

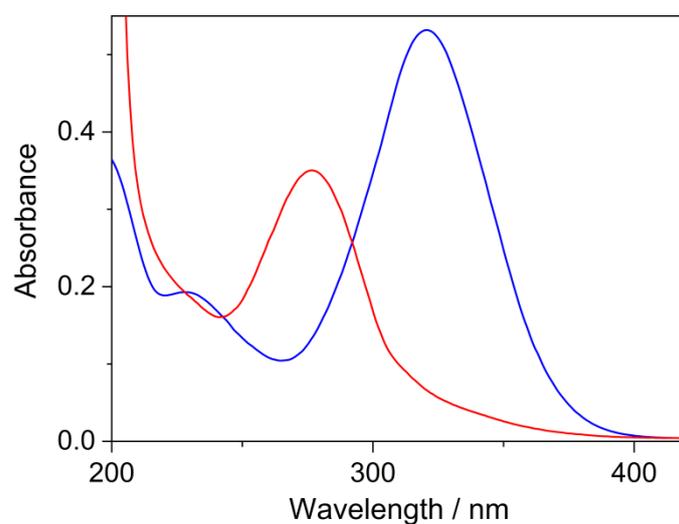
# **Encapsulation of metronidazole in biocompatible macrocycles and structural characterization of its nano spray-dried nanostructured composite**

**Mirella Mirankó <sup>1</sup>, Mónika Megyesi <sup>2</sup>, Zsombor Miskolczy <sup>2</sup>, Judit Tóth <sup>1,2</sup>, Tivadar Feczko <sup>1,2,\*</sup> and László Biczók <sup>2,\*</sup>**

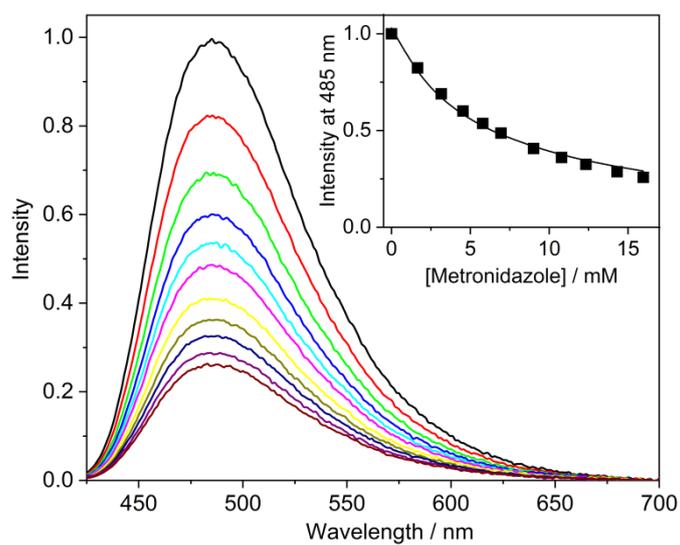
<sup>1</sup> University of Pannonia, Faculty of Engineering, Research Institute of Biomolecular and Chemical Engineering, 8200 Veszprém, Egyetem u. 10., Hungary;  
miranko@mukki.richem.hu (Mi.M); toth@mukki.richem.hu (J.T); feczko@mukki.richem.hu (T.F.)

<sup>2</sup> Research Centre for Natural Sciences, Institute of Materials and Environmental Chemistry, Eötvös Loránd Research Network (ELKH), P.O. Box 286, 1519 Budapest, Hungary;  
megyesi.monika@ttk.hu (Mó.M.); miskolczy.zsombor@ttk.hu (Z.M.); biczok.laszlo@ttk.hu (L.B.)

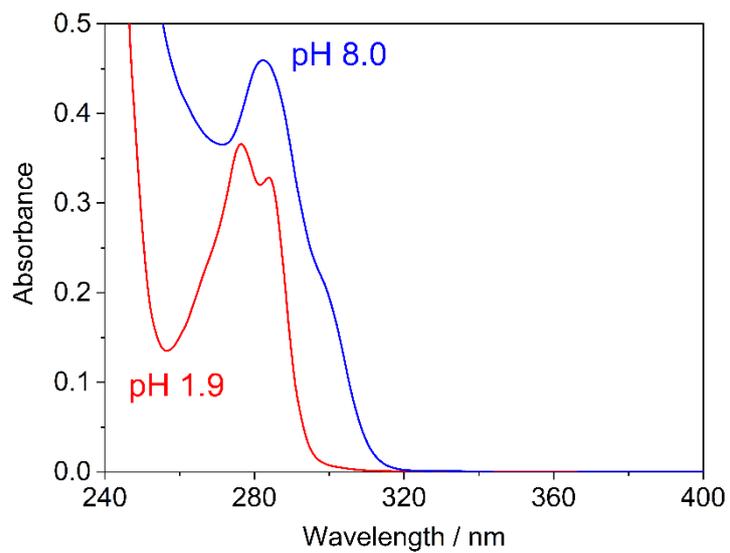
\* Correspondence: feczko@mukki.richem.hu (T.F.); biczok.laszlo@ttk.hu (L.B.)



**Figure S1** Absorption spectra of metronidazole in water (blue) and in 0.1 N H<sub>2</sub>SO<sub>4</sub> solution (red)



**Figure S2** Alteration of the fluorescence spectra in the aqueous solution of 1.0  $\mu$ M dehydrocorydaline and 3.0  $\mu$ M CB7 at 0, 1.7, 3.2, 4.5, 5.8, 6.9, 9.0, 10.8, 12.3, 14.3, and 16.0 mM metronidazole concentrations (pH = 7.5, excitation wavelength 420 nm). Inset: fluorescence intensity at 485 nm as a function of metronidazole concentration.



**Figure S3** Absorption spectra of 80  $\mu$ M 4-sulfonatocalix[4]arene (SCX4) in water at pHs 1.9 and 8.0

## Evaluation of the binding constant of MetH<sup>+</sup> confinement in CB7

Protonation of CB7 is also an unimportant process at pH 3 because it has an equilibrium constant of only  $\sim 166 \text{ M}^{-1}$  [1]. The mass balance equations are:

$$[\text{MetH}^+ - \text{CB7}] + [\text{Met} - \text{CB7}] + [\text{MetH}^+] + [\text{Met}] = [\text{Met}]_T \quad (\text{S1})$$

$$[\text{MetH}^+ - \text{CB7}] + [\text{Met} - \text{CB7}] + [\text{CB7}] = [\text{CB7}]_T \quad (\text{S2})$$

The measurements were performed at equal total concentrations of metronidazole and CB7,  $[\text{CB7}]_T = [\text{Met}]_T$ . Therefore, (S2) and (S1) gives

$$[\text{MetH}^+] + [\text{Met}] = [\text{CB7}] \quad (\text{S3})$$

Based on the definition of the equilibrium constant of the proton dissociation from MetH<sup>+</sup> ( $K_a$ ), the following relationship is derived:

$$[\text{MetH}^+] = \frac{[\text{Met}][\text{H}^+]}{K_a} \quad (\text{S4})$$

Substitution (S4) into (S3) gives

$$[\text{Met}] \left( 1 + \frac{[\text{H}^+]}{K_a} \right) = [\text{CB7}] \quad (\text{S5})$$

The definition of the equilibrium constant of MetH<sup>+</sup>–CB7 complex formation ( $K_p$ ) gives:

$$[\text{MetH}^+ - \text{CB7}] = K_p [\text{MetH}^+] [\text{CB7}] \quad (\text{S6})$$

Substitution (S4) into (S6) results in

$$[\text{MetH}^+ - \text{CB7}] = K_p \frac{[\text{Met}][\text{H}^+]}{K_a} [\text{CB7}] \quad (\text{S7})$$

Substitution (S5) into (S7) leads to

$$[\text{MetH}^+ - \text{CB7}] = K_p \frac{[\text{H}^+]}{K_a} \left( 1 + \frac{[\text{H}^+]}{K_a} \right) [\text{Met}]^2 \quad (\text{S8})$$

The definition of the equilibrium constant of Met–CB7 complex formation ( $K$ ) gives:

$$[\text{Met} - \text{CB7}] = K [\text{Met}] [\text{CB7}] \quad (\text{S9})$$

Substitution (S5) into (S9) provides

$$[\text{Met} - \text{CB7}] = K \left( 1 + \frac{[\text{H}^+]}{K_a} \right) [\text{Met}]^2 \quad (\text{S10})$$

Substitution (S5), (S8), and (S10) into (S2) and rearrangement leads to

$$\left(K + K_p \frac{[H^+]}{K_a}\right) \left(1 + \frac{[H^+]}{K_a}\right) [Met]^2 + \left(1 + \frac{[H^+]}{K_a}\right) [Met] - [CB7]_T = 0 \quad (S11)$$

where  $K_p$  is a fitting parameter,  $[H^+] = 10^{-3}$  M,  $K_a = 4.365 \times 10^{-3}$  M was determined by spectrophotometric titration, and  $K = 1000$  M<sup>-1</sup> based on the experiments with dehydrocorydaline fluorescent probe.

$$q = \left(1 + \frac{[H^+]}{K_a}\right) = 1.2291 \quad (S12)$$

The solution of (S11) quadratic equation using (S12) provides  $[Met]$  at each  $[CB7]_T$  concentration.

$$[Met] = \frac{-q + \sqrt{q^2 + 4(K + K_p[H^+]/K_a)q[CB7]_T}}{2(K + K_p[H^+]/K_a)q} \quad (S13)$$

Using these values,  $[MetH^+]$ ,  $[Met-CB7]$  and  $[MetH^+-CB7]$  can be calculated by (S4), (S10), and (S8). In our experiments, the apparent molar absorption coefficient ( $\varepsilon$ ) was measured at 320 nm as a function of the total CB7 concentration. The result is expressed by the following relationship:

$$\varepsilon = \varepsilon_1 \frac{[Met]}{[Met]_T} + \varepsilon_2 \frac{[MetH^+]}{[Met]_T} + \varepsilon_3 \frac{[Met-CB7]}{[Met]_T} + \varepsilon_4 \frac{[MetH^+-CB7]}{[Met]_T} \quad (S14)$$

where  $\varepsilon_1$ ,  $\varepsilon_2$ ,  $\varepsilon_3$ , and  $\varepsilon_4$  represent the molar absorption coefficient of Met, MetH<sup>+</sup>, Met-CB7, and MetH<sup>+</sup>-CB7, respectively. Independent experiments provided  $\varepsilon_1 = 8730$  M<sup>-1</sup> cm<sup>-1</sup>,  $\varepsilon_2 = 780$  M<sup>-1</sup> cm<sup>-1</sup>,  $K = 1000$  M<sup>-1</sup>, which were kept constant in the nonlinear least-squares analysis of the experimental data. Starting with the initial estimates of  $K_p$ ,  $\varepsilon_3$  and  $\varepsilon_4$ , we calculated  $[Met]$ ,  $[MetH^+]$ ,  $[Met-CB7]$ ,  $[MetH^+-CB7]$  and  $\varepsilon$  by (S13), (S4), (S10), (S8) and (S14), respectively. Then, the iterations were repeated until the best fit of  $\varepsilon$  was achieved. The optimized function gave  $K_p = 1.9 \times 10^5$  M<sup>-1</sup>,  $\varepsilon_3 = 8800$  M<sup>-1</sup> cm<sup>-1</sup> and  $\varepsilon_4 = 620$  M<sup>-1</sup> cm<sup>-1</sup> values.

## Evaluation of the binding constant of MetH<sup>+</sup> confinement in SCX4

Berberine (B) was used as a fluorescent probe for the determination of the binding constant of MetH<sup>+</sup> association with SCX4. Since the total concentration of B ([B]<sub>T</sub> = 5.0 μM) was much lower than the total SCX4 concentration ([SCX4]<sub>T</sub> = 243 μM), B-SCX4 complex can be neglected in the mass balance equations.

$$[SCX4]_T = [SCX4] + [Met - SCX4] + [MetH^+ - SCX4] \quad (S15)$$

$$[Met]_T = [Met] + [MetH^+] + [Met - SCX4] + [MetH^+ - SCX4] \quad (S16)$$

(S15) and (S16) lead to

$$[Met]_T - [SCX4]_T = [Met] + [MetH^+] - [SCX4] \quad (S17)$$

Based on the definition of the equilibrium constant of the proton dissociation from MetH<sup>+</sup> ( $K_a$ ) in water we obtain

$$[Met] = \frac{[MetH^+]K_a}{[H^+]} \quad (S18)$$

(S17) and (S18) give

$$[Met]_T - [SCX4]_T = \left( \frac{K_a}{[H^+]} + 1 \right) [MetH^+] - [SCX4] \quad (S19)$$

We define the following constant

$$a = \frac{[H^+]}{K_a + [H^+]} \quad (S20)$$

Substitution of (S20) into (S19) provides

$$[MetH^+] = a([Met]_T - [SCX4]_T + [SCX4]) \quad (S21)$$

The definition of the equilibrium constant of Met-SCX4 and MetH<sup>+</sup>-SCX4 complex formation ( $K$  and  $K_p$ ) gives

$$[Met - SCX4] = K[SCX4][Met] \quad (S22)$$

$$[MetH^+ - SCX4] = K_p[SCX4][MetH^+] \quad (S23)$$

Substitution (S18) into (S22) gives

$$[Met - SCX4] = K[SCX4] \frac{[MetH^+]K_a}{[H^+]} \quad (S24)$$

Substitution (S23) and (S24) into (S15) results in

$$[SCX4]_T = [SCX4] + K[SCX4] \frac{[MetH^+]K_a}{[H^+]} + K_p[SCX4][MetH^+] \quad (S25)$$

Substitution (S21) into (S25) provides

$$[SCX4]_T = [SCX4] \left\{ 1 + \left( \frac{KK_a}{[H^+]} + K_p \right) a([Met]_T - [SCX4]_T + [SCX4]) \right\} \quad (S26)$$

The following quantity is defined:

$$b = \frac{KK_a}{[H^+]} + K_p \quad (S27)$$

(S27) and (S26) lead to

$$0 = -[SCX4]_T + [SCX4] \{ 1 + ba([Met]_T - [SCX4]_T) \} + b[SCX4]^2 \quad (S28)$$

The solution of the quadratic equation is

$$[SCX4] = \frac{-\{1+ba([Met]_T-[SCX4]_T)\} + \sqrt{\{1+ba([Met]_T-[SCX4]_T)\}^2 + 4b[SCX4]_T}}{2b} \quad (S29)$$

The total concentration of berberine fluorescent probe is the sum of its unbound and SCX4-complexed fraction.

$$[B]_T = [B] + [B - SCX4] \quad (S30)$$

Using (S30) and the definition of the binding constant of B–SCX4 complex formation ( $K_B = 3720 \text{ M}^{-1}$ ) [2], the following relationship is obtained:

$$\frac{[B-SCX4]}{[B]_T} = \frac{K_B[SCX4]}{1+K_B[SCX4]} \quad (S31)$$

The fluorescence intensity ( $I$ ) is proportional to the contribution arising from the fraction of the SCX4-complexed and free berberine:

$$I = \varphi_1 \frac{K_B[SCX4]}{1+K_B[SCX4]} + \varphi_2 \frac{1}{1+K_B[SCX4]} \quad (S32)$$

where  $\varphi_1$  and  $\varphi_2$  parameters are related to the efficiency of fluorescence emission for complexed and free berberine. The fluorescence quantum yields are 0.018 for B–SCX4 complex at pH 2 [2] and 0.00047 for free B in D<sub>2</sub>O [3]. Therefore, the following relationship is employed:

$$\varphi_2 = \frac{0.00047}{0.018} \varphi_1 = 0.026\varphi_1 \quad (\text{S33})$$

[SCX4] is calculated by (S29) and  $\varphi_1$ ,  $K$ , and  $K_p$  parameters were optimised using  $[\text{H}^+] = 0.01$  M and  $K_a = 4.365 \times 10^{-3}$  M. The latter quantity was obtained from the  $\text{p}K_a = 2.36$  value of  $\text{MetH}^+$  deprotonation in water determined by spectrophotometric titrations.

## References

- [1] S. Zhang, L. Grimm, Z. Miskolczy, L. Biczók, F. Biedermann, W.M. Nau, Binding affinities of cucurbit[n]urils towards cations, *Chem. Commun.* 55 (2019) 14131-14134.
- [2] M. Megyesi, L. Biczók, Considerable fluorescence enhancement upon supramolecular complex formation between berberine and p-sulfonated calixarenes, *Chem. Phys. Lett.* 424 (2006) 71-76.
- [3] J.J. Inbaraj, B.M. Kukielczak, P. Bilski, S.L. Sandvik, C.F. Chignell, Photochemistry and photocytotoxicity of alkaloids from goldenseal (*hydrastis canadensis* l.) 1. Berberine, *Chem. Res. Toxicol.* 14 (2001) 1529-1534.