J Incl Phenom Macrocycl Chem (2011) 69:167–172
DOI 10.1007/s10847-010-9827-z

ORIGINAL ARTICLE

# Inclusion complex formation of α- and β-cyclodextrins with riboflavin and alloxazine in aqueous solution: thermodynamic study

**Irina V. Terekhova · Marina N. Tikhova · Tatyana V. Volkova · Roman S. Kumeev · German L. Perlovich**

Received: 26 April 2010 / Accepted: 3 July 2010 / Published online: 17 July 2010


**Abstract** Possibility of encapsulation of riboflavin and alloxazine by α- and β-cyclodextrins in aqueous solution was studied by $^1$H NMR and solubility methods. Thermodynamic parameters of 1:1 inclusion complex formation (K, $\Delta_c G^0$, $\Delta_c H^0$ and $\Delta_c S^0$) were obtained and analyzed in terms of influence of reagent's structure on complexation process. It was shown that α-cyclodextrin displays low binding affinity to riboflavin and alloxazine. On the contrary, β-cyclodextrin forms with riboflavin and alloxazine more stable inclusion complexes. Binding is accompanied by the negative enthalpy and entropy changes that are determined by predominance of van der Waals interactions and possible H-bonding. The presence of ribityl substituent in riboflavin molecule prevents the deep penetration of this compound into macrocyclic cavity. Proposed on the basis of $^1$H NMR data the partial insertion of the hydrophobic part of riboflavin and alloxazine molecules into the β-cyclodextrin cavity causes the enhancement of aqueous solubility of the encapsulated substances. In comparison with α-cyclodextrin, the solubilizing effect of β-cyclodextrin is more pronounced due to its higher binding affinity to alloxazine and riboflavin.

**Keywords** Inclusion complex formation · Cyclodextrin · Riboflavin · Alloxazine · Thermodynamics

## Introduction

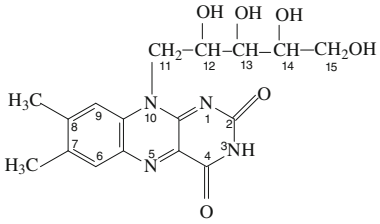
During the last decades industrial application of bioencapsulation is growing [1]. Bioencapsulation is a technology of envelopment of biologically active compounds in microcapsules with the purpose to protect the enclosed substances from the surrounding environment, to increase their efficiency and to receive the controlled-release materials [1, 2]. A number of encapsulating materials (polymers, vesicles, micells, hydrogels) are designed and proposed in the recent years [1–5]. Cyclodextrins (CDs) are of particular interest in this regard [6–11].

CDs are the cyclic oligosaccharides enzimatically produced from starch and composed of six (α-CD), seven (β-CD) and eight (γ-CD) glucose units linked by glycosidic bonds. The shape of CD molecule is similar to truncated cone with a hydrophobic interior and hydrophilic exterior formed by hydroxyl groups. Ability of the hydrophobic cavity to encapsulate different molecules due to noncovalent interactions determines the numerous practical applications of CDs. In particular, CDs are widely used as inert and nontoxic encapsulating materials and solubilizing additives in pharmaceutical formulations, cosmetics and food [6–10]. Physicochemical properties (e.g., stability, solubility, odor, etc.) and bioactivity of the encapsulated molecules can be considerably improved owing to inclusion complexation with CDs.

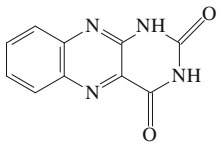
The purpose of this work was to study on inclusion complex formation of CDs with riboflavin and alloxazine. Riboflavin (vitamin $B_2$, 7,8-dimethyl-10-(1′-D-ribityl)isoalloxazine) belongs to the family of flavins. It plays an important role in the living organisms and is widely used in pharmaceutical and vitamin preparations [12]. Bioactivity of riboflavin can be reduced owing to its low aqueous solubility and instability [13–15]. It is well known that under illumination with visible light the biological activity of riboflavin is reduced. Photolysis of riboflavin in alkali and acidic media involves first of all the degradation of ribityl chain and this flavin is easily transformed into alloxazines.

I. V. Terekhova (✉) · M. N. Tikhova · T. V. Volkova · R. S. Kumeev · G. L. Perlovich
Institute of Solution Chemistry, Russian Academy of Sciences, 153045 Ivanovo, Russian Federation
e-mail: ivt@isc-ras.ru

Alloxazines and their derivatives being the products of biochemical, chemical and photochemical decomposition of flavins are present in most biological tissues and in many food supplements [16–19]. Our interest in alloxazine was driven mainly by its similarity to riboflavin. Alloxazine molecule contains no side groups and, therefore, the influence of methyl and ribityl substituents on complex formation of riboflavin with CDs can be evaluated.



riboflavin



alloxazine

There are some publications, in which systems for riboflavin stabilization have been considered [20–24]. Loukas et al. [21, 22] proposed to use the multilamellar liposomes for incorporation of riboflavin in pure form and in the form of CD complex. CDs as a complexing systems that also provide moderate stability against the external factors (light and oxygen) were investigated [23, 24]. Wang et al. [23, 24] showed that inclusion complex formation with $\beta$-CD has a significant effect on riboflavin reduction. To the best of our knowledge, only stability constants of inclusion complexes of CDs with riboflavin have been reported in literature [23, 25–28], and information on the enthalpy and entropy of complex formation is absent. Additionally, interactions of CDs with alloxazine were not studied. The aim of present work was to carry out thermodynamic study on complex formation of $\alpha$- and $\beta$-cyclodextrins with riboflavin and alloxazine in aqueous solution. Our specific interest in alloxazine was arisen due to its structure which is similar to that found in riboflavin and other biologically active isoalloxazines. Thereby, the comparative investigation of binding of CDs with alloxazine and riboflavin was undertaken in order to show the role of ribityl- and methyl-substituents in complex formation process.

## Experimental

### Chemicals

Riboflavin (Sigma), alloxazine (Aldrich), $\alpha$-CD (Aldrich) and $\beta$-CD (Fluka) were used as received. CDs were stable crystalline hydrates, the water content in which was taken into consideration during calculation of concentration. All solutions were prepared by weight on the basis of double-distilled water.

### Solubility study

An excess amount of riboflavin (or alloxazine) was added to the aqueous solutions of CDs, concentrations of which were varied from 0 to 0.04 mol $kg^{-1}$ for $\alpha$-CD and from 0 to 0.014 mol $kg^{-1}$ for $\beta$-CD. Experiment was done at different temperatures (298–315 K). The solutions were placed in plastic vials, which were covered with aluminum foil in order to prevent the possible degradation of riboflavin and alloxazine by light. The solutions were agitated for 72 h at constant temperature. After equilibration, the solutions were centrifuged and analyzed spectrophotometrically. Measurements were carried out at several wavelengths corresponding to sharp peaks and the average result was taken.

### UV–vis spectroscopy

Absorption spectra were recorded at 298 K on a Cary 50 (Varian) spectrophotometer using 1 cm quartz cuvettes. Stoichiometry of the complexes was determined by Job's method [29]. According to this method, solutions of CD and riboflavin (or alloxazine) of equal concentrations (0.1 mmol $kg^{-1}$) were mixed in this way that the total molar concentration of CD and riboflavin (or alloxazine) is held constant, but their mole fractions are varied. A measurable parameter (absorbance) that is proportional to complex formation is plotted against the mole fractions of these two components. The extremum on the plot corresponds to the stoichiometric ratio of the two species.

### $^1$H NMR

$^1$H NMR spectra were recorded using a Bruker-AV-500 spectrometer with the operating frequency at 500 MHz. Chemical shifts were measured at several temperatures (293–315 K) relative to external cyclohexane. The solutions containing a constant concentration of riboflavin (0.28 mmol $kg^{-1}$) and variable concentration of CDs (0–0.04 mol $kg^{-1}$ for $\alpha$-CD and 0–0.015 mol $kg^{-1}$ for $\beta$-CD) were prepared in deuterated water of 99.9% isotopic purity.

## Results and discussion

The fact that CDs can enhance solubility of many compounds is explained by formation of water-soluble inclusion complexes in which the hydrophobic guest molecules are placed in the apolar CD interior [8–10]. To examine the solubilization of riboflavin and alloxazine by CDs the phase-solubility diagrams were obtained (Fig. 1). As can be seen from Fig. 1, solubility of riboflavin and alloxazine is greatly increased in the presence of $\beta$-CD. On the contrary, the solubilizing effect of $\alpha$-CD with respect to

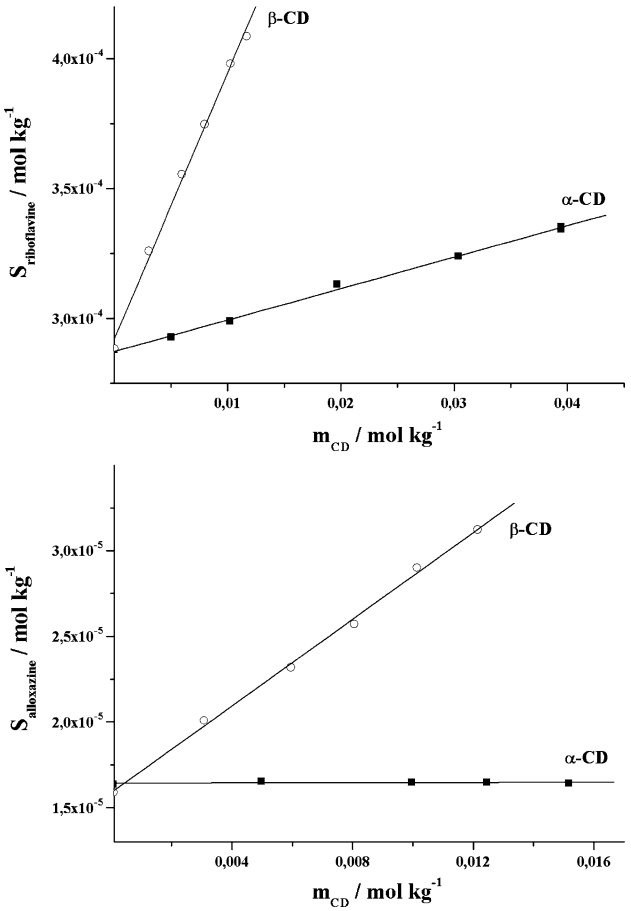



**Fig. 1** Phase-solubility diagrams for complexation of cyclodextrins with riboflavin (*top*) and alloxazine (*bottom*) in water at 298 K

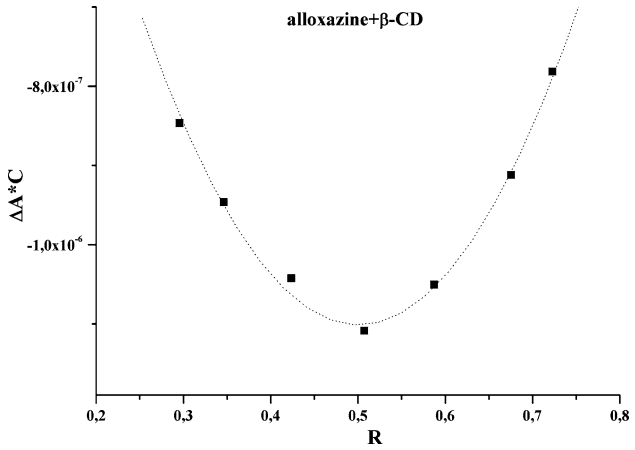



**Fig. 2** Job's plot for complex formation of $\beta$-CD with alloxazine in water at 298 K

**Table 1** Stability constants of $\beta$-CD complexes with riboflavin and alloxazine at different temperatures

| T/K | K/kg mol$^{-1}$ | | |
|---|---|---|---|
| | Riboflavin/$\beta$-CD | | Alloxazine/$\beta$-CD |
| | Solubility | $^1$H NMR | Solubility |
| 293 | – | 34 ± 2 | – |
| 303 | 35 ± 2 | 30 ± 2 | 70 ± 5 |
| 308 | 33 ± 2 | – | – |
| 310 | – | – | 65 ± 5 |
| 313 | 30 ± 2 | 26 ± 2 | – |
| 315 | – | – | 54 ± 4 |

riboflavin is shown slightly and it is practically absent in case of alloxazine. These results indicate that more strong binding of riboflavin and alloxazine with $\beta$-CD takes place.

According to method of Higuchi and Connors [30], the observed in our work linear increase of aqueous solubility of riboflavin and alloxazine with CD concentration is described by the solubility diagram of $A_L$-type. The $A_L$ curve corresponds to 1:1 complex formation [30]. This stoichiometric ratio of the complexes was additionally confirmed by Job's method [29]. Job's plot illustrated in Fig. 2 as an example for system alloxazine + $\beta$-CD exhibits the minima at mole ratio 0.5 reflecting the formation of 1:1 complexes. The similar plots were obtained for all systems under study.

Stability constants of 1:1 complexes can be calculated from the slope of solubility diagrams as reported by Higuchi and Connors [30]:

$$K = \frac{\text{slope}}{S_0(1 - \text{slope})} \quad (1)$$

where $K$ is stability constant; $S_0$ is solubility of riboflavin (or alloxazine) in pure water.

Enthalpy and entropy changes of complex formation were derived from temperature-dependent solubility measurements using the following equation:

$$\ln K = -\frac{\Delta H^0}{RT} + \frac{\Delta S^0}{R} \quad (2)$$

Stability constants at different temperatures are given in Table 1, and van't Hoff plots are shown in Fig. 3. Thermodynamic parameters of complex formation at 298 K are summarized in Table 2.

It was found that complex formation between α-CD and alloxazine is absent. One can assume that diameter of α-CD cavity (4.7–5.3 Å [31]) is too small for incorporation of alloxazine molecule. However, the partial inclusion of such large molecules as anthracene into α-CD cavity has been detected by Blyshak et al. [32]. Moreover, calculation of the geometric parameters of alloxazine was performed using Avogadro computer program and applying B3LYP ab initio method. As it was obtained, the length of the benzene ring (~5 Å) corresponds to α-CD cavity and the partial insertion of alloxazine can be possible. Nevertheless, the binding is

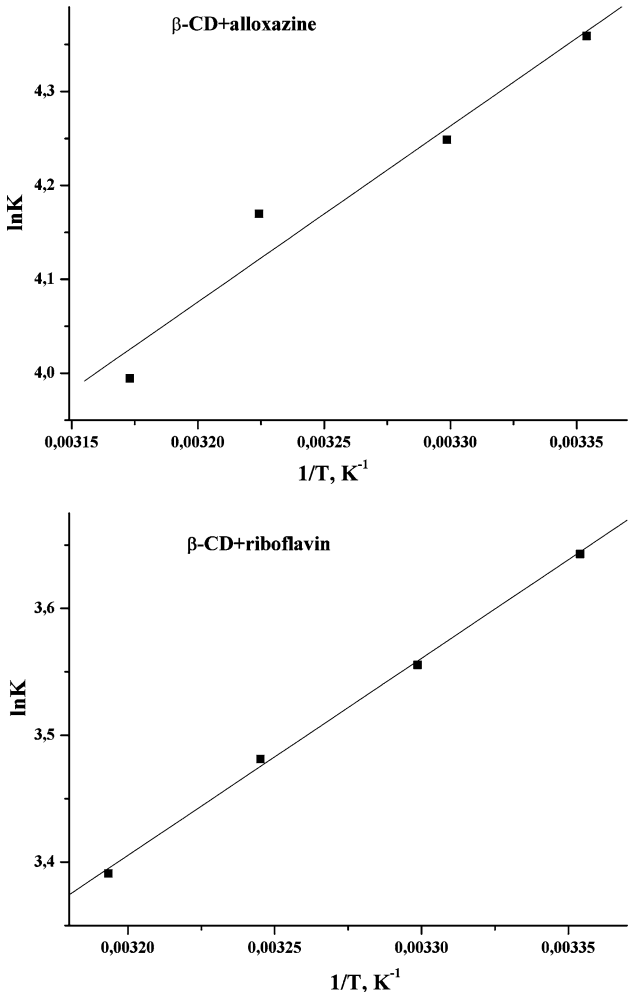



**Fig. 3** The plot of lnK versus 1/T for complexation of *β*-CD with riboflavin (*bottom*) and alloxazine (*top*) in water

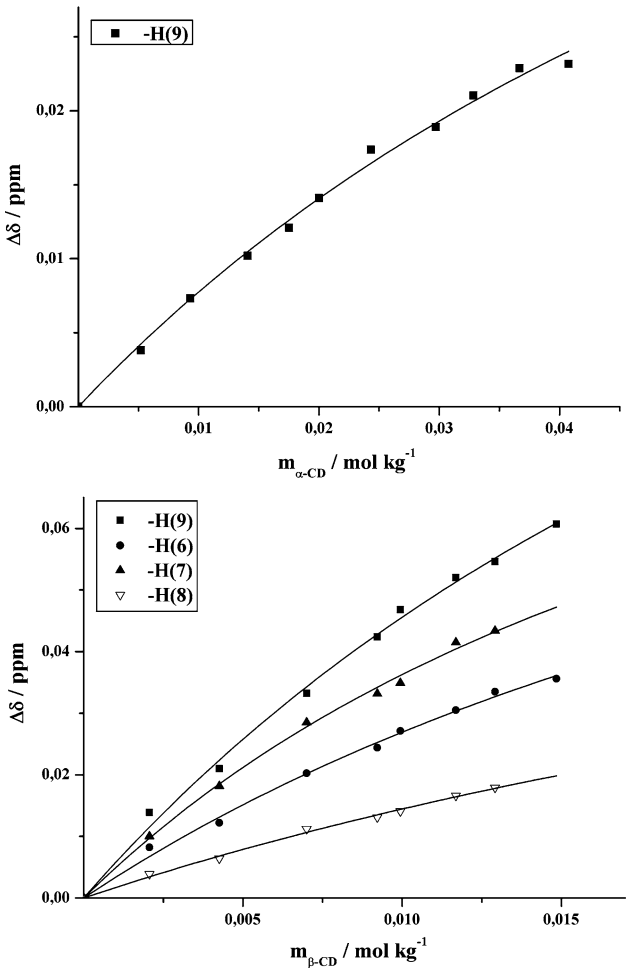



**Fig. 4** Chemical shift changes of riboflavin protons versus the cyclodextrin concentration at 298 K

not observed in this system. We suppose that alloxazine molecule is highly hydrated in aqueous medium owing to possible interactions of water molecules with the nitrogen atoms in the aromatic rings. As it is well known, hydration competes with complex formation and prevents it.

The weak molecular complex is formed between α-CD and riboflavin (Table 2). Determination of the enthalpy and entropy changes was not possible due to very low stability constant of riboflavin/α-CD complex. We suggest that binding in this case is realized via methyl side groups presented in the riboflavin structure. Two $CH_3$-groups can enter the apolar cavity of α-CD. According to this binding mode, the ribityl substituent is placed outside the α-CD cavity and can participate in hydrogen bonding with hydroxyls surrounding the cavity.

To confirm the proposed binding mode, the $^1$H NMR spectrum of riboflavin in the presence of α-CD was

**Table 2** Thermodynamic parameters of 1:1 complex formation of *β*-cyclodextrin with riboflavin and alloxazine in water at 298 K

| Complex | K/kg mol$^{-1}$ | $\Delta_cG^0$ (kJ mol$^{-1}$) | $\Delta_cH^0$ (kJ mol$^{-1}$) | $T\Delta_cS^0$ (kJ mol$^{-1}$) |
|---|---|---|---|---|
| | *Solubility method* | | | |
| Alloxazine/*β*-CD | 78 ± 5 | −10.8 | −15.6 ± 1.2 | −4.8 |
| Riboflavin/α-CD | 3.9 ± 0.2 | −3.4 | – | – |
| Riboflavin/*β*-CD | 38 ± 2 | −9.0 | −11.9 ± 0.4 | −3.9 |
| | *$^1$H NMR* | | | |
| Riboflavin/α-CD | 6 ± 1 | −4.4 | – | – |
| Riboflavin/*β*-CD | 32 ± 1 | −8.6 | −10.1 ± 1.0 | −1.5 |

**Table 3** Complexation-induced chemical shifts of riboflavin protons at 298 K

| Complex | $\Delta_c\delta$/ppm | | | |
|---|---|---|---|---|
| | H(6) | H(7) | H(8) | H(9) |
| α-CD/riboflavin | 0.05 ± 0.01 | 0.05 ± 0.01 | 0.03 ± 0.01 | 0.06 ± 0.01 |
| β-CD/riboflavin | 0.12 ± 0.01 | 0.14 ± 0.01 | 0.09 ± 0.01 | 0.15 ± 0.01 |

recorded and analyzed. The $^1$H NMR spectrum of riboflavin in $D_2O$ shows the signals of protons H(6), H(7), H(8), H(9) H(11), H(12), H(13), H(14) and H(15). Addition of α-CD results in the overlapping of several signals of CD protons with the signals of riboflavin protons belonging to ribityl side group. Therefore, only signals of H(6), H(7), H(8) and H(9) protons were examined upon addition of different amounts of α-CD. The chemical shift changes of riboflavin proton H(9) as a function of α-CD concentration are illustrated in Fig. 4 (top view).

Complexation-induced chemical shift changes ($\Delta_c\delta$) and stability constants of the complexes (K) were evaluated from these dependences by nonlinear curve fitting procedure [33]. Values of K and $\Delta_c\delta$ are given in Tables 2 and 3, respectively. Thermodynamic parameters of β-CD complex formation with riboflavin were derived from temperature dependences of K (Table 1) applying Eq. 2. A good agreement of thermodynamic parameters obtained from $^1$H NMR and solubility methods should be noted herein (Table 2).

The observed in the presence of α-CD downfield shift of signals of H(6)-H(9) riboflavin protons (Table 3) points out that benzene ring with methyl-groups is inserted into macrocyclic cavity. However, the small $\Delta_c\delta$ values ($\leq$0.06 ppm) indicate a weak interaction of riboflavin with α-CD.

The increase of β-CD cavity size results in formation of more stable complexes. It can be seen from Table 2, stability constants of β-CD complexes are generally larger than those for α-CD complexes. More significant downfield shifts of riboflavin protons were also observed for β-CD complex formation (Table 3). These facts confirm that alloxazine and riboflavin penetrate deeper into the larger β-CD cavity than into the smaller α-CD cavity. As it follows from Table 2, complex formation of β-CD with alloxazine and riboflavin is characterized by the negative enthalpy and entropy changes. Since the enthalpy contribution to the free energy is dominant, the binding is enthalpy driven. The negative $\Delta_cH^0$ and $\Delta_cS^0$ are mainly caused by prevalence of van der Waals interactions. In β-CD complexes the aromatic rings of both guest molecules are in close contact with the cavity walls and intermolecular forces are rather strong. The binding strength is maximal for β-CD and less for α-CD due to more snug fit for β-CD. Moreover, formation of the hydrogen bonds between β-CD external surface and polar groups of the guests can be possible. Thus, van der Waals interactions and H-bonding are the main forces responsible for the complexation process. These interactions restrict the flexibility of reagents giving the entropy loss (Table 1).

Comparative analysis of the thermodynamic parameters of complex formation of β-CD with alloxazine and riboflavin shows that binding is more enthalpy favorable with the former one (Table 2). Stability constant of alloxazine/β-CD complex is approximately two times higher than K for riboflavin/β-CD complex. More probably, the deeper inclusion of alloxazine molecule into β-CD cavity occurs and it is prevented by the bulky ribityl side group in the case of riboflavin. We suppose that stronger attractive interactions caused by the deeper penetration of alloxazine result in higher exothermicity of binding. More negative $\Delta_cS^0$ obtained for this system is consistent with this suggestion.

## Conclusions

Thermodynamic study on 1:1 complex formation of α-CD and β-CD with riboflavin and alloxazine in aqueous solution was carried out by $^1$H NMR and solubility techniques. Experimental results showed that, in comparison with α-CD, β-CD displays higher binding affinity to riboflavin and alloxazine forming with them more stable enthalpy-stabilized inclusion complexes. The van der Waals interactions and H-bonding were proposed as driving forces of complex formation. Availability of ribitiyl substituent in riboflavin molecule prevents the deeper insertion of this compound into macrocyclic cavity and results in decreasing of the exothermicity of binding. The considerable enhancement of aqueous solubility of riboflavin and alloxazine in the presence of β-CD is caused by the deeper penetration of the hydrophobic moiety of guest molecules into the host cavity. The solubilizing efficiency of β-CD was found to be higher than those of α-CD. Thus, β-CDs are more suitable for encapsulation of riboflavin and alloxazine in aqueous media.

**Acknowledgments** This work was supported by the Russian Foundation for Basic Research (grant N$^o$09-03-97563). We are grateful to E. Gorbachev for help with calculations.

## References

1. De Vos, P., Bučko, M., Gemeiner, P., et al.: Multiscale requirements for bioencapsulation in medicine and biotechnology. Biomaterials **30**, 2559–2570 (2009)

2. Champagne, C.P., Fustier, P.: Microencapsulation for the improved delivery of bioactive compounds into foods. Curr. Opin. Biotechnol. **18**, 184–190 (2007)
3. Branco, M.C., Schneider, J.P.: Self-assembling materials for therapeutic delivery. Acta Biomater. **5**, 817–831 (2009)
4. Sagar, G.H., Arunagirinathan, M.A., Bellare, J.R.: Self-assembled surfactant nano-structures important in drug delivery: a review. Indian J. Exp. Biol. **45**, 133–159 (2007)
5. Goldberg, M., Langer, R., Jia, X.Q.: Nanostructured materials for applications in drug delivery and tissue engineering. J. Biomater. Sci. Polym. Ed. **18**, 241–268 (2007)
6. Szente, L., Szejtli, J.: Cyclodextrins as food ingredients. Trends Food Sci. Technol. **15**, 137–142 (2004)
7. Szeitli, J., Szente, L.: Elimination of bitter, disgusting tastes of drugs and foods by cyclodextrins. Eur. J. Pharm. Biopharm. **61**, 115–125 (2005)
8. Loftsson, T., Duchêne, D.: Cyclodextrins and their pharmaceutical applications. Int. J. Pharm. **329**, 1–11 (2007)
9. Vyas, A., Saraf, S., Saraf, S.: Cyclodextrins based novel drug delivery systems. J. Incl. Phenom. Macrocycl. Chem. **62**, 23–42 (2008)
10. Del Valle, E.M.M.: Cyclodextrins and their uses: a review. Process Biochem. **39**, 1033–1046 (2004)
11. Szejtli, J.: In: Davies, J.E.D., MacNicol, D.D., Vögtle, F., Attwood, J.L. (eds.) Comprehensive Supramolecular Chemistry: Cyclodextrins, vol. 3. Oxford, Elsevier (1996)
12. Massey, V.: The chemical and biological versatility of riboflavin. Biochem. Soc. Trans. **28**, 283–296 (2000)
13. Heelis, P.F.: The photophysical and photochemical properties of flavins (isoalloxazines). Chem. Soc. Rev. **11**, 15–39 (1982)
14. Saleh, A.M.: Stability of riboflavin in solubilized systems. Pharmazie. **29**, 474–478 (1974)
15. Smith, E.C., Metzler, D.E.: The photochemical degradation of riboflavin. J. Am. Chem. Soc. **85**, 3285–3288 (1963)
16. Chastain, J., McCormick, D.B.: In: Muller, F. (ed.) Chemistry and Biochemistry of Flavoenzymes, pp. 196–200. CRC Press, Boston (1991)
17. Sikorska, E., Khmelinskii, I.V., Williams, S.L., et al.: Spectroscopy and photophysics of 6,7-dimethyl-alloxazine: experimental and theoretical study. J. Mol. Struct. **697**, 199–205 (2004)
18. Szymusiak, H., Konarski, J., Koziol, J.: An INDO/S MO study of alloxazine and its monomethyl derivatives. J. Chem. Soc. Perkin Trans. **2**, 229–236 (1990)
19. Komasa, J., Rychlewski, J., Koziol, J.: Electronic structure of alloxazine and its methyl derivatives. J. Mol. Struct. (Theochem). **170**, 205–212 (1988)
20. Habib, H.J., Asker, A.F.: Photostabilization of riboflavin by incorporation into liposomes. J. Parent Sci. Technol. **45**, 124–127 (1991)
21. Loukas, Y.L., Jayasekera, P., Gregoriadis, G.: Characterization and photoprotection studies of a model $\gamma$-cyclodextrin-included photolabile drug entrapped in liposomes incorporating light absorbers. J. Phys. Chem. **99**, 11035–11040 (1995)
22. Loukas, Y.L.: A Plackett–Burman screening design directs the efficient formulation of multicomponent DRV liposomes. J. Pharm. Biomed. Anal. **26**, 255–263 (2001)
23. Wang, X.-M., Chen, H.-Y.: A spectroelectrochemical study of the interaction of riboflavin with $\beta$-cyclodextrin. Spectrochim. Acta A **51**, 599–605 (1996)
24. Wang, X.-M., Yan, M.-D., Zhu, J., Chen, H.-Y.: The surface-enhanced Raman spectroelectrochemical study on the interaction between $\beta$-cyclodextrin and the electrochemically generated radical intermediate of flavin. J. Electroanal. Chem. **451**, 187–192 (1998)
25. Roy, D.K., Deb, N., Ghosh, B.C., Mukherjee, A.K.: Inclusion of riboflavin in $\beta$-cyclodextrin: a fluorimetric and absorption spectrometric study. Spectrochim. Acta A **73**, 201–204 (2009)
26. Loukas, Y.L., Vraka, V., Gregoriadis, G.: Use of nonlinear least-squares model for the kinetic determination of the stability constant of cyclodextrin inclusion complexes. Int. J. Pharm. **144**, 225–231 (1996)
27. Loukas, Y.L.: Multiple complex formation of unstable compounds with cyclodextrins: efficient determination and evaluation of the binding constant with improved kinetic studies. Analyst **122**, 377–381 (1997)
28. Loukas, Y.L.: Multiple complex formation of fluorescent compounds with cyclodextrins: efficient determination and evaluation of the binding constant with improved fluorometric studies. J. Phys. Chem. B **101**, 4863–4866 (1997)
29. Job, P.: Ann. Chim. **9**, 113–203 (1928)
30. Higuchi, T., Connors, K.A.: Phase-solubility techniques. Adv. Anal. Chem. Instrum. **4**, 117–212 (1965)
31. Saenger, W., Jacob, J., Gessler, K., et al.: Structures of the common cyclodextrins and their larger analogues—beyond the doughnut. Chem. Rev. **98**, 1787–1802 (1998)
32. Blyshak, L.A., Warner, I.M., Patonay, G.: Evidence for non-inclusional association between $\alpha$-cyclodextrin and polynuclear aromatic hydrocarbons. Anal. Chim. Acta **232**, 239–243 (1990)
33. Terekhova, I.V., Kumeev, R.S., Alper, G.A.: Inclusion complex formation of $\alpha$- and $\beta$-cyclodextrins with aminobenzoic acids in aqueous solution studied by $^1$H NMR. J. Incl. Phenom. Macrocycl. Chem. **59**, 301–306 (2007)