quenching (static versus dynamic) [38]. For static quenching, the fluorescence lifetime of the investigated molecule remains unchanged in the presence of a quencher, while for dynamic quenching $\tau_0/\tau \neq 1$. In order to further confirm the quenching mechanism, time-resolved emission spectra of BSA upon gradual addition of I and II were recorded. The lifetime parameters presented in Table 4 show that fluorescence decays consist of a fast (~ 1 ns (10%)) and a slow (~ 5.6 ns (90%)) components, with an average time ~ 5.2 ns. The nonexponential kinetic traces of the native BSA has been described previously and most probably they can be assigned to the presence of two Trp moieties at distinct conformational states [21]. It is clearly seen that the average lifetime of BSA is practically unchanged in the presence of different concentrations of I and II, which confirms our previous conclusions drawn from the analysis of the steady-state spectroscopic measurements described above i.e., static quenching mechanism between BSA and I (or II). Moreover, the high value of the quenching rate constant at room temperature

($k_q(\mathbf{I}) = K_{SV}(\mathbf{I}) / \langle \tau_0 \rangle = 3.6 \times 10^{12} \text{ M}^{-1} \text{ s}^{-1}$, $k_q(\mathbf{II}) = K_{SV}(\mathbf{II}) / \langle \tau_0 \rangle = 3.4 \times 10^{12} \text{ M}^{-1} \text{ s}^{-1}$), which is much higher than typical diffusion-controlled quenching rate constants ($2 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$) excludes dynamic character of the quenching process (diffusion controlled reaction).

Table 4. BSA fluorescence lifetimes as a function of **I** and **II** concentration at room temperature.

Molecule	Molecule Concentration 10 ⁻⁵ (M)	τ_1 (ns)	A_1 (%)	τ_2 (ns)	A_2 (%)	$\langle \tau \rangle$ (ns)	r^2
I	0	0.98	6.6	5.59	93.4	5.28	0.99
	0.4	0.98	7.8	5.70	92.2	5.35	0.99
	0.8	0.93	10.4	5.64	89.6	5.15	0.99
	1.2	0.87	12.8	5.49	87.2	4.89	0.99
	1.6	0.91	10.8	5.55	89.2	5.05	0.99
	2.0	0.91	13.2	5.56	86.8	4.94	0.99
II	0	0.98	6.6	5.59	93.4	5.28	0.99
	0.4	0.97	7.2	5.58	92.8	5.25	0.99
	0.8	1.01	10.8	5.68	89.2	5.18	0.99
	1.2	0.96	11.5	5.53	88.5	5.02	0.99
	1.6	0.83	10.4	5.40	89.6	4.93	0.99
	2.0	0.97	9.6	5.70	90.4	5.24	0.99

2.4.2. Thermodynamic Parameters

To support the above conclusions i.e., the number of binding sites per protein molecule (n) and binding constant value, we also analysed the fluorescence quenching data using Scatchard equation (Equation (4)). As seen in Figure 8, the linear dependence between $\log\left(\frac{I_0 - I}{I}\right)$ and $\log[\mathbf{I}]$ (and $\log[\mathbf{II}]$) is obtained at four temperatures studied. The slope and the intercept values were therefore obtainable and used to calculate n and K_b . The results are assembled in Table 3. The calculated, temperature-dependent K_b values confirm that the fluorescence quenching has a static character, in agreement with the expectation that the binding constant for the static quenching decreases with increasing temperature. Moreover, K_b values correlate well with the alternatively calculated binding constant (K_{BH}) using Benesi–Hildebrand relation (see Table 3). Finally, n values (found to be about 1 for both studied systems) further confirm the 1:1 stoichiometry of the complexes.

In order to understand the role of different types of non-covalent interactions, namely, van der Waals forces, multiple hydrogen-bonding, hydrophobic, and electrostatic interactions, in the binding of **I** (or **II**) with BSA, related thermodynamic characteristics were estimated by using the van't Hoff equation (Equation (5)). The values of ΔH and ΔS are determined from the slope and intercept of the plot of $\ln K_b$ versus $1/T$ for four different temperatures and presented in Table 3. The negative free energy change (ΔG) value determined for both systems indicates that the interaction of BSA with two studied dyes is a spontaneous reaction. Moreover, from the magnitude and the sign of the ΔS and ΔH thermodynamic parameters ($\Delta S < 0$, $\Delta H < 0$ and $|\Delta H| \gg |\Delta S|$), it can also be deduced that: (i) BSA-**I** and BSA-**II** interaction is an exothermic reaction (negative enthalpy change, $\Delta H < 0$); (ii) reaction is more enthalpy-driven (negative entropy change ($\Delta S < 0$) is balanced by highly negative enthalpy change ($\Delta H < 0$) i.e., $|\Delta H| \gg |\Delta S|$); (iii) hydrogen bonding and weak van der Waals forces play a crucial role in methyl benzoate derivative-BSA interactions.

3. Materials and Methods

3.1. Reagents and Materials

Bovine serum albumin (BSA, min. 98%, from Sigma-Aldrich, St. Louis, MO, USA) and tryptophan (Trp, 98%, from Sigma-Aldrich), tyrosine (Tyr, 98%, from Sigma-Aldrich), phenylalanine (Phe, 98%, from Sigma-Aldrich) and phosphate buffer solution (pH 7.4) were used as received. The methyl *o*-methoxy *p*-methylaminobenzoate-I and methyl *o*-hydroxy *p*-methylaminobenzoate-II have been synthesized and purified by Gormin [44–46]. The purity of dyes was controlled by thin layer chromatography.

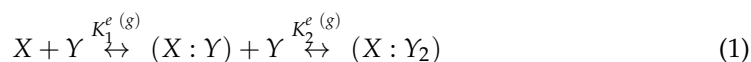
3.2. Apparatus and Methods

3.2.1. Steady-State and Time-Resolved Measurements

Absorption and fluorescence spectra were recorded using, respectively, a Shimadzu UV-2401 PC spectrophotometer and Shimadzu RF-5301 PC spectrofluorometer. The emission spectra were corrected for the spectral response of the photomultiplier (Hamamatsu R-928) and monochromator pass, but not for reabsorption which was negligible in these samples. Time-resolved emission spectra were taken with the streak camera (C4334-01 Hamamatsu) and 2501 S spectrograph system (Bruker Optics). Solid state Nd: YAG laser (PL 2143A/SS EKSPLA) and optical parametric generator (PG 401/SH EKSPLA) were used as the picosecond excitation light pulses source [47].

3.2.2. Determination of Stoichiometry and Binding Constants

The determination of the ground (*g*) and excited (*e*) state binding constant and stoichiometry of the formed complex



can be determined by analysing the changes in the absorbance and/or fluorescence intensity of the molecule *X* in the presence of *Y*. From the fluorescence (absorption) titration data, the binding constant was determined by Benesi–Hildebrand equation [48]:

$$\frac{1}{A - A_0} = \frac{1}{K_n^g(A_1 - A_0)} \cdot \frac{1}{[Y]^n} + \frac{1}{A_1 - A_0} \quad (2a)$$

$$\frac{1}{I - I_0} = \frac{1}{K_n^e(I_1 - I_0)} \cdot \frac{1}{[Y]^n} + \frac{1}{I_1 - I_0} \quad (2b)$$

In Equation (2), *n* is binding stoichiometry, I_0 (A_0), I (A) and I_1 (A_1) are the fluorescence intensities (or absorbances) of the molecule *X*, respectively in the absence of *Y*, intermediate and at infinite concentration of *Y*. Thus, the $1/(I - I_0)$ (or $1/(A - A_0)$) should follow a linear dependence on $1/[Y]^n$ for the correct stoichiometry (*n*).

The binding stoichiometry was also determined from Job's method (continuous variation method) [43]. Job plots were constructed by plotting the difference in absorbance and/or fluorescence intensity (ΔA (ΔI)) of *X* observed in the presence of *Y* against the mole fraction of the dye *X* (x_x). The value of mole fraction of *X* at the maximum deviation yields the stoichiometry of the formed complex i.e., stoichiometry of *X*:*Y* complex is 1:2 if $x_x = 0.33$; 1:1 if $x_x = 0.5$; 2:1 if $x_x = 0.66$ etc.

3.2.3. Analysis of Fluorescence Quenching Data

In order to determine the extent and mechanism of the fluorescence quenching, the results of spectroscopic measurements are analysed using Stern–Volmer equation [38]:

$$\frac{I_0}{I} = 1 + k_q \tau_0 [Q] = 1 + K_{SV} [Q] \quad (3)$$

where I_0 and I are the fluorescence intensities in the absence and presence of different concentration of quencher ($[Q]$), respectively. k_q , τ_0 and K_{SV} are bimolecular quenching constant, average fluorescence lifetime of the molecule in the absence of quencher and Stern-Volmer quenching constant, respectively.

Additionally, using fluorescence quenching data, Scatchard equation (Equation (4)) was employed to estimate the binding constant (K_b) and the number of binding sites per protein molecule (n) [49–51]:

$$\log \frac{I_0 - I}{I} = \log K_b + n \log [Q] \quad (4)$$

3.2.4. Analysis of Thermodynamic Parameters

The related thermodynamic characteristics were determined from the van't Hoff equation [52]:

$$\ln K_b = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \quad (5)$$

where K_b is the binding constant at temperature T and R is universal gas constant, ΔH and ΔS are the changes in enthalpy and entropy of the binding process. The Gibb's free energy change (ΔG) during the binding processes was calculated by using the equation:

$$\Delta G = \Delta H - T \Delta S \quad (6)$$

at each corresponding temperature.

3.2.5. Analysis of Supramolecular Solvation Dynamics

The supramolecular solvation dynamics was analysed by the decay of the solvation correlation function $c(t)$, defined by Maroncelli and Fleming as [39,40]:

$$c(t) = \frac{\tilde{\nu}(t) - \tilde{\nu}(\infty)}{\tilde{\nu}(0) - \tilde{\nu}(\infty)} \quad (7)$$

where $\tilde{\nu}(t)$, $\tilde{\nu}(0)$, and $\tilde{\nu}(\infty)$ are the wavenumbers of the fluorescence band maximum at times t , 0 , and ∞ . The decay of the solvation correlation function, $c(t)$, was fitted to an exponential decay:

$$c(t) = \sum_{i=1}^N A_i e^{-t/\tau_i} \quad (8)$$

4. Conclusions

In the present work, an attempt was made to understand the nature and mechanisms of interactions between two selected methyl benzoate derivatives (**I** and **II**) and bovine serum albumin. In order to clarify the role of Trp residue of BSA in the **I**-BSA and **II**-BSA interaction phenomenon, **I**-Trp and **II**-Trp systems were also investigated. Experimental results show that the investigated interactions (with both BSA and Trp) are mainly based on the ground- and excited-state complex formation processes. Both molecules form a stable complex with BSA and Trp (with 1:1 stoichiometry) in the ground and excited states. The binding constant was in the order of 10^4 M^{-1} . Moreover, our experimental data clearly indicate that mainly Trp residue of BSA is involved in the process of specific interaction between **I** (or **II**) and BSA.

The fluorescence quenching studies along with time-resolved fluorescence measurements show that the binding of the **I** and **II** causes fluorescence quenching of BSA through a static mechanism, revealing a 1:1 interaction. The binding reaction is associated with the negative ΔS , ΔH and ΔG which implies that the hydrogen bonding and van der Waals forces play major roles in stabilizing the complex. Moreover, the reaction is spontaneous, exothermic and more enthalpy-driven. Finally, our studies have shown that solvation dynamics, which occurs in subpicosecond time scale in neat solvents of different polarities,

is slowed down significantly when organic molecule is transferred to BSA cavity. In future works, separate molecular docking studies will be used to confirm the most probable binding site with BSA.

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Spectroscopic studies on the supramolecular interactions of methyl benzoate derivatives with *p*-sulfocalix[6]arene macrocycles

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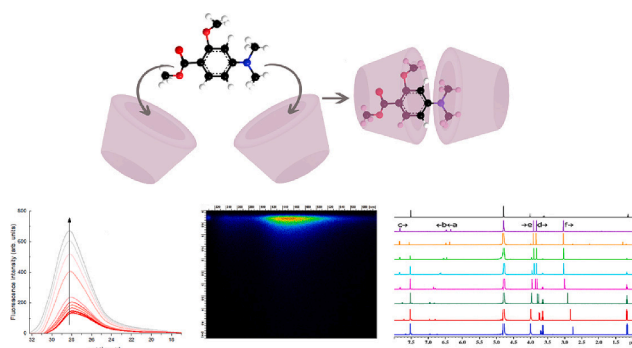
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HIGHLIGHTS

- Methyl benzoate derivatives interact with SCA[6] to form stable inclusion complexes.
- ESIPT and TICT processes are clearly different if the molecule migrates to hydrophobic nanocavity.
- 1:1 complex is formed for I-SCA[6] and II-SCA[6] systems, while the stoichiometry of the III-SCA[6] and IV-SCA[6] is 1:2.
- Solvation dynamics of pure water is markedly different from that of confined water molecule in SCA[6] system.

GRAPHICAL ABSTRACT



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ABSTRACT

This paper is a continuation of our previous research and aims to further investigate and elucidate the nature and mechanisms of noncovalent supramolecular interactions between four methyl benzoate derivatives (I-IV), which are capable of exhibiting Twisted Intramolecular Charge Transfer (TICT) and/or Excited State Intramolecular Proton Transfer (ESIPT)-type behavior, and chemical and biological nanocavities. Photophysical and photochemical properties of molecules I-IV in aqueous solution in the presence of well-recognized macrocyclic host *p*-sulfocalix[6]arenes (SCA[6]) have been studied using steady-state, time-resolved and ¹H NMR spectroscopic techniques. The changes in the ground- and excited-state spectroscopic characteristics (absorption and fluorescence spectra, time-resolved fluorescence spectra, fluorescence decay times and ¹H NMR spectra) undergo significant modifications upon encapsulation of the investigated methyl benzoate derivative in the macromolecular cavity. For the two compounds (I and II), the interactions with the macrocycles with a hydrophobic SCA[6] cavity lead to the formation of stable inclusion complexes with 1:1 stoichiometry, both in the ground and excited state, while the stoichiometry of the III-SCA[6] and IV-SCA[6] complexes in the ground and excited states is 1:2. The values of the equilibrium constants have been determined from the spectroscopic data using Benesi-Hildebrand and nonlinear regression procedures. The location of the organic molecule inside the SCA[6] has been investigated by ¹H NMR experiments. The changes in macrocyclic compound-induced NMR chemical shifts clearly indicate that the chemical structure of inclusion complexes is very different for methyl benzoate

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derivative-SCA[6] and methyl benzoate derivative-CB[7] systems. Finally, we have shown, using time-dependent fluorescence Stokes shift, that very fast solvation dynamics of pure water is markedly different from that of the confined water molecule in SCA[6] system.

1. Introduction

Since 1987, when Lehn, Cram and Pedersen shared the Nobel Prize for their fundamental research in guest–host systems, a rapid growth of interest of many research groups in supramolecular chemistry has been observed [1–6]. Over the past few decades, the theoretical and experimental investigations of various research groups have focused on both purely cognitive and application aspects of host–guest chemistry, which finally resulted in the award of the 2016 Nobel Prize to Sauvage, Feringa and Stoddart for research on molecular machines [7]. Considering that, reversible association processes are mainly stabilized by weak non-covalent interactions such as electrostatic, van der Waals, hydrogen bonding, π – π stacking, hydrophobic effects and ion–dipole interactions among others, considerable efforts have been paid to understand the mechanisms of such processes leading to the formation of host–guest systems at the molecular level. In parallel, efforts have also been made to apply various host–guest supramolecular systems in nanomedicine, pharmacy, nanoelectronics, molecular machines or various functional materials [8–13].

Among various macrocyclic compounds with a defined cavity such as cyclodextrins (CDs), cucurbit[n]urils (CBs), calixarenes (CAs), pillar[n]arene, cyclophanes, crown ethers first three of these have gained huge attention, especially for their biomedical and pharmaceutical applications. Low toxicity, excellent biocompatibility friendly to the biological environment make these macrocyclic compounds crucial in the development and application of biomedical materials. These three most popular families of macrocycles with a hydrophobic cavity and a hydrophilic external surface are container- or basket-shaped, consist of a broad upper rim and a narrower lower rim, such as CDs and CAs, or with a symmetrical inner cavity, such as CBs [3]. The upper rim of well-defined hydrophobic interior cavity of CDs contains secondary hydroxyl groups while the narrower is lined with primary hydroxyl groups. Characterized by a highly symmetrical structure, CBs compounds have two identical portal ends lined with carbonyl groups. CAs, corn-shaped with a hydrophobic cavity which dimensions depend on the number of incorporated phenolic units, also possess two rims. The lower rim contains phenolic oxygen and thus has a hydrophilic property, while the upper rim is hydrophobic due to the presence of methyl groups [14]. Unfortunately, in contrast with water-soluble CDs, the poor water solubility of CAs greatly limits their biomedical applications. To address this problem, researchers have developed a way to make CAs water-soluble by sulfonation at the rims of CAs. Currently, water-soluble calixarenes of the *p*-sulfonato type (*p*-sulfocalix[n]arenes (SCA[n])) are well-known for their ability to form host–guest inclusion complexes with cationic and neutral guest species, as well as supramolecular building blocks in crystal engineering [15].

The use of various classes of chemical and biological macromolecules in the study of the ground and excited state processes leading to the formation of host–guest complexes has been the subject of our great research interest over the last decade [4,16–24]. In order to be able to understand the influence of the chemical and biological macromolecules, specifically α -, β - and γ -cyclodextrins, cucurbit[7]uril and bovine serum albumin (BSA), on the photochemical processes of the guest molecule, we investigated the correlation between the host and guest structure and processes of inclusion complex formation. Recently we have investigated the spectroscopic and photochemical properties of organic compounds representing an interesting from the spectroscopic point of view class of molecules which are capable of exhibiting Twisted Intramolecular Charge Transfer (TICT) and/or Excited State Intramolecular Proton Transfer (ESIPT)-type behavior (some methyl

benzoate derivatives) in neat solvents of different polarity, binary solvent mixtures, chemical (CDs, CBs) and biological (BSA) macromolecules [25–30]. Our scientific interest in these compounds has focused not only on their interesting, microenvironment-dependent photophysical and photochemical properties, but also on their potential in applications e.g., TICT and ESIPT fluorescence probes, metal ion sensors, active materials in dry xerographic toners or lasing medium in proton transfer lasers [31–36].

Our spectroscopic studies have shown that both the ESIPT and TICT processes for methyl benzoate derivatives are clearly different if the molecule migrates from a polar protic medium (water) to a non-polar, hydrophobic nanocavity. Interestingly, we found clear differences in inclusion complex formation between both α -, β - and γ -cyclodextrins as well as cucurbit[7]uril [25–29]. We have shown that both the size and shape of the macrocycle affect not only the geometry, structure, stoichiometry, and binding strength but also photoinduced intramolecular processes such as ESIPT and TICT taking place inside the cavity. To obtain further insight into the nature and mechanisms of noncovalent interaction between some methyl benzoate derivatives and macrocyclic hosts and to understand fully the mechanisms of ESIPT and TICT processes taking part inside different macromolecular cavities, the focus of this study is to test the influence of *p*-sulfocalix[6]arenes on the TICT and ESIPT reactions of some methyl benzoate derivatives incorporated into SCA[6] hydrophobic cavity. Summarizing, in light of the above-mentioned applications of the studied methyl benzoate derivatives, macrocyclic compounds as well as supramolecular systems based on host – guest interactions, our work may find application in the rational design of tailor-made self-assembled systems containing macromolecular cavities.

2. Experimental details

The four investigated methyl benzoate derivatives (I–IV) have been synthesized and purified by Gormin et al. [37,38]. The purity of molecules was controlled by thin layer chromatography. Self-aggregation was also studied in the steady-state, time-resolved and NMR spectroscopic experiments and showed that no self-aggregation occurred when investigated molecules were dissolved in different liquid media at concentrations below 10^{-4} M [39–41].

p-Sulfocalix[6]arene (SCA[6]) were purchased from Alfa Aesar ($\geq 99\%$) and used without further purification. Steady-state absorption and corrected fluorescence spectra were recorded using Shimadzu UV-2401 PC spectrophotometer and Shimadzu RF-5301 PC spectrofluorometer, respectively. Time-resolved emission spectra were measured applying a streak camera (C4334-01 Hamamatsu) and 2501 S spectrograph (Bruker Optics). Solid state Nd: YAG laser (PL 2143A/SS EKSPLA) and optical parametric generator (PG 401/SH EKSPLA) system were used as pulsed excitation light source [42].

^1H NMR spectra of I–IV in 99.95% $^2\text{H}_2\text{O}$ (purchased from DEUTERO GMBH, Kastellaun, Germany) were recorded with Bruker Avance III 500 MHz spectrometer at 300 K. Chemical shifts were referenced to the internal standard of acetone (δ_{H} 2.225).

3. Results and discussion

3.1. Ground-state inclusion complexes

Steady-state and time-resolved spectroscopic techniques are commonly used to evaluate the nature and mechanisms of interactions between organic molecules, the photophysical and photochemical

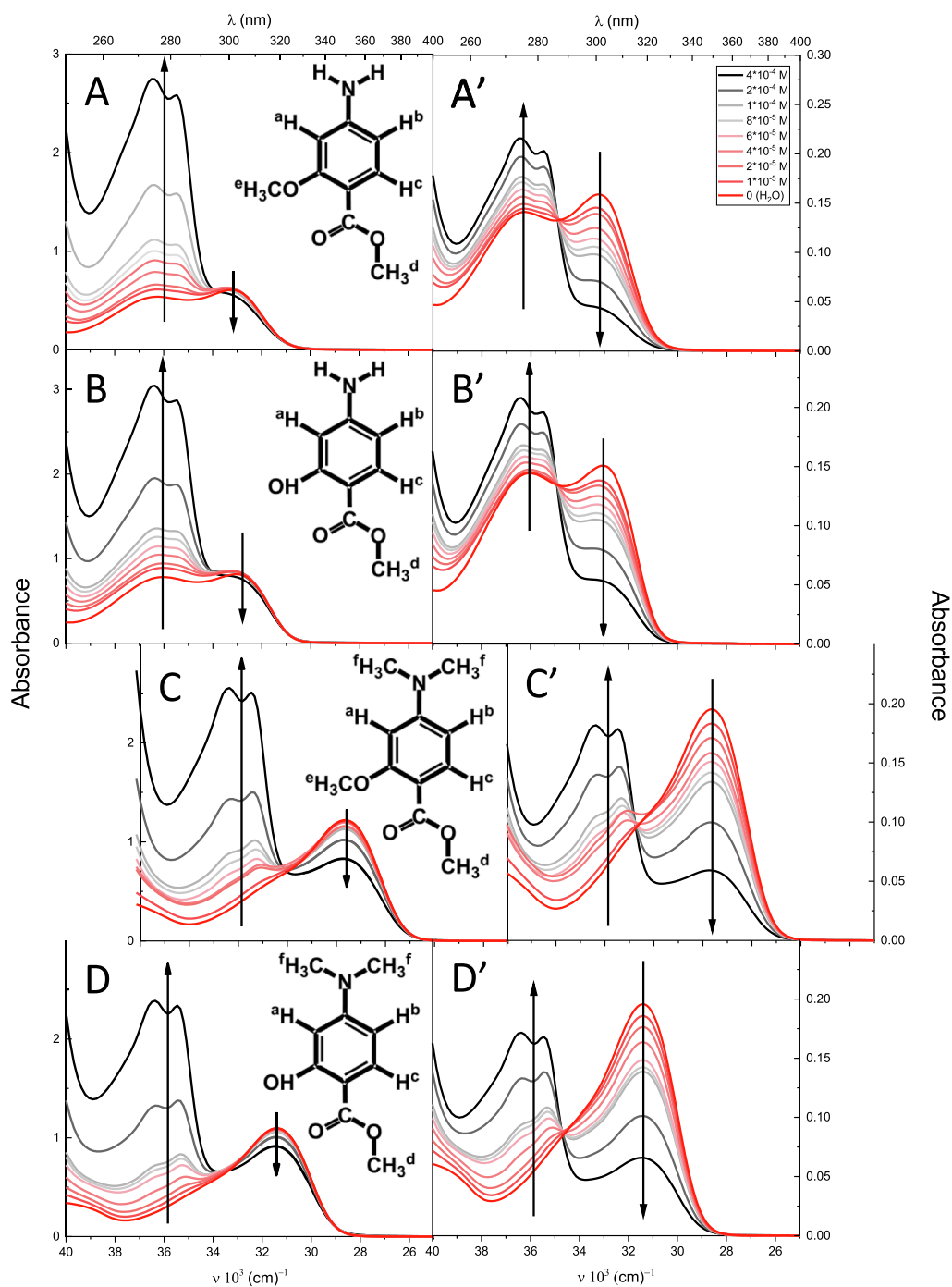


Fig. 1. Absorption (left panel) and normalized absorption (right panel) spectra of I (A, A'), II (B, B'), III (C, C') and IV (D, D') ($c = 5 \cdot 10^{-5}$ M) in aqueous solutions containing different concentration of SCA[6].

properties of which strongly depend on microenvironment, and macrocyclic compounds. For a such system the host–guest complexation generally leads to significant changes in the absorption and/or emission properties of organic molecules incorporated into a non-polar cavity. As in the case of the investigated methyl benzoate derivatives with CDs, CB [7] and BSA systems [25–30], substantial changes have also been observed in the absorption and fluorescence spectral behavior of I-IV upon the addition of different concentrations of SCA[6]. As can be seen in Fig. 1, the addition of SCA[6] to the aqueous solution of the molecules under study causes changes in both the molar absorption coefficient and the shape of the analyzed electronic bands in the 200–400 nm spectral range. This ground-state spectral behavior suggests a significant change

in the immediate neighborhood (solvation shell) of the studied molecule, which may be caused by the incorporation of the molecule inside the non-polar cavity. It should be noted that, for all studied systems, the addition of SCA[6] to methyl benzoate derivative in the aqueous solution led to very similar changes in the absorbance behavior of the investigated compound, i.e., upon increasing the concentration of SCA [6] the molar absorption coefficient of the short-wavelength absorption band located at around 275 nm increases and simultaneously the long-wavelength band located at around 300 nm decreases. Moreover, the presence of the isosbestic point between these two absorption (left panel of Fig. 1) and normalized absorption (right panel of Fig. 1) bands clearly indicates the existence of an equilibrium between methyl benzoate

Table 1

Equilibrium constants of I-IV in aqueous solution with SCA[6] determined using Benesi-Hildebrand and nonlinear regression procedure, and additionally a comparison of results for the ground- and excited-state complexation of the molecules under study with cyclodextrins (α , β and γ), cucurbit[7]urils (CB[7]), and bovine serum albumin (BSA).

Molecule	Medium	$K_1^g (M^{-1}) / K_2^g (M^{-1})$	$K_1^e (M^{-1}) / K_2^e (M^{-1})$
I	SCX[6]	640 ^a / -	660 ^a / -
		530 ^b / -	550 ^b / -
		300 ^d / -	680 ^c / -
		490 ^e / -	
	CB[7]	1301 ($\pm 3\%$) ^a	1642 ($\pm 9\%$) ^a
		1531 ($\pm 7\%$) ^b	1626 ($\pm 3\%$) ^b
	α -CD	NSC ^a	15 (± 2) ^a
	β -CD	329 ^a	687 ^a / 41 ^a
	γ -CD	32 (± 3) ^a	3 (± 0.8) ^a / 16 (± 2) ^a
	II	BSA	38 (± 2) ^b
(1.9 \pm 0.1) $\times 10^4$ ^a			(2.1 \pm 0.1) $\times 10^4$ ^a
610 ^a / -			930 ^a / -
630 ^b / -			1200 ^b / -
SCX[6]		350 ^d / -	1440 ^c / -
		480 ^e / -	
CB[7]		1176 ($\pm 17\%$) ^a	2153 ($\pm 12\%$) ^a
α -CD		840 ($\pm 17\%$) ^b	4572 ($\pm 21\%$) ^b
β -CD		NSC ^a	587 (± 63) ^a / 35 (± 5) ^a
γ -CD		153 ^a	210 (± 20) ^b / 5 (± 2) ^b
III	BSA	89 ^b	418 ^a / 25 ^a
		66 (± 7) ^a	564 ^b / 119 ^b
		68 (± 6) ^b	187 (± 80) ^a / 47 (± 20) ^a
		(2.3 \pm 0.1) $\times 10^4$ ^a	379 (± 50) ^b / 15 (± 2) ^b
	SCX[6]	1250 ^a / 550 ^b	1040 ^a / 700 ^a
		910 ^b / 450 ^b	1630 ^b / 1230 ^b
	CB[7]	1500 ^d / 370 ^d	1150 ^c / 550 ^c
		1090 ^e / 390 ^e	
	β -CD	1053 ($\pm 3\%$) ^a	1677 ($\pm 15\%$) ^a
	IV	SCX[6]	1647 ($\pm 3\%$) ^b
-			106 ^f
CB[7]		1490 ^a / 550 ^a	1950 ^a / 1010 ^a
		1520 ^b / 500 ^b	2080 ^b / 1470 ^b
α -CD	1390 ^d / 620 ^d	1750 ^c / 310 ^c	
β -CD	1720 ^e / 410 ^e	2250 ($\pm 12\%$) ^a	
	1925 ($\pm 6\%$) ^b	4231 ($\pm 16\%$) ^b	
	-	38 ^f / 52 ^f	
	-	326 ^f / 1520 ^f	

NSC – No stable complex.

^a Determined from the steady-state spectroscopic data using Benesi-Hildebrand procedure.

^b Determined from the steady-state spectroscopic data using nonlinear least-squares regression procedure.

^c Determined from the time-resolved spectroscopic data using Benesi-Hildebrand procedure.

^d Determined from the ¹H NMR spectroscopic data using Benesi-Hildebrand procedure.

^e Determined from the ¹H NMR spectroscopic data using nonlinear least-squares regression procedure.

^f From [25,28].

derivative and methyl benzoate derivative-SCA[6] ground-state complex.

Since for all investigated systems evident changes in the SCA[6]-dependent absorption spectra occur, the absorbance values of the long-wavelength absorption band were used to determine the ground-state equilibrium constants and stoichiometry between I and IV and SCA[6] by plotting the Benesi-Hildebrand graph, using a formula [43,44]:

$$\frac{1}{X - X_0} = \frac{1}{K_n^{g(e)}(X_1 - X_0)} \cdot \frac{1}{[SCA[6]]^n} + \frac{1}{X_1 - X_0} \quad (1)$$

where n is the stoichiometry of the host-guest inclusion complex, X

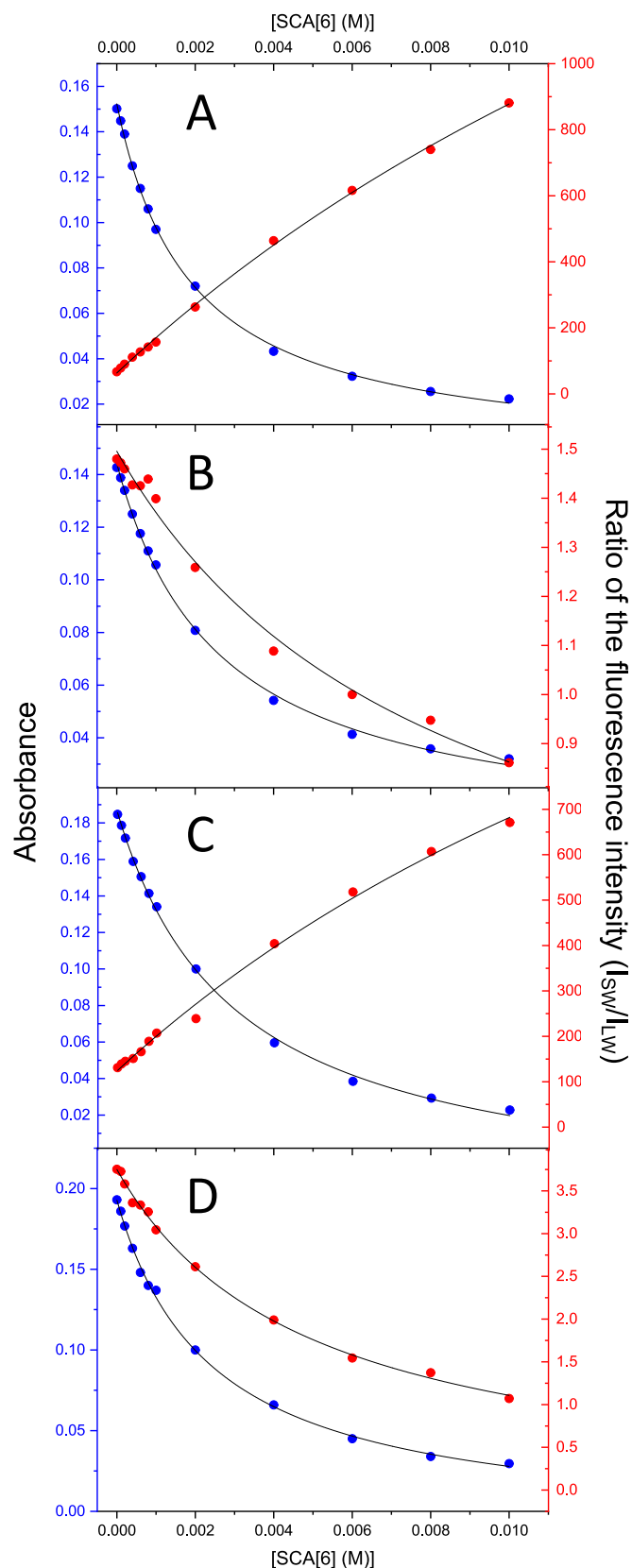


Fig. 2. Plots of the absorbance (red symbols) and ratio of the fluorescence intensity of the short-wavelength (SW) and the long-wavelength (LW) bands (blue symbols) versus SCA[6] concentration ([SCA[6]]) for molecule I (A), II (B), III (C) and IV (D).

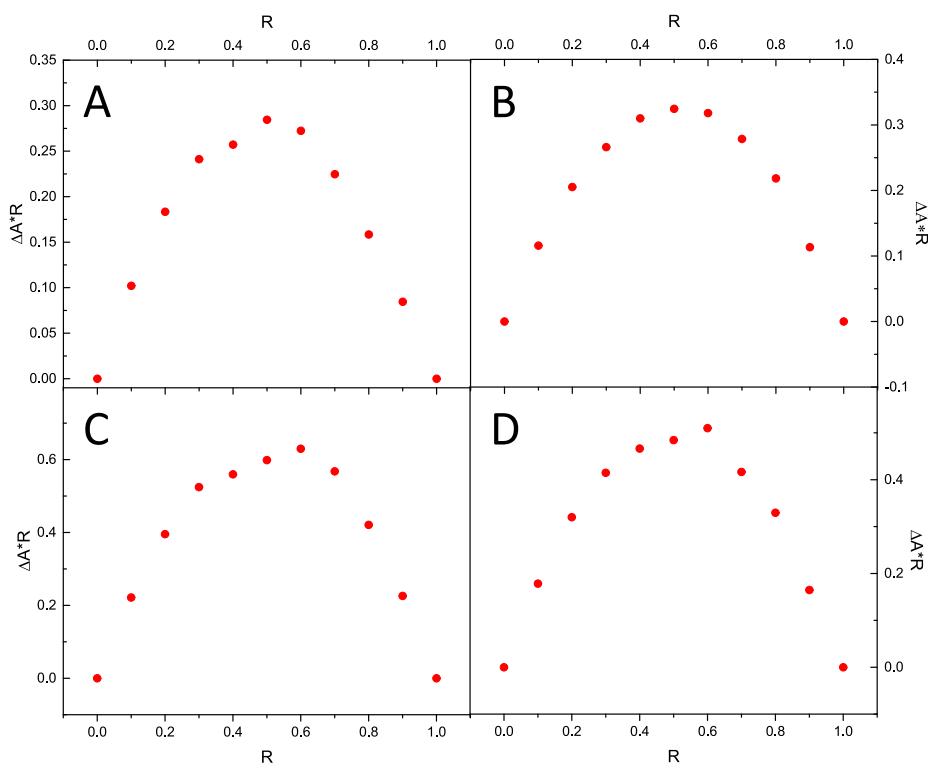


Fig. 3. Steady-state absorption job plots for the investigated methyl benzoate derivative-SCX[6] systems (I-SCA[6] (A), II-SCA[6] (B), III-SCA[6] (C), IV-SCA[6] (D)).

represents the selected spectroscopic characteristic i.e., A_0 , I_0 , τ_0 , δ_0 ; A_1 , I_1 , τ_1 , δ_1 and A , I , τ , δ are the absorbances, fluorescence intensities, fluorescence lifetimes and ^1H NMR shifts of the guest (in the absence of SCA[6]), in 1: n complex (when all of the guest molecules are complexed) and at the particular concentration.

For molecules I and II, the $1/(A - A_0)$ follows a linear dependence on $[\text{SCA}[6]]^{-1}$ (see Fig. SM1 (Supplementary Materials)), which indicates that these two organic compounds incorporate inside the SCA[6] host cavity forming an inclusion complex with a 1:1 stoichiometry and ground-state equilibrium constant (K_1^g) equals 640 and 610 M^{-1} for I and II, respectively (see Table 1). On the other hand, the Benesi-Hildebrand dependence for III and IV deviates from linearity which indicates that molecules III and IV can interact with SCA[6] forming the ground-state inclusion complexes with stoichiometry 1:2, besides the 1:1 host-guest absorbing complexes. The analysis of the absorbance variation provides the equilibrium constants K_1^g and K_2^g for III-SCA[6] system as 1250 and 550 M^{-1} and for IV-SCA[6] as 1490 and 550 M^{-1} .

It is important to note that the equilibrium constants can also be determined using nonlinear least-squares regression analysis. Moreover, it has been shown that nonlinear regression provides more reliable K estimates than the Benesi-Hildebrand method [45–47]. The ground (or excited) state equilibrium constants ($K_1^{g(e)}$, $K_2^{g(e)}$) can be determined from spectroscopic data using formulas:

$$X = \frac{X_0 + X_1 K_1^{g(e)} [\text{SCA}[6]]}{1 + K_1^{g(e)} [\text{SCA}[6]]} \quad \text{in the case of 1 : 1 complex} \quad (2)$$

and

$$X = \frac{X_0 + X_1 K_1^{g(e)} [\text{SCA}[6]] + X_2 K_1^{g(e)} K_2^{g(e)} [\text{SCA}[6]]^2}{1 + K_1^{g(e)} [\text{SCA}[6]] + K_1^{g(e)} K_2^{g(e)} [\text{SCA}[6]]^2} \quad \text{in the case of 1 : 2 complex} \quad (3)$$

Using preliminary parameters estimates obtained from the Benesi-Hildebrand method, the dependence of absorbance (A) versus SCA[6]

concentration ($[\text{SCA}[6]]$) for all the investigated systems were constructed (Fig. 2) and on their basis the equilibrium constants were determined. Table 1 presents equilibrium constants values determined using Benesi-Hildebrand and nonlinear regression procedure, and additionally a comparison of results for the ground-state complexation of four methyl benzoate derivatives (I–IV) with selected chemical and biological nanocavities (i.e., cyclodextrins (α , β and γ), cucurbit[7]urils, *p*-sulfocalix[6]arene and bovine serum albumin). First of all, the differences in the equilibrium constants obtained by these two methods are noticeable, which confirms the previous observations that (i) the Benesi-Hildebrand method gives an estimation rather than proper values and (ii) non-linear rather than linear regression methods should be used to obtain reliable results [48]. Moreover, the most noticeable observation for the investigated ground-state inclusion complexes in different microcavities is that the size and shape of the macrocycle affect stoichiometry and binding strength. In the case of all the investigated systems with chemical and biological carriers, methyl benzoate derivatives in the ground state form complexes with 1:1 stoichiometry, excluding the III-SCA[6] and IV-SCA[6] systems in which a single molecule III (and IV) is trapped in a capsule composed of two SCA[6] cavities. On the other hand, the ground-state binding strength of the investigated molecules in the hydrophobic interior of SCA[6] is slightly weaker than those for the methyl benzoate-CB[7] systems and significantly weaker than that of the methyl benzoate-BSA systems.

In order to confirm the stoichiometry of the ground-state inclusion complexes, the continuous variation method, known as the Job plot method was applied [49,50]. The difference in absorbance recorded at the location corresponding to the maximum of the LW absorption band in the absence and presence of SCA[6] was plotted as a function of the mole fraction of I–IV ($R = [M]/([M] + [\text{SCA}[6]])$), keeping a constant total concentration of reactants. As can be seen in Fig. 3, for two methyl benzoate derivatives i.e., I and II, Job's plot shows a maximum located at a molar fraction of $R = 0.5$, which confirms the formation of 1:1 inclusion complexes, while for III and IV maximum observed at about $R = 0.6$ confirms the formation of ground-state inclusion complexes with 1:2

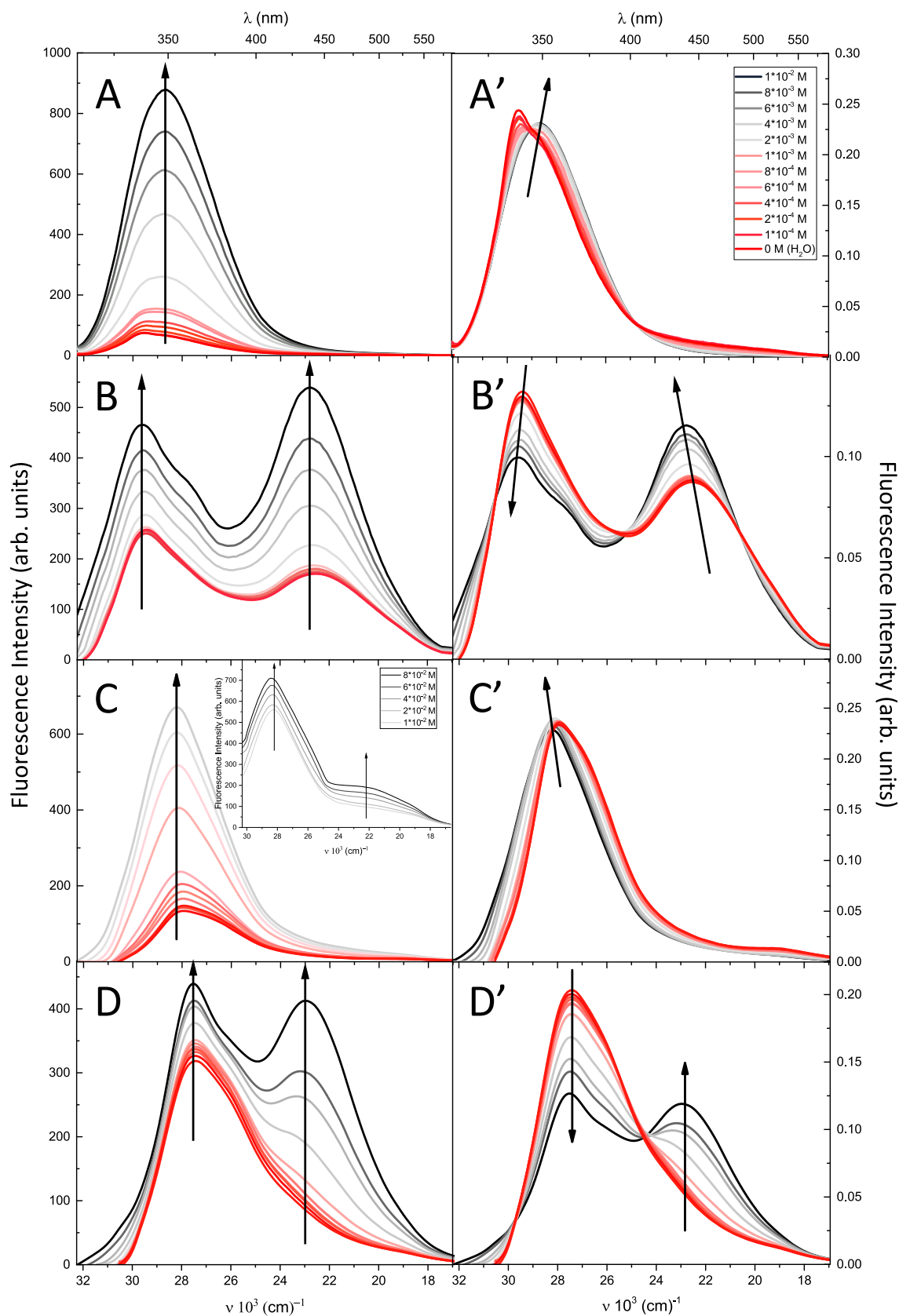
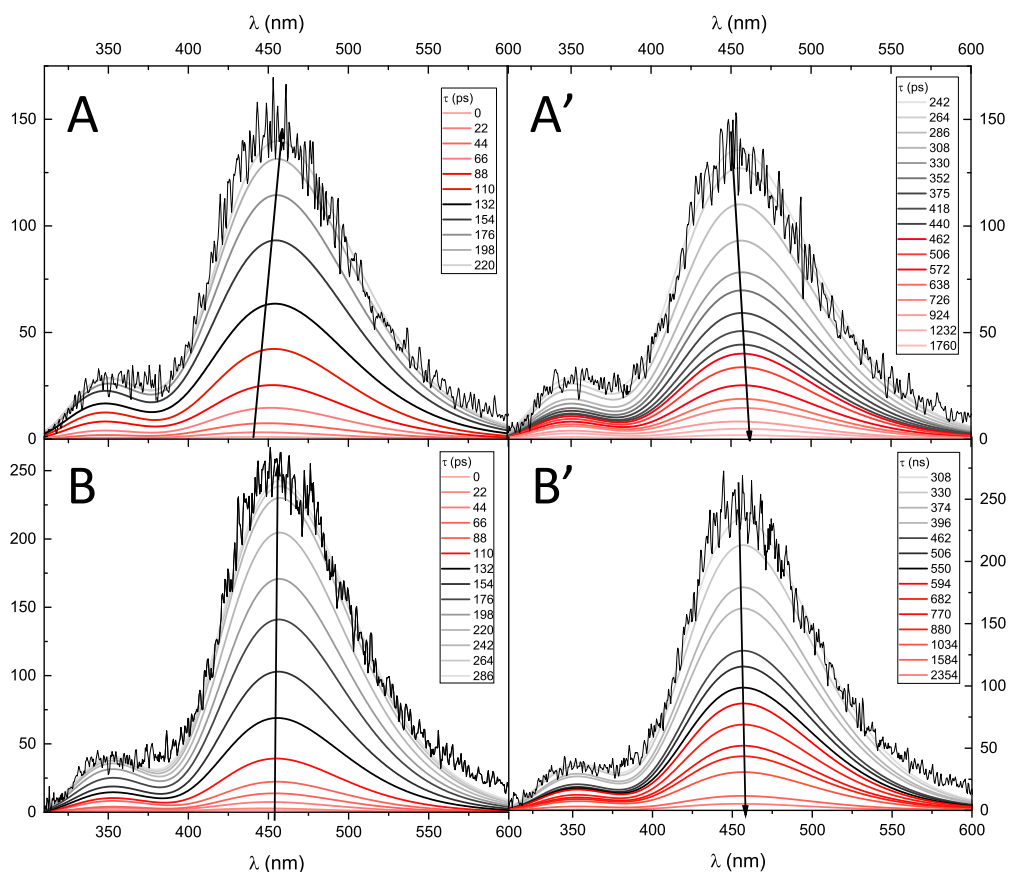


Fig. 4. Fluorescence (left panel) and normalized fluorescence (right panel) spectra of I (A, A'), II (B, B'), III (C, C') and IV (D, D') ($c = 5 \cdot 10^{-5}$ M) in aqueous solutions containing different concentration of SCA[6].

Table 2Fluorescence lifetimes of the molecules under study ($c = 10^{-4}$ M) in aqueous solutions containing different concentrations of SCA[6].

Molecule	SCA[6] concentration (10^{-4}) [M]	τ_1 [ns]	A_1 [%]	τ_2 [ns]	A_2 [%]	τ_3 [ns]	A_3 [%]	χ^2
I	0	0.11	100	–	–	–	–	1.14
	1	0.12	100	–	–	–	–	1.07
	5	0.16	100	–	–	–	–	1.28
	10	0.17	100	–	–	–	–	1.07
	50	0.21	100	–	–	–	–	1.15
II	0	0.17	100	–	–	–	–	1.09
	1	0.18	37	0.85	63	–	–	1.03
	5	0.20	24	0.71	76	–	–	1.13
	10	0.22	5	0.55	95	–	–	1.25
	50	0.24	21	0.54	79	–	–	1.20
III	0	0.17	100	–	–	–	–	1.05
	1	0.18	100	–	–	–	–	1.09
	5	0.21	100	–	–	–	–	1.14
	10	0.23	5	–	–	2.61	95	1.13
	50	0.25	8	–	–	2.91	92	1.01
IV	0	0.17	100	–	–	–	–	1.12
	1	0.17	76	0.45	34	–	–	1.20
	5	0.22	44	0.67	56	–	–	1.05
	10	0.24	20	0.65	42	2.35	38	1.10
	50	0.27	16	0.42	51	2.31	33	1.21

**Fig. 5.** Time-resolved emission spectra of II (A, A') and IV (B, B') ($c = 5 \cdot 10^{-5}$ M) in aqueous solution with SCA[6] ($1 \cdot 10^{-3}$ M) at various times after excitation.

stoichiometry.

3.2. Excited-state inclusion complexes

Understanding the molecular mechanisms involved in the formation of the methyl benzoate derivative-SCA[6] inclusion complexes in the

excited state, first of all, requires the knowledge of the mechanisms of photoinduced intramolecular charge (electron and/or proton) transfer processes occurring in homogeneous media. The ground- and excited-state behavior of the molecules under study in neat solvents of different polarity has been studied using steady-state and time-resolved spectroscopic techniques and quantum-chemical calculations

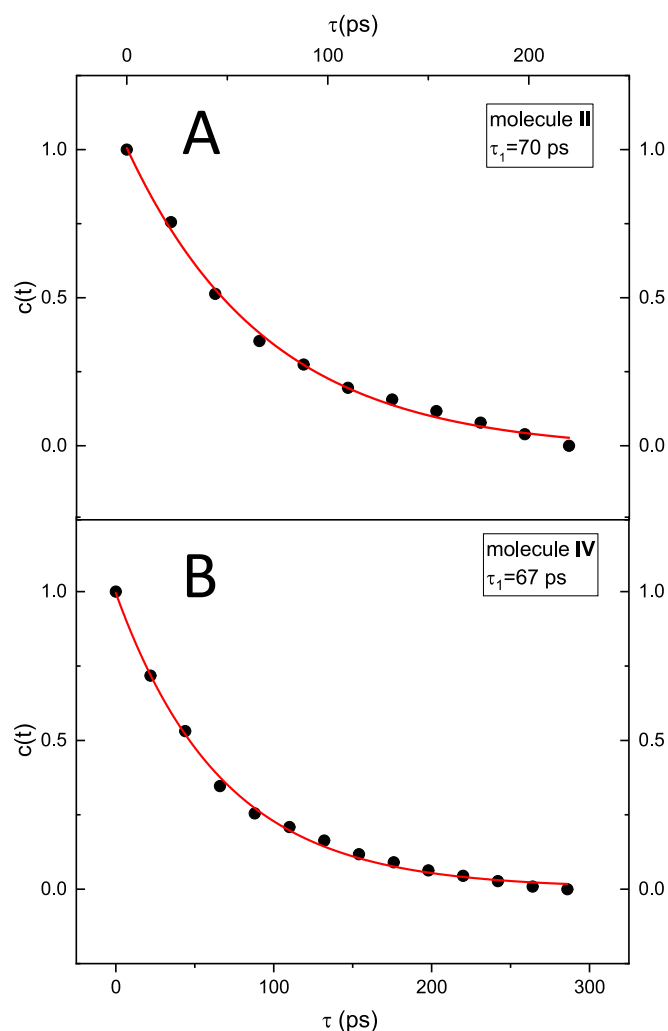


Fig. 6. Temporal behaviour of $c(t)$ function for the long-wavelength band of II (A) and IV (B) in aqueous solution with SCA[6] ($c_{\text{SCA}[6]} = 5 \cdot 10^{-4}$ M).

[37–41,51–55]. Experimental and theoretical studies have shown that the fluorescence spectrum of I dissolved in polar aprotic medium possesses a single, short-wavelength, locally-excited band, while emission of II consists of two partially overlapping bands i.e., LE and ES IPT. It is important to note that both ES IPT and TICT processes are responsible for the long-wavelength emission of molecule IV, while for molecule III, besides the short-wavelength, LE fluorescence, the long-wavelength TICT emission can also occur. Moreover, our previous experimental and theoretical data clearly indicate that, in the polar protic media, a specific interaction, i.e., hydrogen bonding between methyl benzoate derivatives and protic solvents, is occurring in addition to the non-specific dipole–dipole interaction.

In order to get a more comprehensive understanding of the excited-state molecular processes between methyl benzoate derivatives and SCA [6], fluorescence (left panel) and normalized fluorescence (right panel) spectra of I–IV in aqueous solutions containing different concentrations of SCA[6] are recorded and presented in Fig. 4. It is clearly seen that for all the investigated systems addition of SCA[6] to I–IV resulted in a significant increase in fluorescence intensity, along with the presence of isosbestic points (normalized fluorescence spectra), and slight blue-shift in the location of the LE fluorescence band maximum (~ 5 nm). Such changes in the fluorescence spectral behavior in the presence of macrocyclic compounds indicate the clear difference of the microenvironment (solvation shell) surrounding molecules I–IV. The increase in SCA[6]-dependent fluorescence intensity is fully understandable if we

assume that the electronically excited molecule embedded inside the SCA[6] is devoid of intermolecular hydrogen bonds (methyl benzoate derivative–water) that quench the fluorescence. Moreover, the hypsochromic shift of the LE band also indicates that the investigated organic molecule has changed its microenvironment (reduction in polarity) from the polar aqueous medium to a more hydrophobic macrocyclic cavity. Moreover, the presence of the isosbestic point in normalized fluorescence spectra suggests that the excited-state complexes are formed for all the systems studied.

When analyzing the influence of SCA[6] on the emission spectra of methyl benzoate derivatives, particular attention should be paid to the spectral behavior of the molecules exhibiting TICT- and ES IPT-behavior. Kasha and co-workers have shown [40] that molecules I and III cannot exhibit ES IPT because of the presence of the methoxy group ortho to the ester group containing the carbonyl. In contrast, molecules II and IV contain an *o*-hydroxy group which can H bond to the carbonyl of the ester substituent, and which upon excitation is capable of an intramolecular proton transfer to the carbonyl group. Moreover, the presence of the *p*-dimethylamino group (III and IV) permits TICT to be observed when activated in a suitable dielectric environment. It has been shown in our previous papers that the long-wavelength TICT emission of III and IV is highly sensitive to external hydrogen bonding perturbation, which can compete with the photoinduced excited-state intramolecular charge transfer process, causing a decrease in TICT emission yield. The significantly increased LE fluorescence, with a slight enhancement of TICT fluorescence of III and IV (located at ~ 450 nm and 525 nm for III and IV, respectively) upon the addition of SCA[6] indicates incomplete isolation of the studied molecule from the aqueous environment through complete entrapment of the guest molecule in a capsule composed of one or two SCA[6] cavities. Moreover, the significant increase in the LE and ES IPT fluorescence intensity of molecules II and IV with a simultaneous slight enhancement of TICT fluorescence for molecules III and IV confirms the partial immersion of molecules III and IV in the hydrophobic micropockets of SCA[6] i.e., the geometrical structure of the formed host–guest inclusion complex does not favor the process of complete electron transfer, accompanied by the rotation of dimethylamine group. It is also important to note that, the above data show that the structural geometry of the III-SCA[6] and IV-SCA[6] inclusion complexes formed in the excited state may be different from that of III-CB[7] and IV-CB[7] complexes in terms of the orientation of the guest molecule [26].

As the changes of the emission spectra recorded in aqueous solutions containing different concentrations of the macrocyclic compound are relatively large, the spectral data were used, according to the Benesi-Hildebrand plot, to estimate the preliminary equilibrium constants of investigated excited-state methyl benzoate derivative-SCA[6] inclusion complexes. As can be seen in Fig. SM2 (Supplementary Materials), for I-SCA[6] and II-SCA[6] systems, the plots of $1/[I_{\text{SW}}/I_{\text{LW}}] - (I_{\text{SW}}/I_{\text{LW}})_0$ versus $[\text{SCA}[6]]^{-1}$ (I_{SW} and I_{LW} are the intensity of the short-wavelength (SW) and the long-wavelength (LW) fluorescence bands) demonstrate linear dependence, which indicates the formation of well-defined inclusion complexes in the excited state with a stoichiometry of 1:1, while for III-SCA[6] and IV-SCA[6] systems departure from linearity is evident. Assuming that only 1:1 inclusion complexes are being formed for I-SCA[6] and II-SCA[6] and both, 1:1 and 1:2 for III-SCA[6] and IV-SCA[6] and using the preliminary equilibrium constants determined from Benesi-Hildebrand dependence ($K_1^e = 660$ and 930 M^{-1} for I and II, respectively and K_1^e and K_2^e for III-SCA[6] system as 1040 and 700 M^{-1} and for IV-SCA[6] as 1950 and 1010 M^{-1} , see Table 1) excited-state equilibrium constants were then determined by using the nonlinear formula of Eqs. (2) and (3) (see Fig. 2). The excited-state equilibrium constants are presented in Table 1. Similar to the situation observed for the ground-state equilibrium constants, it is clearly seen that the excited-state K values determined using the Benesi-Hildebrand method slightly differ from those calculated using the nonlinear regression procedure. This behavior confirms our and other research group's observations that

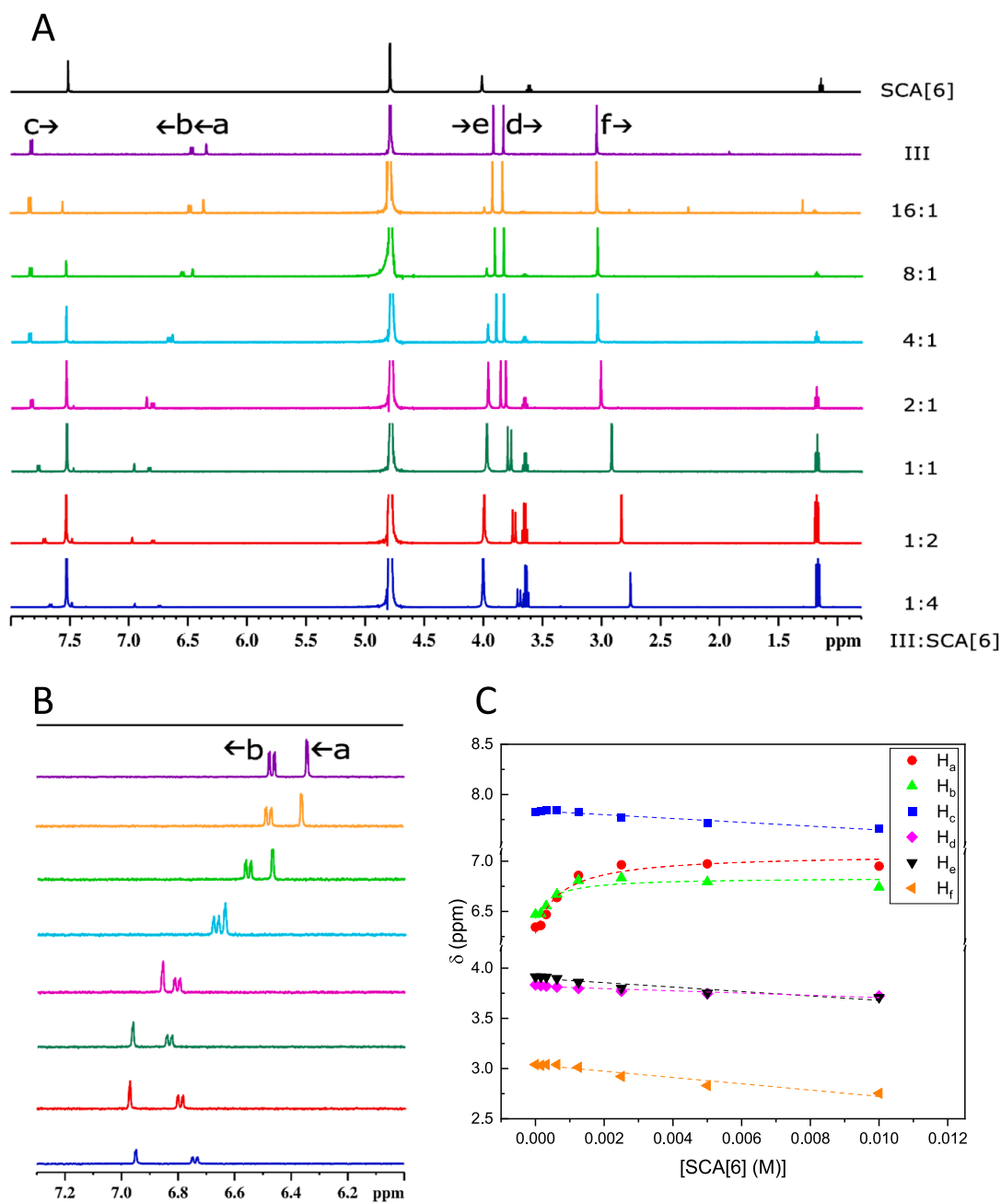


Fig. 7. ^1H NMR spectra (500 MHz) of **III** in the absence and presence of SCA[6] (A), chemical shifts of H_a and H_b protons covering the range of 6.2–7.2 ppm (B), and chemical shifts profiles of H_a – H_f for **III** upon titration with SCA[6] (C).

quantitative data analysis using only linear regression methods is an oversimplification.

Analyzing the data collected in Table 1 one can state that, the excited-state and ground-state equilibrium constants for I-SCA[6] system are of a similar order, while the excited-state equilibrium constants for the other three systems studied are slightly higher than the ground-state equilibrium constants, which suggests a higher binding affinity to SCA[6] in the excited state. As in the case of the ground-state equilibrium constants, the magnitudes of the equilibrium constants for the investigated methyl benzoate derivative-SCA[6] systems are slightly weaker than those for inclusion complexes with CB[7] and CDs, and significantly weaker than those for complexes with BSA.

It is important to note that, the continuous variation method (see Fig. SM3) (Supplementary Materials) clearly shows that for I-SCA[6]

and II-SCA[6] systems, Job's curve maximum occurs at a molar fraction of about 0.5, which confirms the aforementioned 1:1 stoichiometry in the excited state, while maximum observed at 0.6 for III-SCA[6] and IV-SCA[6] confirms the formation of excited-state inclusion complexes with stoichiometry 1:2.

4. Time-resolved studies

As time-resolved emission spectra measurements belong to very sensitive indicators suitable for monitoring photoinduced intra- and intermolecular processes in different homogeneous and heterogeneous media, we also used this spectroscopic technique (that measures both the fluorescence spectra at various times after excitation as well as the fluorescence decay times in a single experiment) to explore excited-state

Table 3

The complexation-induced chemical shifts of H_a-H_f protons for molecules under study upon titration with SCA[6].

M:SCX [6]	H _a (ppm)	H _b (ppm)	H _c (ppm)	H _d (ppm)	H _e (ppm)	H _f (ppm)
I	6.490	6.423	7.747	3.840	3.856	–
16:1	6.497	6.428	7.755	3.827	3.860	–
8:1	6.540	6.472	7.766	3.819	3.859	–
4:1	6.626	6.560	7.776	3.790	3.848	–
2:1	6.760	6.699	7.774	3.756	3.808	–
1:1	6.844	6.794	7.742	3.700	3.737	–
1:2	6.862	6.823	7.681	3.650	3.650	–
1:4	6.834	6.806	7.614	3.590	3.665	–
II	6.280	6.374	7.707	3.894	–	–
16:1	6.290	6.383	7.711	3.880	–	–
8:1	6.333	6.417	7.725	3.870	–	–
4:1	6.423	6.480	7.740	3.840	–	–
2:1	6.575	6.582	7.743	3.795	–	–
1:1	6.713	6.654	7.702	3.732	–	–
1:2	6.787	6.672	7.617	3.663	–	–
1:4	6.808	6.645	7.488	3.620	–	–
III	6.344	6.470	7.826	3.834	3.915	3.041
16:1	6.361	6.478	7.831	3.826	3.915	3.033
8:1	6.469	6.555	7.840	3.822	3.911	3.041
4:1	6.638	6.671	7.844	3.812	3.898	3.041
2:1	6.859	6.808	7.829	3.799	3.861	3.013
1:1	6.964	6.835	7.772	3.771	3.801	2.922
1:2	6.974	6.795	7.714	3.747	3.752	2.831
1:4	6.951	6.742	7.662	3.722	3.710	2.754
IV	6.428	6.457	7.765	3.906	–	3.012
16:1	6.251	6.458	7.769	3.901	–	3.009
8:1	6.259	6.462	7.771	3.891	–	3.005
4:1	6.391	6.533	7.788	3.860	–	3.013
2:1	6.554	6.600	7.783	3.810	–	2.996
1:1	6.755	6.644	7.730	3.750	–	2.930
1:2	6.861	6.616	7.637	3.656	–	2.838
1:4	6.890	6.569	7.560	3.540	–	2.766

processes in investigated host–guest systems. The fluorescence lifetimes and preexponential coefficients of the studied methyl benzoate derivatives upon the gradual addition of SCA[6] are presented in Table 2. Before undertaking the investigation of the excited-state interactions between investigated molecules and SCA[6], it is necessary to remind that our previous time-resolved fluorescence measurements on I-IV in water have shown that fluorescence decay fits a two-exponential function with time constants of fast component $\tau_{\text{HB}} \sim 10\text{--}40$ ps (emission of the excited hydrogen-bonded (HB) methyl benzoate derivative-H₂O complex) and slow one $\tau_{\text{UB}} \sim 200\text{--}400$ ps (emission of an unbound molecule (UB)). Unfortunately, due to the temporal resolution of our apparatus ($\tau_{\text{TR}} \sim 50$ ps), in our current studies, fluorescence decay for I-IV in H₂O can be reasonably well fitted by a single exponential function. Therefore, the fluorescence lifetime τ_1 of the investigated substance in water represents the average amount of time the two existing species (τ_{UB} (unbound) and τ_{HB} (hydrogen-bonded complex)) remain in the excited state prior to their return to the ground state.

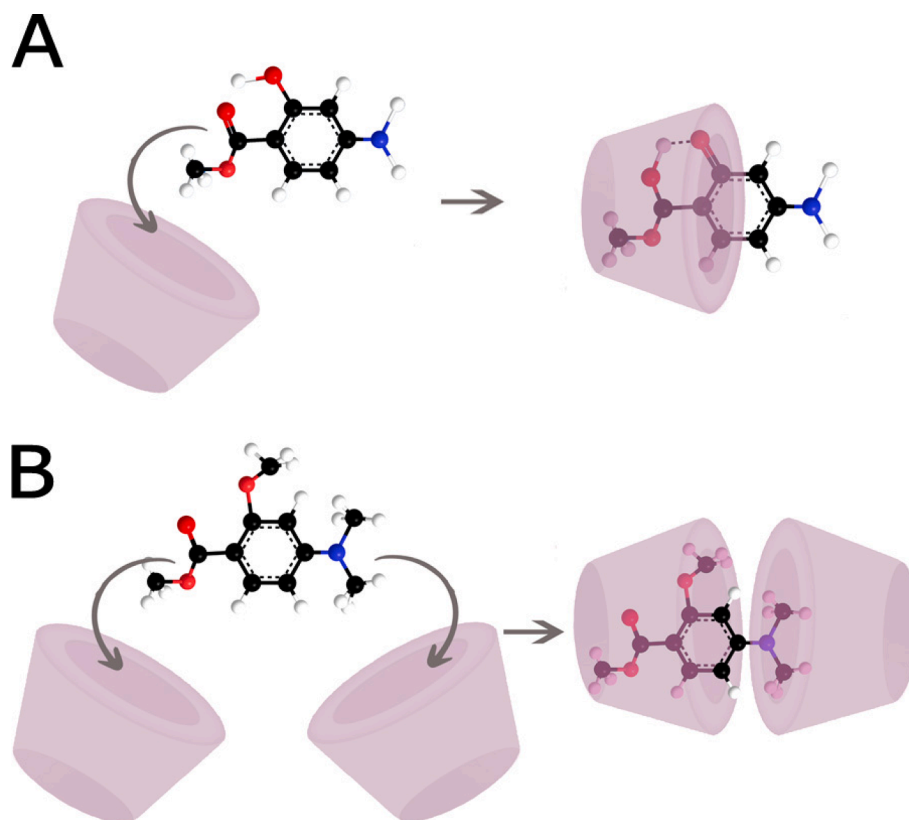
In the case of molecule I in H₂O and aqueous solutions containing different concentrations of SCA[6], the fluorescence intensity decay remains monoexponential. The value of fluorescence decay time component τ_1 slightly increases with increasing SCA[6] concentration, which suggests that molecule I is encapsulated in the macrocyclic host. SCA[6] cavity at the molecular level protects the fluorophore encapsulated in the host cavity from direct contact with the neighboring water molecules, which reduces the intermolecular (I-H₂O) hydrogen bonding interactions, and therefore enhancement of the fluorescence intensity and lifetime of I in the presence of the SCA[6]. It is clearly seen that whereas fluorescence decay of molecule II (which is capable of exhibiting ESIPT-type behavior in some media) in H₂O can be reasonably well

fitted by monoexponential function, those in the presence of SCA[6] could be well reproduced with the sum of two exponential functions: fast τ_1 (170–240 ps) and slow τ_2 (~ 0.6 ns) decay components. According to our recent results [26,29,30], we can state that τ_2 component (which is close to the lifetime of ESIPT tautomer reported for this molecule in polar aprotic solvents) can be assigned to the emission from ESIPT state, whereas τ_1 component corresponds to the average lifetime of two existing species i.e., free molecule II and II-H₂O hydrogen-bonded complex. The increase in lifetime τ_1 and preexponential factor A_2 (corresponding to decay of ESIPT tautomer) with increasing SCA[6] concentration can be also interpreted in terms of encapsulation of II in the SCA[6]. Incorporation of molecule II into the interior of SCA[6] (i) reduces the efficiency of deactivation via nonradiative channels (solute–solvent hydrogen bonding interaction), and hence, the value of fluorescence lifetime of free molecule II increases, (ii) reduces polarity as compared to H₂O and increases the possibility of ESIPT process.

As stated above on the basis of the steady-state experimental results, the presence of SCA[6] in the aqueous solution of III slightly facilitates the rotation of the dimethylamino group and thereby enhances the probability of emission from the TICT state. The fluorescence decay characteristics of III in the presence of SCA[6] confirm the conclusions drawn from the evolution of the steady-state emission spectra. Fluorescence decay profiles of III registered in the absence of SCA[6] and in the presence of SCA[6] at low concentration ($c_{\text{SCA}[6]} < 1 \cdot 10^{-3}$ M) fit reasonably well to monoexponential function, whereas at the highest concentration, fluorescence decay can be approximated by a two-exponential function with decay times: τ_1 (170–250 ps) (small contribution) and τ_3 (~ 2.7 ns) (major component). It is important to note that, the decay time of the major component (τ_3) is close to the lifetime of TICT emission in the aprotic medium [28,38–41]. Therefore, the decay component τ_3 should be attributed to the emission from the TICT state.

Similarly to the fluorescence kinetics of methyl benzoate derivative showing LE, ESIPT and TICT fluorescence (molecule IV) with CB[7], in the absence of SCA[6] fluorescence decay can be reasonably well fitted by monoexponential function, in the presence of SCA[6] at low concentration ($c_{\text{SCA}[6]} < 1 \cdot 10^{-3}$), the decay fits two-exponential function with time constants of τ_1 (170–270 ps) and τ_2 (~ 0.6 ns), whereas at the highest concentration ($c_{\text{SCA}[6]} > 1 \cdot 10^{-3}$), three fluorescent species contribute to the observed total emission. The above steady-state and time-resolved results combined with our previous investigations [25,26,28–30] and detailed analysis of literature reports [38–41] unanimously point that the investigated ESIPT- and TICT-type methyl benzoate derivative (IV) in the presence of SCA[6] shows also the spectroscopically inhomogeneous system in which twisted intramolecular charge transfer (TICT) process occurs in addition to the LE and ESIPT processes. Thus, the possibility of photoinduced rotation of the dimethylamino group is possible due to the presence of a macrocyclic host and slow component τ_3 can be assigned to the emission from the TICT state.

To support the formation of well-defined excited-state methyl benzoate derivative-SCA[6] inclusion complexes, their stoichiometry and equilibrium constant values, the analysis of fluorescence kinetics of I-IV according to the Benesi-Hildebrand model was performed. Using data obtained from the time-resolved fluorescence titration experiment, the Benesi – Hildebrand plots were constructed (see Fig. SM4 (Supplementary Materials)), the respective K_1^{ex} and K_2^{ex} values were calculated, and presented in Table 1. As seen in Fig. SM4 (Supplementary Materials), a linear dependence ($1/(\tau - \tau_0)$ versus $[\text{SCA}[6]]^{-1}$) is obtained for two investigated systems (I-SCA[6] and II-SCA[6]) over the whole range of $[\text{SCA}[6]]$, while for III-SCA[6] and IV-SCA[6] the dependence of ($1/(\tau - \tau_0)$ versus $[\text{SCA}[6]]^{-1}$) can be drawn as two independent straight lines. This behavior confirms qualitative conclusions drawn from the evolution of the steady-state emission spectra (according to Benesi-Hildebrand, nonlinear regression and Job procedures) i.e., the formation of 1:1 excited-state inclusion complexes between the I (and II) and



Scheme 1. Schematic representation of the guest-SCA[6] inclusion complexes for methylamino derivatives (I, II) (A) and dimethylamino derivatives (III, IV) (B).

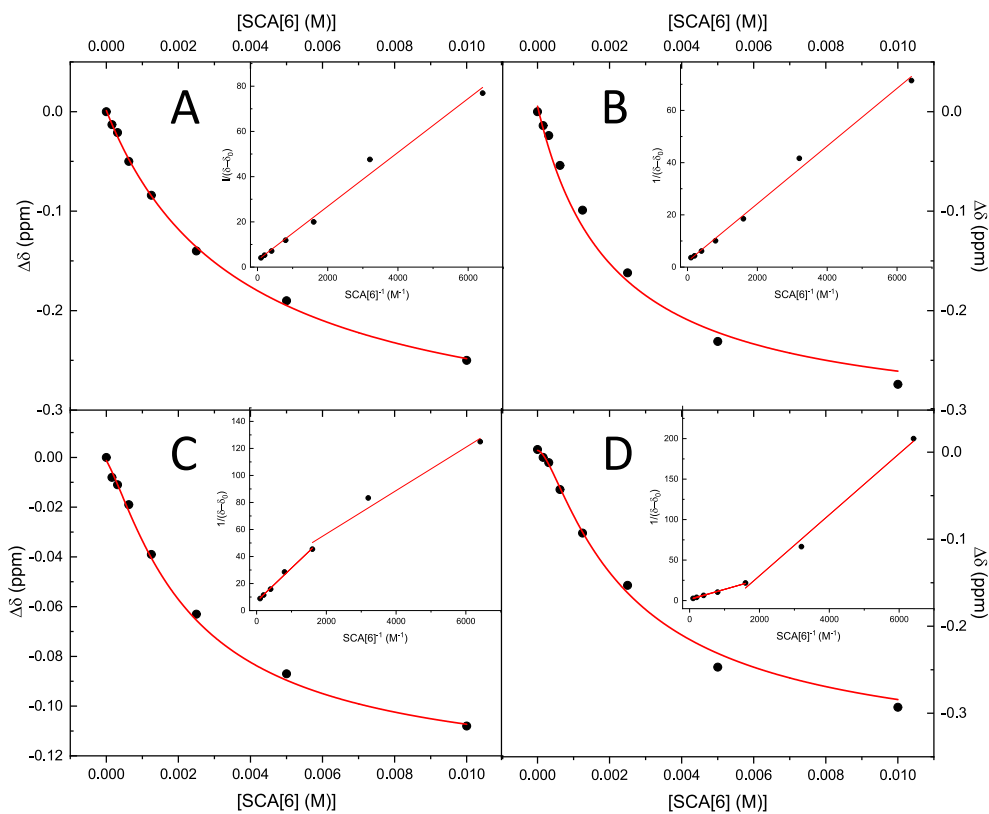


Fig. 8. Chemical shifts profiles of h_d for I (A), II (B), III (C) and IV (D) upon titration with SCA[6]. Inset: Benesi-Hildebrand plot for H_d proton.

SCA[6], while the stoichiometry of the III-SCA[6] and IV-SCA[6] complexes in the excited states is 1:2. The equilibrium constant, determined from the ratio of the corresponding intercept and slope of Benesi-Hildebrand plots on the basis of the evolution of the spectral and temporal data is substantially different. This difference is understandable in that the decay time of the short component τ_1 of methyl benzoate derivatives (interpreted as the average lifetime of two existing species (unbound (free) molecule and hydrogen-bonded complex) in aqueous solutions containing different concentrations of SCA[6] cannot be precisely determined in the streak camera experiment because it is much shorter than the temporal resolution of the setup. Thus, the results of the time-resolved spectroscopic measurements (according to Benesi-Hildebrand plot) provide an estimate rather than a proper equilibrium constant of the inclusion complex.

As mentioned in the introduction, we have also recently undertaken time-resolved spectroscopic studies to understand the solvent dynamics around the methyl benzoate derivatives encapsulated within selected chemical (CDs, cucurbit[7]urils) and biological (BSA) nanocavities in an aqueous medium. It has been shown that solvation time in solvent (bulk water and other polar solvents) shell is ultrafast and occurs in a time scale < 1 ps [56–58], whereas, in investigated macromolecular systems solvation dynamics of water molecules significantly slows down as a consequence of the formation of inclusion complexes between nanocavities and I-IV. In order to analyze quantitatively how the relaxation dynamics is changed when the organic molecule is relocated from the bulk water to the SCA[6] cavity, time-resolved emission spectra are monitored. Unfortunately, the time constant of the solvation dynamics of all molecules under study cannot be precisely determined in the streak camera experiment because of the limited time resolution of our setup (~ 50 ps). Thus, only from the time-dependent long-wavelength fluorescence spectral behavior of II and IV, we can directly obtain information on the solvation dynamics. Fig. 5 shows the temporal evolution of the fluorescence spectrum of II and IV in an aqueous solution with SCA[6] ($c_{\text{SCA}[6]} = 10^{-4}$ M) at various times after excitation. Since the presented in Fig. 5 spectroscopic data show large time-dependent Stokes shift of the long-wavelength fluorescence band (455 cm^{-1} (II) and 460 cm^{-1} (IV)), it was tempting to construct solvent correlation function $c(t)$, describing the solvation dynamics, introduced by Maroncelli and Fleming as [56]:

$$c(t) = \frac{\tilde{\nu}(t) - \tilde{\nu}(\infty)}{\tilde{\nu}(0) - \tilde{\nu}(\infty)} \quad (4)$$

where $\tilde{\nu}(t)$, $\tilde{\nu}(0)$, and $\tilde{\nu}(\infty)$ are the fluorescence maxima locations at times t , 0, and ∞ . The temporal behavior of normalized solvation correlation parameter $c(t)$ is very similar for the two investigated systems (II-SCA[6] and IV-SCA[6], see Fig. 6). In both cases very good fit can be obtained to a single exponential function with the decay component of $\tau_{\text{solv.}}$ (II) = 67 ps and $\tau_{\text{solv.}}$ (IV) = 70 ps. As expected very fast solvation dynamics of pure water is markedly different from that of the confined water molecule in SCA[6] system. The time scale of the investigated processes in the presence of SCA[6] is a least of 2 orders of magnitude slower than the relaxation of water molecules in ordinary bulk water. This unusually large slowdown of the solvation dynamic is understandable in terms of the two types of water molecules (bound and free) in the vicinity of supramolecular systems proposed by Nandi and Bagchi [57]. They suggested that the bound (those which are hydrogen bonded to the macromolecules) and free (placed at a distance from macromolecules and formed 3-dimensional hydrogen bond network) water molecules are dynamically exchanged between these two states of water. According to the above-mentioned model and Bhattacharyya's and co-worker's previous works [59], we can state that water molecules inside the SCA[6] are immobilized and solvation dynamics requires an "outward" jump of the molecule from the hydrophobic micropocket so as to experience solvation by "free" water molecules outside the cavity. It should be also noted that this dynamic exchanged model developed by Nandi and Bagchi is also confirmed by computer simulations [58].

Theoretical calculations clearly show that hydrogen bonding between macrocyclic compounds and water is much stronger than water-water specific interactions.

5. NMR studies

In order to obtain more insight into the formed host-guest (methyl benzoate derivative-SCA[6]) inclusion complex structures, ^1H NMR spectroscopic technique was used. The NMR-titration experiments in D_2O have been performed keeping constant the methyl benzoate derivative (I-IV) concentration ($c = 2.5 \cdot 10^{-3}$ M), whereas the SCA[6] concentration has been gradually increased from 0 to $1 \cdot 10^{-2}$ M. The complexation-induced ^1H chemical shifts of H_a - H_f (see numbering of protons in Fig. 1) i.e., δ_{a-f} as a function of the SCA[6] concentration) are presented in Fig. 7 and Figs. SM5-7 (Supplementary Materials) and Table 3. To gain insight into the structure of the formed host-guest inclusion complex, it is necessary to assume that, in general, the upfield shift of the proton resonances is due to the magnetic shielding provided by the aromatic walls of the interior of macrocyclic compounds and thus it is experimental evidence for the inclusion of fluorophore into SCA[6] cavity, while the downfield shifts suggest that the proton is rather outside the cavity [60,61]. Analyzing the data presented in Fig. SM7 (Supplementary Materials Figs. 5-7) and Table 3 one can state that upfield (encapsulation-induced) chemical shift of the proton resonances occurs for peaks H_c , H_d and H_e for all investigated molecules and H_f for III and IV, while for two aromatic protons (H_a and H_b) is observed a visible downfield shift. These changes in SCA[6]-induced chemical shifts clearly indicate that the benzene ring is only partially inside the SCA[6] cavity (H_c , H_d and H_e benzene region are located in SCA[6] cavity), while the two other protons (H_a and H_b) are exposed to the bulk water. It is important to note that, in the case of III and IV, the upfield shift of H_f proton in the presence of SCA[6] clearly suggests that dimethylamino group ($-\text{N}(\text{CH}_3)_2$) is incorporated into SCA[6] cavity forming an inclusion complex with a 1:2 stoichiometry, which confirms the conclusions drawn from the steady-state spectroscopic measurements. Scheme 1 presents the most probable structures of the methyl benzoate derivative-SCA[6] inclusion complexes created on the basis of our measurements. For the convenience of data presentation, schematic representation of the guest-SCA[6] inclusion complexes were classified into the two following groups: *p*-methylamino derivatives (I and II) and *p*-dimethylamino derivatives (III and IV). As can be seen, the substituent (methylamino versus dimethylamino) in the benzene ring of the investigated molecules clearly influences the stoichiometry and structure (orientation of the molecule inside the host's cavity) of formed inclusion complexes. Interestingly, the changes in macrocyclic compound-induced NMR chemical shifts are very different for methyl benzoate derivative-SCA[6] and methyl benzoate derivative-CB[7] systems. In our previous paper [26], we have shown that the dimethylamino group is located outside of the CB[7] cavity and molecules III and IV form inclusion complexes with a 1:1 stoichiometry.

To explore quantitatively the host – guest complex formation between I and IV and SCA[6], the NMR titration data were analyzed using Benesi-Hildebrand and nonlinear relations. As protons located via encapsulation inside the host cavity exhibit the largest upfield shifts, the NMR titration data obtained for the most sensitive proton to changes in its immediate environment, H_d , were analyzed based on the Benesi-Hildebrand and nonlinear regression procedure. For the all systems under study, the Benesi-Hildebrand dependence for the H_d proton is shown in Fig. 8. As expected, the I-SCA[6] and II-SCA[6] inclusion complexes were confirmed to bind in a 1:1 ratio, while, the non-linearity of the double reciprocal plot for III-SCA[6] and IV-SCA[6] confirms the formation of higher-order (1:2) complexes. As can be seen in Table 1, a nonlinear fit of the ^1H NMR data to Eqs. (2) and (3) (see Fig. 8) allowed us to determine the equilibrium constants whose values are in good agreement with the data obtained in the ground-state optical spectroscopy titration experiments.

6. Conclusion

The steady-state, time-resolved and ^1H NMR spectroscopic studies clearly indicate that all investigated methyl benzoate derivatives interact with SCA[6] to form stable inclusion complexes both in the ground and in the excited state. From our investigations, it is inferred that a 1:1 host–guest complex is formed for I-SCA[6] and II-SCA[6] systems, while for III-SCA[6] and IV-SCA[6], a single molecule III (and IV) is trapped in a capsule composed of two SCA[6] cavities. Moreover, it was shown that the photoinduced intramolecular processes such as ESIPT and TICT are strongly dependent on the stoichiometry and structure (orientation of the methyl benzoate derivative inside the host's cavity) of formed inclusion complexes i.e., ESIPT takes place when the benzene ring is located inside the SCA[6] cavity. The observed changes in the time-dependent fluorescence Stokes shift point toward the fact that the dynamics of pure water is markedly different from that of the confined water molecule in SCA[6] system i.e., water molecules located inside the SCA[6] exhibit markedly slower solvation dynamics. Overall, our present (in combination with our previous) studies offer a detailed outlook into the nature and mechanisms of noncovalent interaction between some methyl benzoate derivatives and chemicals (CDs, cucurbit[7]urils, SCA[6]) [25–29] and biological (BSA) [30] macrocyclic hosts. In conclusion, we believe that the results obtained in this work may be important in relation to the applications of inclusion complexes in pharmacy and medicine (binding, stabilization and release of drug molecules in host–guest systems) or TICT and ESIPT fluorescence probes.

CRedit authorship contribution statement

Karolina Baranowska: Conceptualization, Visualization, Investigation, Resources, Writing – original draft, Methodology, Software. **Michał Mońka:** Investigation. **Agnieszka Kowalczyk:** Investigation. **Zbigniew Kaczyński:** Writing – review & editing. **Piotr Bojarski:** Writing – review & editing. **Marek Józefowicz:** Conceptualization, Software, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.saa.2023.123131>.

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