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THIACALIX[4]ARENE-FUNCTIONALIZED VESICLES AS PHOSPHORESCENT INDICATORS FOR PYRIDOXINE DETECTION IN AQUEOUS SOLUTION.

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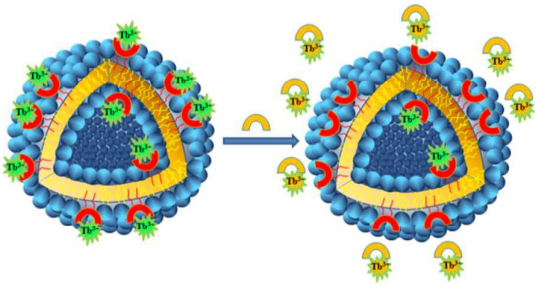
Amphiphilic tetracarboxylate derivatives of p-tert-butylthiacalix[4]arene were obtained by click reactions of the corresponding azido derivatives with acetylene dicarboxylic acid. Embedding of the amphiphilic tetraacids in DPPC vesicles was studied by DLS, AFM, turbidity technique and by probing with merocyanine 540. The obtained DPPC-calixarene vesicles are effective antenna for Tb(III) ion luminescence. It allows the use of DPPC-calixarene-Tb(III) vesicles for the selective detection of analytes with a higher affinity for Tb(III) due to cation removing from the calixarenes in the DPPC bilayer. It was found that pyridoxine hydrochloride can be selectively detected at the 7μM concentration in the presence of 100 fold excess of different biologically important molecules, like amino acids, adenosine phosphates, sugars, amines and ammonium salts.

Introduction

Multifunctional phospholipid vesicles have received considerable interest due to their use in molecular recognition, drug delivery, cell mimics, catalysis and many other applications[1,2]. Molecular recognition of membrane-embedded receptors plays a key role in different biological processes associated with the cell signalling or transmitting of signals across cell membranes[3-5]. A better understanding of these processes can provide improved insights into related processes in biology and can be exploited for the development of supramolecular receptors for analytical applications. Fluorescent signalling is preferred in analyte detection due to the achievable high sensitivity[6].

Recently, König et al. reported a novel strategy for the modular construction of luminescent sensors on the base of unilamellar vesicular membranes, which serve as self-assembled supporting matrix for amphiphilic metal complex receptors and fluorescent reporter dyes[7-9]. Amphiphilic fluorophores and binding sites are expected to cluster on the surface resulting in fluorescence quenching of the amphiphilic dyes. Binding of analyte molecules to the surface-accessible receptors induces a reorganization of the membrane components and leads to a change of emission intensity. In this paper, we report vesicle-supported luminescent sensors using the selective removal of interface-bound terbium ions by the analyte as a new detection principle (**Scheme 1**).



Scheme 1. Schematic representation of "stripping" principle of analyte detection based on calixarene-Tb(III) decorated vesicles.

Thiacalix[4]arene derivatives have a great potential as artificial amphiphilic ligands due to their unique properties: variety of stereoisomeric configurations, easy functionalization and preorganization effect[13-15]. It has been shown that some amphiphilic calixarene derivatives are able to form vesicles themselves [16-18].

Recently[19] we synthesized an universal "clickable" platform based on thiacalix[4]arene (Scheme 2). To achieve a wide variety of binding sites as well as amphiphilic properties, a thiacalix[4]arene's scaffold adopting *1,3-alternate* conformation was selected. Selective functionalization of the

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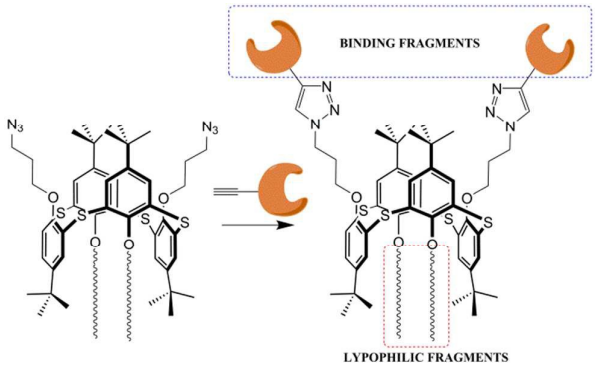
macrocycle lower rim by Mitsunobu reaction creates two molecular domains with quite different properties located on opposite sides of the macrocycles plane. One of them has lipophilic properties due to the introduction of long chain alkyl substituents. The other side of the macrocycle contains the binding sites that are easily introduced by click reactions.



Scheme 2. Schematic representation of "clickable" calixarene platform with the spatial separation of lipophilic and binding fragments.

We present here the embedding of amphiphilic tetra-carboxyl thiacalix[4]arene ligands with different lipophilicity into the DPPC bilayer, the study of their colloidal stability and photophysical properties upon binding of Tb-ion and the use of the modified vesicles as luminescent receptors for the recognition of some biologically important molecules.

Experimental

Materials and sample preparation

All reagents were purchased from either Acros or Sigma-Aldrich and used without further purification. DPPC was supplied by Avanti Polar Lipids (Alabaster, AL). Solvents were purified by standard methods[20]. Parent p-*tert*-butylthiacalix[4]arene was synthesized according to literature methods[21].

TLC was done using "Silufol UV 254" with UV lamp VL-6.LC (6W –254 nm tube). Elemental analysis was performed with automated CHNS/O analyzer «Perkin Elmer PE 2400 series 2». Microwave-assisted reactions were carried out in microwave reactor CEM MARS 5 with Glass Chem vials. NMR experiments were recorded at Bruker Avance 400 Nanobay with $CDCl_3$ (δ:H 7.26 ppm) as internal standard. MALDI mass-spectra were recorded at UltraFlex III TOF/TOF with PNA matrix, laser Nd:YAG, λ=355 nm.

Synthetic procedures

Compounds **2**, **3**[22] and **4**[19] were synthesized according to literature procedures.

General procedure for synthesis of compounds 5-7

Compounds 2-4 (2.02 mmol), diethyl azodicarboxylate (1.6 ml, 0.010 mol), triphenylphosphine (2.12 g, 8.09 mmol), and 3-bromo-1-propanol (1.12 g, 8.09 mmol) were dissolved in 30 ml of dry toluene under inert atmosphere. The reaction mixture was stirred at 70 Cº for 24 h, and then solvent was evaporated in *vacuo*. The crude products were washed twice with ethanol to give 5-7 as white powder.

5,11,17,23-Tetra-*tert*-butyl-25,27-dibutyloxy-26,28-di-3-bromopropyloxy-2,8,14,20-tetrathiacalix[4]arene **5**

^{1}H NMR (400 MHz, $CDCl_3$, 25°C) δ: 0.79 (t, 6H, Me, J 9.4 Hz), 0.88–0.92 (m, 4H, CH_2), 1.06–1.15 (m, 4H, CH_2), 1.28 (s, 18H, CMe_3), 1.31 (s, 18H, CMe_3), 1.56-1.61(m, 4H, CH_2), 3.06 (t, 4H, CH_2Br, J 6.32 Hz), 3.70-3.84 (m, 8H, CH_2), 4.00 (t, 4H, OCH_2, J 5.16 Hz), 7.32 (s, 4H, H_{Ar}), 7.33 (s, 4H, H_{Ar}).

Found (%): C, 60.28; H, 6.91; S, 11.88. $C_{54}H_{74}Br_2O_4S_4$.

Calculated (%): C, 60.32; H, 6.94; S, 11.93.

MALDI-TOF: m/z: 1074,28 $[M]^+$, 1297,27 $[M+Na]^+$. Yield 76%

5,11,17,23-Tetra-*tert*-butyl-25,27-dioctyloxy-26,28-di-3-bromopropyloxy-2,8,14,20-tetrathiacalix[4]arene **6**

^{1}H NMR (400 MHz, $CDCl_3$, 25°C) δ: 0.87 (t, 6H, Me, J 6.79 Hz), 1.16–1.29 (m, 26H, CH_2, CMe_3), 1.30 (s, 18H, CMe_3) ,1.56-1.66 (m, 4H, CH_2), 3.05 (t, 4H, CH_2Br, J 7.00 Hz), 3.72-3.86 (m, 8H, CH_2), 4.01 (t, 4H, OCH_2, J 6.60 Hz), 7.31 (s, 4H, H_{Ar}), 7.33 (s, 4H, H_{Ar}).

Found (%): C, 62.70; H, 7.60; S, 10.78. $C_{62}H_{90}Br_2O_4S_4$.

Calculated (%): C, 62.71; H, 7.64; S, 10.80.

MALDI-TOF: m/z: 1186,41 $[M]^+$, 1209,40 $[M+Na]^+$. Yield 87 %

5,11,17,23-Tetra-*tert*-butyl-25,27-ditetradecyloxy-26,28-di-3-bromopropyloxy-2,8,14,20-tetrathiacalix[4]arene **7**.

Yield 85%, ^{1}H NMR spectra is in agreement with literature[19]

General procedure for synthesis of compounds 8-10

Compounds **5-7** (0.39 mmol), sodium azide (0.26 g, 3.88 mmol) and 30 ml of dry DMF were placed in a glass vial (Glass Chem vial). The reaction mixture was heated to 140 °C in a microwave oven CEM Mars 5 (400 W) for 1.5 hours. Then it was treated with 10 ml of $CHCl_3$, washed three times with distilled water, dried over $MgSO_4$. After concentration in *vacuo* and precipitation by ethanol the target compounds **8-10** were collected by filtration as white solids.

5,11,17,23-Tetra-*tert*-butyl-25,27-dibutyloxy-26,28-di-3-azidopropyloxy-2,8,14,20-tetrathiacalix[4]arene **8**

^{1}H NMR (400 MHz, $CDCl_3$, 25°C) δ: 0.79 (t, 6H, Me, J 7.32 Hz), 0.89–0.97 (m, 4H, CH_2,), 1.06-1.14 (m, 4H, CH_2), 1.28 (s, 18H, CMe_3) , 1.30 (s, 18H, CMe_3) , 1.32-1.38 (m, 4H, CH_2) 2.95 (t, 4H, CH_2N_3, J 7.20 Hz), 3.75-3.86 (m, 8H, CH_2), 3.96 (t, 4H, OCH_2, J 6.90 Hz), 7.31 (s, 4H, H_{Ar}), 7.32 (s, 4H, H_{Ar}).

IR (KBr, 20 ˚C): ν (N_3)= 2096 sm^{-1}.

Found (%): C, 64.87; H, 7.45; S, 12.78. $C_{54}H_{74}N_6O_4S_4$.

Calculated (%): C, 64.89; H, 7.46; S, 12.83.

MALDI-TOF: m/z: 998,47 $[M]^+$, 1021,46 $[M+Na]^+$. Yield 70 %

5,11,17,23-Tetra-*tert*-butyl-25,27-dioctyloxy-26,28-di-3-azidopropyloxy-2,8,14,20-tetrathiacalix[4]arene **9**.

^{1}H NMR (400 MHz, $CDCl_3$, 25℃) δ: 0.79 (t, 6H, Me, J 7.32 Hz), 1.05–1.18 (m, 24H, CH_2), 1.28 (s, 18H, CMe_3) , 1.30 (s, 18H, CMe_3) , 2.96 (t, 4H, CH_2N_3, J 7.11 Hz), 3.76-3.87 (m, 8H, CH_2), 3.96 (t, 4H, OCH_2, J 6.91 Hz), 7.31 (s, 4H, H_{Ar}), 7.33 (s, 4H, H_{Ar}).

IR (KBr, 20 ˚C): ν (N_3)= 2096 sm^{-1}.

Found (%): C, 66.97; H, 8.14; S, 11.47. $C_{62}H_{90}N_6O_4S_4$.

Calculated (%): C, 66.99; H, 8.16; S, 11.54.

MALDI-TOF: m/z: 1110,59 $[M]^+$, 1133,58 $[M+Na]^+$. Yield 70 %

5,11,17,23-Tetra-*tert*-butyl-25,27-ditetradecyloxy-26,28-di-3-azidopropyloxy-2,8,14,20-tetrathiacalix[4]arene **10**.

Yield 62%, ^{1}H NMR spectra is in agreement with literature[19]

General procedure for synthesis of compounds 11-13

Compounds **8-10** (1 g, 0.08 mmol), acetylene dicarboxylic acid (0.88 g, 0.78 mmol) were dissolved in 20 ml of dry acetone under inert atmosphere. This reaction mixture was stirred at 60 Cº for 8 h, and then solvent was evaporated in *vacuo*. The crude product was washed several times with ethanol. The compounds **11-13** were obtained after drying in *vacuo* as white powder.

5,11,17,23-Tetra-*tert*-butyl-25,27-dibutyloxy-26,28-bis[3-(4,5-dicarboxy-1,2,3-triazol-1-yl)propyloxy]-2,8,14,20-tetrathiacalix[4]arene **11**.

^{1}H NMR (400 MHz, $CDCl_3$, 25℃) δ: 0.84 (t, 6H, Me, J 7.16 Hz), 0.87–1.21 (m, 24H, CH_2, CMe_3), 1.24-1.41 (m, 20H, CH_2, CMe_3) , 1.82-2.02 (m, 4H,CH_2), 3.79 (t, 8H, OCH_2, J 8.08 Hz), 4.14-4.31 (m, 4H OCH_2), 4.62-4.75 (m, 4H, $TrzCH_2$), 7.32 (s, 4H, H_{Ar}), 7.42 (s, 4H, H_{Ar}).

Found (%): C, 60.53; H, 6.51; N, 6.57; S, 10.21. $C_{62}H_{78}N_6O_{12}S_4$. Calculated (%): C, 60.56; H, 6.56; N, 6.83; S, 10.43.

MALDI-TOF: m/z: 1226,47 $[M]^+$, 1249,47 $[M+Na]^+$. Yield 74 %.

5,11,17,23-Tetra-*tert*-butyl-25,27-dioctyloxy-26,28- bis[3-(4,5-dicarboxy-1,2,3-triazol-1-yl) propoxy]-]-2,8,14,20-tetrathiacalix[4]arene **12**

^{1}H NMR (400 MHz, $CDCl_3$, 25℃) δ: 0.88 (t, 6H, Me, J 7.16 Hz), 0.93–1.31 (m, 60H, CH_2, CMe_3), 1.61-1.97 (m, 4H,CH_2), 3,76 (t, 8H, OCH_2, J 8.08 Hz), 3,96-4,28 (m, 4H, OCH_2), 4.48-4.63 (m, 4H, $TrzCH_2$), 7.28 (s, 4H, H_{Ar}), 7.36 (s, 4H, H_{Ar}).

Found (%): C, 62.53; H, 8.07; N, 5.02; S, 8.68. $C_{70}H_{94}N_6O_{12}S_4$. Calculated (%): C, 62.66; H, 7.21; N, 6.26; O 14.31, S, 9.56.

MALDI-TOF: m/z: 1338,60 $[M]^+$, 1361,60 $[M+Na]^+$. Yield 70 %.

5,11,17,23-Tetra-*tert*-butyl-25,27-ditetradecyloxy-26,28- bis [3-(4,5-dicarboxy-1,2,3-triazol-1-yl) propyloxy]-2,8,14,20-tetrathiacalix[4]arene **13**.

Yield 61 %, ^{1}H NMR spectra is in agreement with literature[19].

Vesicles preparation

DPPC lipid films were formed from chloroform solutions, dried at 85°C, and left under reduced pressure for a minimum of 2 h to remove all traces of organic solvent. Turbid MLV-suspensions were prepared by adding 20mM TRIS buffer, 150mM NaCl, pH 7.4 to the films and heating for 60 min at 60°C (above the phase transition temperature). SUV-dispersions were obtained by extrusion through 100 nm-pore size polycarbonate membranes with a Mini-Extruder from Avanti. Buffer was prepared using Milli-Q water. Concentration of DPPC stock dispersion was typically 0.7 mM. For binary system 10 mol % (or less in some experiments) of calixarene were added to the chloroform solutions during preparation the lipid films.

Spectrophotometric measurements

UV–VIS absorption spectra were recorded using a spectrophotometer LAMBDA 35 (Perkin Elmer production, USA) using 10 mm quarts cuvettes.

Turbidity measurements.

The dependence of optical density at wavelengths of 400 nm on temperature was recorded using LAMBDA 35 spectrophotometer equipped with Huber thermostat. The temperature was varied in the range from 25 to 55°C, heating rate was 0.1°C /min. The obtained plots were mathematically treated using Van´t-Hoff's two-state model[23]. In accordance with this model the main temperature of phase transition of the lipid bilayer corresponds to the inflection point of the respective turbidity plot.

Dynamic light scattering (DLS) measurements.

Dynamic light scattering measurements were performed by Malvern Zetasizer equipment at 25 °C using 1 cm disposable polystyrene fluorescence cuvettes (VWR). 4 MW He–Ne laser with 633 nm wavelength acted as radiation source. The analysis of the obtained signal was performed on the basis of frequency and phase analysis of scattered light using software supplied with the device.

Fluorescence spectroscopy.

Emission spectra of Merocyanine 540 (0.007 mM) in the absence and presence of vesicles were recorded with a Fluorolog FL-221 spectrofluorimeter (HORIBA Jobin Yvon) in the range of 540 to 660 nm and excitation wavelength 530 nm with 3 nm slit. In the case of Tb (III) in the range of 450 to 650 nm and excitation wavelength 330 nm with 2 nm slit. All measurements were carried out using 10 mm quarts cuvettes.

Atomic-force microscopy

AFM was made on Multimode V (Veeco, USA) in tapping mode using cantilever RTESP (Veeco), (phosphorus doped Si, k= 20-80N/m, f=247-306 kHz.). Mica was coated by 20 µl of vesicles solution (diluted up to 0.7 µM) and then dried at 80 °C for 3 hours.

Results and Discussion

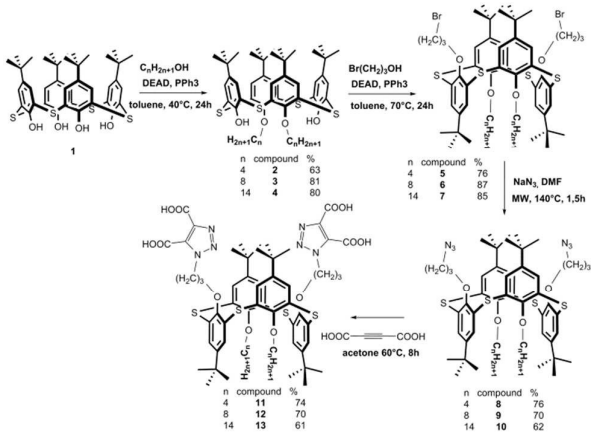
There are only few studies devoted to the incorporation of calixarene derivatives into phospholipid bilayers. Schrader et al. constructed a chromatic vesicle by the incorporation of amphiphilic phosphonates and ammonium calixarenes into phospholipids in the presence of polydiacetylene, which could be used to specifically recognize proteins through electrostatic interactions[24]. Ungaro et al. reported that the DNA condensation properties of amphiphilic guanidinium calixarene and the corresponding transfection efficiency increases significantly upon co-assemble with phospholipid[25]. Wang et al. designed and fabricated multifunctional liposomes consisting of phospholipids and two kinds of amphiphilic *p*-sulfonatocalix[4]arenes as operational targeted drug delivery carriers[26]. The application of calixarenes derivatives in membrane transport of acetylcholine[27] and chloride ions[28,29] across a phospholipid bilayer has been described. Miscibility of calixarene[30, 31] and calix[4]resorcinarene[32] derivatives with natural phospholipid monolayers was also studied.

It should be noted that in all presented results classical calix[4]arene derivatives in the *cone* configuration were used. However, the *1,3-alternate* configuration has several advantages: flexibility in the comparison with *cone*, that allows to adjust to the molecular geometry of substrates as well as a good synthetic availability by the selective lower rim functionalization. Thus, for the first time we explore the embedding of *1,3-alternate* thiacalix[4]arene derivatives into phospholipid vesicles.

One of the objectives of the study is to investigate the influence of hydrophobicity of the thiacalixarene derivatives on the embedding onto DPPC phospholipid vesicles; therefore, compounds **11-13** with different alkyl chain length were synthesized.

Synthesis of thiacalix[4]arene tetraacids.

Disubstituted thiacalix[4]arene derivatives **2-4** (Scheme 3) were synthesized from parent macrocycle 1 using Mitsunobu conditions reaction in good yields.



Scheme 3. General synthetic procedure for the synthesis of calixarene tetra-acids **11-13**

The corresponding di-substituted thiacalix[4]arene derivatives **2-4** were treated with 3-bromo-1-propanol in Mitsunobu conditions to give tetra-substituted derivatives **5-7**. Two singlets of thiacalixarene aromatic protons in ^{1}H NMR and cross-peaks between aromatic and methylene protons of alkyl and bromoalkyl fragments in NOESY spectra clearly indicate that compounds **5-7** adopt an *1,3-alternate* configuration. Tetra-substituted derivatives **5-7** were reacted with sodium azide to give azides 8-10 by nucleophilic substitution in a microwave reactor. The structure of compounds **8-10** was determined by NMR, IR and MALDI-TOF spectrometry. The band of the azido groups at 2096 cm^{-1} in the IR spectrum and the corresponding molecular ion peak indicate on the formation of a bis-azido derivative. The *1,3-alternate* conformation of **8-10** was confirmed by 1D and 2D NMR experiments as it was done for precursors **5-7**. Thiacalixarene tetraacids **11-12** were synthesized from azides **8-9** by azide-alkyne cycloaddition with acetylene dicarboxylic acid. Compound **13** was synthesized by us previously[19] in similar manner.

Functionalization of DPPC vesicles with calixarene tetraacids

Calixarenes **11-13** are water insoluble and didn't form any stable micelles or vesicles according to DLS data. Therefore, their embedding in phospholipid vesicles is the path of its solubilization in aqueous solutions. The vesicles CA-4_DPPC, CA-8_DPPC and CA-14_DPPC modified by synthesized amphiphilic calixarenes **11-13**, respectively, were obtained by the well-established film-hydration method[33] from a mixture of commercially available synthetic phospholipid 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and calixarene chloroform solutions (from 0.1 to 10 mol% with respect to DPPC).

The resulting multilamellar vesicles (MLVs) were homogenized by extrusion at temperature higher that T_m (41.5^{o}C) of DPPC to yield small unilamellar vesicles (SUVs). According to dynamic light scattering data (Table 1) all calixarene-modified vesicles have sizes within 100-150 nm and there is no dependence of the vesicle size

on the calixarene amounts embedded. Thus, we can assume that the addition of even larger concentrations of up to 10% of calixarene does not destroy the phospholipid vesicles.

Table 1. DLS-data for the vesicles containing amphiphilic ligands **11-13**[*]

Calixarene	D, nm	PDI	D, nm	PDI	D, nm	PDI
mol%	**CA4_DPPC**		**CA8_DPPC**		**CA14_DPPC**	
0%			112±3 (PDI 0.21)			
0.1%	101±2	0.236	111±1	0.313	113±4	0.215
0.5%	130±1	0.144	140±2	0.253	115±4	0.243
0.7%	109±2	0.168	107±4	0.234	135±5	0.266
1%	109±2	0.218	101±2	0.291	124±6	0.425
3%	119±1	0.130	112±1	0.304	116±4	0.386
5%	131±3	0.120	105±2	0.289	115±2	0.282
7%	110±1	0.124	112±1	0.260	133±2	0.211
10%	122±2	0.161	121±1	0.326	111±2	0.237

[*]C(DPPC) = 0,7 mM; C(TRIS) = 20mM; C(NaCl) = 150 mM; pH = 7,4

Embedding of amphiphilic ligands into lipid membranes can be monitored through changes in the temperature of the main phase transition from the liquid crystalline phase to the gel phase or melting point (T_m) of membranes. The transition is accompanied by changes in the lipid chain orientation (ordered or disordered) and lattice order (solid or liquid). To determine T_m turbidity measurements are often used[23]. Calculated T_m values using a two state transition thermodynamic approach are presented in Table 2.

Table 2. Temperature of the main phase transition T_m of CA-4_DPPC, CA-8_DPPC and CA-14_DPPC vesicles determined by turbidity measurements[*]

Calixarene	T_m, °C		
mol%	**CA4_DPPC**	**CA8_DPPC**	**CA14_DPPC**
0%		41.50±0.01	
1%	40.60±0.01	40.90±0.02	40.73±0.03

[*]C(DPPC) = 0.7 mM; C(TRIS) = 20 mM; C(NaCl) = 150 mM; pH = 7.4

According to the data obtained (Table 2) 1% of embedded calixarene with tetra, octa and tetradecyl alkyl substituents' results in a small decrease of the main phase transition temperature of DPPC. However, the increase of the calixarene concentration to 10 mol % leads to a significant temperature decrease especially in the case of octa- and tetradecyl alkyl derivatives. It is known[23,34,35] that the T_m decrease of DPPC even for tenths of a degree suggests that the neighboring phospholipids are packed less tightly due to the incorporation of amphiphilic molecules into bilayer and is often used for the creation of temperature-dependent drug release liposome containers[36].

The main driving force to transfer the surfactants from bulk solution to lipid membrane is hydrophobic interactions[37]. Respectively, our results can be well explained in respect that long alkyl chain derivatives have stronger hydrophobic interactions with lipophilic part of the lipid membrane. Such behavior is characteristic for other anionic, cationic and non-ionic surfactants: alkyl sulphates[37], alkyltrimethylammonium bromides[37] , long alkyl-chain alcohols[38] that also depress the DPPC main phase transition temperature with increase of the alkyl chain length.

The formation of the CA14_DPPC vesicles was confirmed by AFM observations, as shown in Fig. 1. The samples were prepared by dropping a small amount of aqueous solution onto a mica surface and evaporating the solvent. It can be seen that nanoparticles with 75 nm diameter and 26 nm height were formed, which survive the sample drying process. The lack of full flattening of vesicles into bilayer is in good agreement with literature data for DPPC vesicles[39,40] and vesicles of DPPC with embedded cholesterol[41] and β-carotene[42].

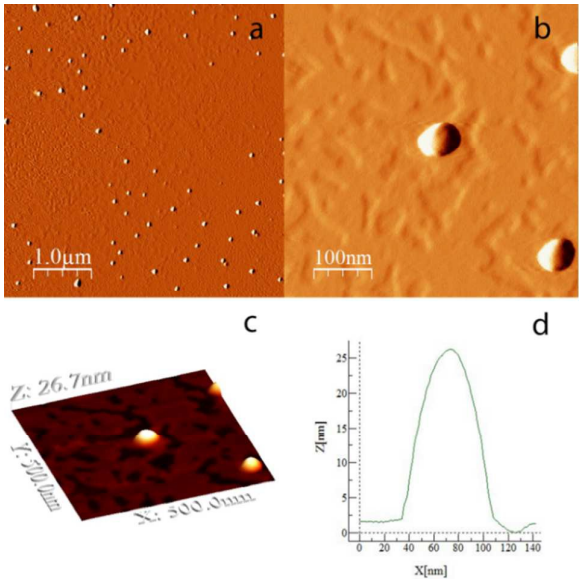


Fig. 1 AFM topview with 5 μm scan range (**a**), 0.5μm scan range (**b**); three-dimensional view (**c**) and size profile (**d**) of CA14_DPPC vesicles with 10% mol calixarene load.

Thus, calixarene tetraacids **11-13** incorporate onto DPPC vesicles and change the T_m of DPPC. The T_m decreases in the series of CA4_, CA8_, CA14_DPPC, indicating that last amphiphile has the strongest interactions with the DPPC bilayer.

MC-540 probing of calixarene - functionalized vesicles

Further evidence of calixarene incorporation into the DPPC vesicle was obtained by probing with a negative charged merocyanine dye MC 540 (5-[(sulfonyl-2(3H)-benzoxazoylidine)-2-butenyldene]-l,2-dibutyl-2-thiobarbarbituric acid)[43]. Its optical properties depend strongly on the environment. The dye is particularly sensitive to changes of polarity: its absorbance and fluorescence emission in water solution is lower than in hydrocarbon solvents[44]. Those properties, combined with the ability to interact with lipid bilayers and synthesized calixarenes **11-13** ($COOH...O^-$ hydrogen bonding) make this dye very promising for the investigation of calixarene embedded vesicles.

Dye MC 540 in DPPC liposomes shows a double-band absorption spectrum peaking at 568 nm (a monomer peak) and at 532 nm (a dimer peak) (**Fig. 2 a**)[46]. The addition of calixarene tetra acids **11-13** leads to an increase of the monomeric form of MC-540. The ratio of absorption at 568/532 nm in the case of MC-540 in non-functionalized DPPC vesicles is 1.2 and changes to 1.5-1.6 in vesicles with embedded calixarene tetra acids. We assume that the calixarene associates with MC-540 in the DPPC bilayer thereby preventing the formation of dimeric or other aggregated forms of MC-540. According to the absorption data the amount of the monomeric form remains constant in the series of vesicles containing C4-C8-C14 amphiphiles (**Fig. 2 a**).

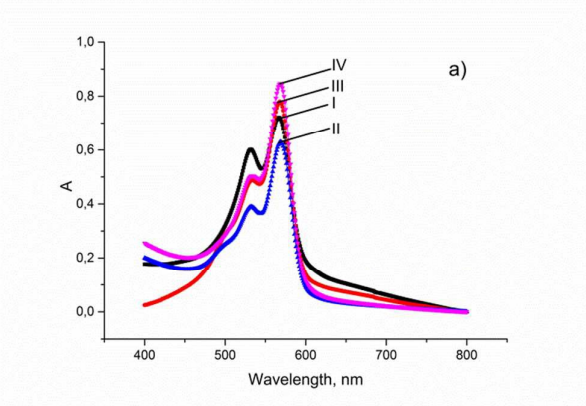


Fig. 2 UV-VIS (**a**) and emission (**b**) spectra for vesicles: MC540-DPPC (I), MC540-CA4-DPPC (II), MC540-CA8-DPPC (III), MC540-CA14-DPPC (IV). Experimental conditions: C(CALIX) = 0,07 mM; C(DPPC) = 0,7 mM; C(MC-540) = 0,007 mM; C(TRIS) = 20mM; C(NaCl) = 150 mM; pH = 7.4, t=25°C.

The incorporation of calixarenes onto MC540-DPPC vesicles leads to a large increase in their fluorescence intensity at 589 nm that corresponds to the MC-540 monomeric form emission (**Fig. 2 b**)[45-47]. There are many factors, such as lipid packing in bilayers, temperature, sample concentration, polarity of the environment, etc., that effect the emission characteristics of MC 540. In the case of lipid vesicles in the gel state ($T<T_m$), the difference in the fluorescence intensities is only due to the difference in the partition characteristics of the dye into the lipid bilayer. The dye is located in the interfacial region of membranes[48,49]. In the presence of the amphiphilic receptor, the dye interacts with them, forming aggregates that are located inside of the membranes hydrophobic region. As a result, the fluorescence intensity of dye increases.

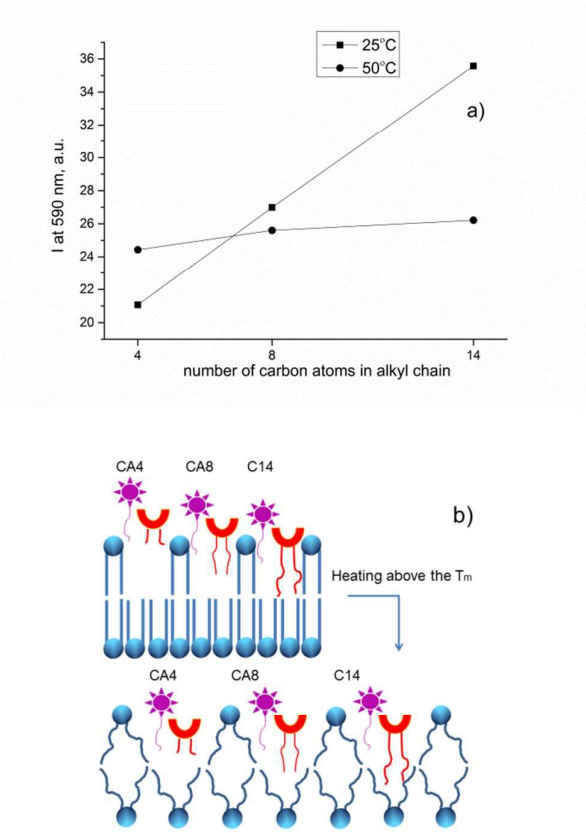


Fig. 3 (**a**) Emission intensity (a.u.) at 590 nm of MC-540 embedded in CA14-DPPC vesicles at 25 and 50°C *vs* number of carbon atoms in calixarene alkyl chains; (**b**) schematic representation of calixarene-MC540 associates distribution in the bilayer below and above the normal transition temperature of pure DPPC T_m.

This phenomenon is in agreement with data on calixarene lipophilicity effects. The emission intensity of MC-540 with calixarene **11** embedded into DPPC vesicles increases twofold in comparison with non-functionalized DPPC vesicles and MC540, in the case of **12** the increase is fivefold and for **13** a ninefold increase is observed (**Fig. 2b**). Moreover, almost a linear dependence of the MC-540 fluorescence intensity on the number of carbon atoms in the calixarene alkyl chains was observed (**Fig. 3a**). More lipophilic calixarenes may allow for a deeper penetration into the bilayer.The fluorescence of MC 540 also depends on the lipid packing (gel- or fluid-phases) in the membrane. A major change occurs around the main phase transition of phosphatidylcholine membranes T_m (41.5°C for DPPC). At the temperature above the T_m the membrane expands laterally, fatty acid side-chain motion increases, while the long-range ordering and semi-crystalline close packing of headgroups disappears (**Fig. 3b**)[50]. Vesicles containing calixarene **13** demonstrate a similar behavior. The fluorescence intensity of

MC540-CA14-DPPC vesicles is enhanced more than three times in the disordered or fluid state (at 50°C) due to the elevated partitioning of the probe into the lipid phase (deeper penetration into the membrane). For this reason, there is practically no effect of the hydrophobic properties of amphiphilic calixarene on the fluorescence at 50 °C (**Fig. 3a**).

The investigation of the absorption and emission spectra of MC540 in calixarene-embedded vesicles provides further evidence of the calixarene incorporation into the DPPC vesicles. An increase of the alkyl chain length leads presumably to deeper embedding of the calixarene-MC540 associates into the DPPC lipid layer and therefore increasing emission intensity in the gel- phase[50], while in the fluid-phase all calixarene-MC540 aggregates are embedded similar (**Fig. 3b**).

Tb(III)-calixarene modified vesicles

For further post-modification of the calixarene-embedded vesicles by Tb(III) ions the tetradecyl calixarene derivative **13** was chosen, because it has the strongest interaction with DPPC vesicles. CA14-DPPC vesicles form luminescent complexes with Tb(III) with a 30-fold emission increase in comparison with the luminescence intensity of non-functionalized DPPC vesicles with Tb(III) (Fig. 4).

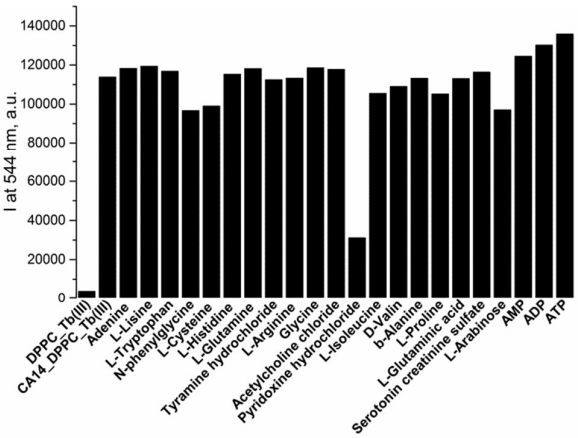
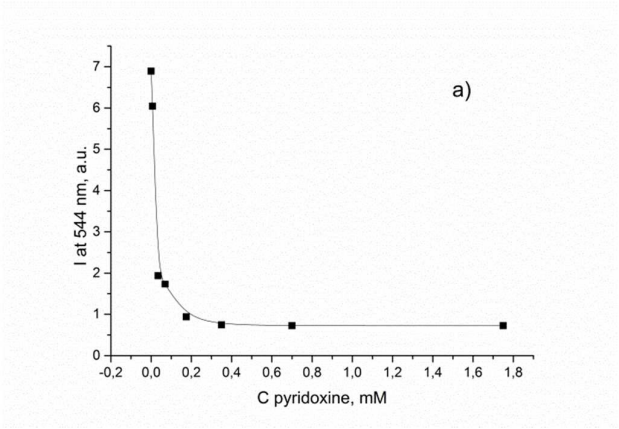


Fig. 4 Emission intensity of DPPC-CA14-Tb(III) vesicles at 544 nm after addition of organic analyte molecules. C [CA14] = 0,07 mM; C[DPPC] = 0,7 mM; C[Tb] = 0,07 mM; C[TRIS] = 20mM; C[NaCl] = 150 mM; pH = 7,4, C[org. guest] = 0.35 mM.

Thus, the triazole ring with attached carboxyl groups is a quite effective sensitizing antenna for Tb(III). The competitive binding of Tb(III)by organic guest molecules forming non-fluorescent complexes can be used for analyte sensing. The investigation of the luminescent response among a series of different biologically important molecules, like amino acids, adenosine phosphates, sugars, amines and ammonium salts, showed that CA14-DPPC-Tb(III) modified vesicles preferentially change their emission properties in the presence of pyridoxine. For the analysis of biological samples it is of importance that other molecules have little or no influence (within 10%) on the Tb(III) luminescence.



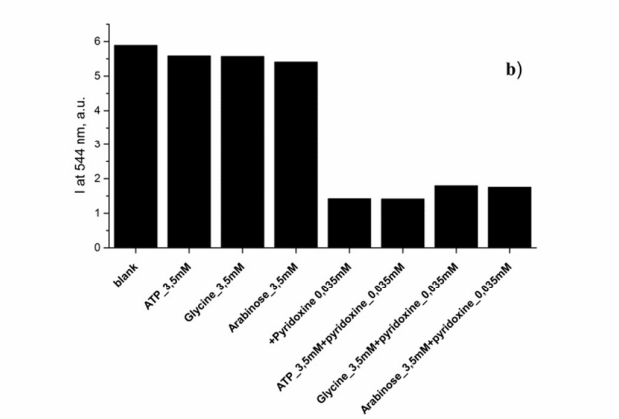


Fig. 5 (**a**) Emission intensity of DPPC-CA14-Tb(III) vesicles at 544 nm vs pyridoxine HCl concentration. C[CA14] = 0,07 mM; C[DPPC] = 0,7 mM; C[Tb] = 0,7 mM; C[TRIS] = 20mM; C[NaCl] = 150 mM; pH = 7,4, C[pyridoxine] = 0,007-7 mM. (**b**) Emission intensity of DPPC-CA14-Tb(III) vesicles at 544 nm before/after addition of different guest mixtures, C[CA14] = 0,07 mM; C[DPPC] = 0,7 mM; C[Tb] = 0,7 mM; C[TRIS] = 20mM; C[NaCl] = 150 mM; pH = 7,4.

It's known that pyridoxine form complexes with lanthanides by coordination of the phenolic oxygen atom[51-53]. Pyridoxine exhibits four interchangeable ionic forms at different pH, i.e. at pH < 5 it occurs as cationic form, at pH 6.8 as neutral form and dipolar ion, and at pH > 8 as anionic form[54]. At the experimental conditions (pH 7.4) there is a sufficient percentage of the anionic form present that can effectively bind the Tb(III) ion (Fig 4a). The ability of pyridoxine to sensitize Tb(III) ion emission is very low in comparison with CA-14.

Titration of CA14-DPPC-Tb(III) with pyridoxine (**Fig. 5, a**) quenches the luminescence of Tb(III) in a concentration depended manner. The response is significant even at low pyridoxine concentration of 7µM, which is useful for analytical applications. It is important to note that the presence of 100 fold excess of another analytes (sugar, amino acid or nucleoside triphosphate) does not affect to the response of the analytical determination of pyridoxine (**Fig. 5, b**).

During the last decades, there has been an increasing interest for the vitamins determination, in particularly, vitamin B_6 [55-57]. It is an essential precursor of active 3-hydroxy-2-methylpyridine derivatives such as pyridoxal and pyridoxamine phosphate coenzymes of a wide variety of enzymes of intermediary metabolism[58]. Vitamin B_6 deficiency leads to a number of various clinical signs and symptoms like eczema and seborrhea dermatitis, cheilosis, glossitis, angular stomatitis, anemia, central nervous system changes[59]. For these reasons, the detection of low vitamin B6 concentrations in plasma (typically nanomoles per liter) is of interest[60].

Widely used methods for vitamins B_6 analysis in biological fluids are based on either time-consuming microbiological, enzyme, radioimmunoassays and chemical assays or HPLC methods including cation exchange and reversed phase separations with pre- or post-column derivatization and fluorimetric detection[61-63]. Recently, a simple, sensitive but not selective method for the determination of vitamin B6 was developed based on the fluorescence quenching of l-cysteine capped CdS/ZnS quantum dots[64]. Synthesized vesicles decorated by Tb(III) ions discover a simple analytical way based on fluorimetric measurements for selective vitamin B_6 detection in biological samples. The application of the well-developed extraction and microextraction technics (liquid-liquid extraction, micro-solid phase extraction, dispersive liquid-liquid micro extraction) for vitamin preconcentration may allow to achieve a nanomolar detection limit[65].

Conclusions

Embedding of tetracarboxylic thiacalixarene derivatives with different alkyl chains in DPPC vesicles leads to a considerable decrease of the main phase transition temperature indicating the embedding of the amphiphilic calixarenes into bilayer. The photophysical properties of the solvatochromic dye MC-540 change dramatically upon binding to DPPC-calixarene vesicles due to the formation of MC-calixarene aggregates on the vesicle surface. The DPPC-calixarene vesicles act as luminescent indicators using the “stripping” of the Tb(III), ions from the calixarenes in the DPPC bilayer by analyte coordination. It was found that pyridoxine hydrochloride selectively removes the Tb(III) ions from the calixarene forming a weakly luminescent complex. Vitamin B_6 can be selectively detected at the 7μM concentration in the presence of 100 fold excess of biologically important molecules, such as amino acids, adenosine phosphates, sugars, amines and ammonium salts.

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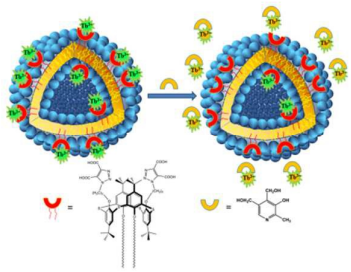
Notes and references

1 K. Ariga, H. Ito, J.P. Hill and H. Tsukube, *Chem. Soc. Rev.*, 2012, **41**, 5800 – 5835.
2 J. Voskuhl and B.J. Ravoo, *Chem. Soc. Rev*., 2009, **38**, 495–505.
3 B. Alberts, A. Johnson and J. Lewis, Molecular Biology of the Cell, Garland Science, New York, 2002.
4 M. Luckey, Membrane Structural Biology, Cambridge University Press, Cambridge, 2008.
5 S. Singer, *Science*, 1992, **255**, 1671 – 1677.
6 R. Martínez-Máñez and F. Sancenón, *Chem. Rev*., 2003, **103**, 4419–4476.
7 A. Müller and B. König, *Org. Biomol. Chem*., 2015, **13**, 1690–1699.
8 B. Gruber, S. Stadlbauer, K. Woinaroschy and B. König, *Org. Biomol. Chem*., 2010, **8**, 3704–3714.
9 S. Banerjee, M. Bhuyan and B. König, *Chem. Commun.*, 2013, **49**, 5681–5683.
10 S.V. Eliseeva and J.-C.G. Bünli, *Chem. Soc. Rev*., 2010, **39** 189–227.
11 O. Laporte and W.F. Meggers, *J. Opt. Soc. Am*., 1925, **11**, 459–460.
12 J. Lakowicz, Principles of Fluorescence Spectroscopy. Quenching of Fluorescence, Kluwer Academic, New York, 1999.
13 Z. Asfari, V. Bohmer, J. Harrowfield and J. Vicens, Calixarenes 2001, Kluver, Netherland, 2001.
14 N. Morohashi, F. Narumi, N. Iki, T. Hattori and S. Miyano, *Chem. Rev*., 2006, **106**, 5291–5316.
15 A. I. Konovalov and I.S. Antipin, *Mendeleev Commun*., 2008, **18**, 229–237.
16 S. Houmadi, D. Coquière, L. Legrand, M.C. Fauré, M. Goldmann, O. Reinaud and S. Rémita, *Langmuir*, 2007, **23**, 4849–4855.
17 M. Strobel, K. Kita-Tokarczyk, A. Taubert, C. Vebert, P.A. Heiney, M. Chami and W. Meier, *Adv. Funct. Mater.*, 2006, **16**, 252–259.
18 Y. Tanaka, M. Miyachi and Y. Kobuke, *Angew. Chem. Int. Ed.*, 1999, **38**, 504–506.
19 V.A. Burilov, R.I. Nugmanov, R.R. Ibragimova, S.E. Solovieva and I.S. Antipin., *Mendeleev Commun*., 2015, **25**, 177–179.
20 W.L.F. Armarego and C.L.L. Chai, Purification of Laboratory Chemicals, Elsevier, New York, 2009.
21 N. Iki, C. Kabuto, T. Fukushima, H. Kumagai, H. Takeya, S. Miyanari, T. Miyashi and S. Miyano, *Tetrahedron*, 2000, **56**, 1437–1443.
22 I. Bitter and V. Csokai, *Tetrahedron Lett*., 2003, **44**, 2261–2265.
23 F. Eker, H.O. Durmus, B.G. Akinoglu, F. Severcan, *J. Mol. Struct*., 1999, **482–483**, 693–697.
24 S. Kolusheva, R. Zadmard, T. Schrader and R. Jelinek, *J. Am. Chem. Soc*., 2006, **128**, 13592−13598.
25 F. Sansone, M. Dudič, G. Donofrio, C. Rivetti, L. Baldini, A. Casnati, S. Cellai and R. Ungaro, *J. Am. Chem. Soc*., 2006, **128**, 14528−14536.
26 Y.-X. Wang, Y.-M. Zhang, Y.-L Wang and Y. Liu, *Chem. Mater*., 2015, **27**, 2848−2854.
27 T. Jin, *Chem. Commun.,* 1999, **1**, 2129–2130.
28 S. Licen, V. Bagnacani, L. Baldini, A. Casnati, F. Sansone, M. Giannetto, P. Pengo and P. Tecilla, *Supramol. Chem.,* 2013, **25**, 631–640.
29 O.Y. Shatursky, L.A. Kasatkina, R.V. Rodik, S.O. Cherenok, A.A. Shkrabak, T.O. Veklich, T.A. Borisova, S.O. Kosterin and V.I. Kalchenko, *Org. Biomol. Chem.*, 2014, **12**, 9811–9821.
30 B. Korchowiec, A. Ben Salem, Y. Corvis, J.-B.R. de Vains, J. Korchowiec and E. Rogalska, *J. Phys. Chem. B*, 2007, **111**, 13231–13242.
31 P. Shahgaldian and A. W. Coleman, *Langmuir*, 2003, **19**, 5261–5265.
32 P. Vitovič, D.P. Nikolelis and T. Hianik, *Biochim. Biophys. Acta, Biomembr.*, 2006, **1758**, 1852–1861.

33 J. Lasch, V. Weissig and M. Brandl, Preparation of Liposomes, ed. V. Torchilin and V.Weissig, Oxford University Press, 2, 2003, 3–29.
34 M. Pantusa, R. Bartucci, D. Marsh and L. Sportelli, *Biochim. Biophys. Acta, Biomembr.*, 2003, **1614**, 165–170.
35 B. Różycka-Roszak, B. Jurczak and K.A. Wilk, *Thermochim. Acta*, 2007, **453**, 27–30.
36 N. Pippa, A. Meristoudi, S. Pispas and C. Demetzos, *Int. J. Pharm.*, 2015, **485**, 374–382.
37 T. Inoue, K. Miyakawa and R. Shimozawa, *Chem. Phys. Lipids*, 1986, **42**, 261–270.
38 L. Lobbecke and G. Cevc, *Biochim. Biophys. Acta*, 1995, **1237**, 59–69.
39 J. Zhou, D. Liang and S. Contera, *Nanoscale*, 2015, **7**, 17102-17108.
40 S. J. Attwood, Y. Choi and Z. Leonenko, *Int. J. Mol. Sci.* 2013, **14**, 3514-3539.
41 S. Kumar, J.H. Hoh, *Langmuir,* 2000, **16**, 9936-9940.
42 D. Augustyńska, M. Jemioła-Rzemińska, K. Burda and K. Strzałka, *Acta Biochim. Pol.*, 2012, **59**, 125–128.
43 F.M. Hamer, The Cyanine Dyes and Related Compounds, John Wiley, New York, 1964.
44 N.S. Dixit and R.A. Mackay, *J. Am. Chem. Soc.*, 1983, **105**, 2928–2929.
45 K.S. Gulliya, Novel chemotherapeutic agents: preactivation in the treatment of cancer and AIDS I, Springer, Verlag Berlin, Heidelberg, 1996.
46 L. Šikurová and R. Franková, *Gen. Physiol. Biophys.*, 1994, **13**, 393–403.
47 M. Langner and S.W. Hui, *Biochim. Biophys. Acta*, 1993, **1149**, 175–179.
48 I.P. Lelkes and I.R. Miller, *J. Membr. Biol.*, 1980, **52**, 1–15.
49 I.P. Lelkes, D. Bach and I.R. Miller, *J. Membr. Biol.*, 1980, **52**, 141–148.
50 P.K. Williamson, K. Mattocks and R.A. Schlegel, *Biochim. Biophys. Acta*, 1983, **732**, 387–393.
51 M.S. El-Ezaby and F.R. El-Eziri, *J. Inorg. Nucl. Chem.*, 1976, **38**, 1901.
52 K.W. Yang, L.F. Wang, J.G. Wu, Zh.Y. Yang, and X. Gao, *J. Inorg. Biochem.*, 1993, **52**, 151–155.
53 M.S. Refat, F.M. Al-Azab, H.M.A. Al-Maydama, R.R. Amin and Y. M.S. Jamil, *Spectrochim. Acta, Part A*, 2014, **127**, 196–215.
54 D.E. Metzler and E.E. Snell, *J. Am. Chem. Soc.*, 1955, **77**, 2431.
55 B. Klejdus, J. Petrlová, D. Potěšil, V. Adam, R. Mikelová, J. Vacek, R. Kizek, V. Kubáň, *Anal. Chim. Acta*, 2004, **520**, 57–67.
56 O. Heudi, T. Kilinc, and P. Fontannaz, *J. Chromatogr. A*, 2005, **1070**, 49–56.
57 P.F. Chatzimichalakis, V.F. Samanidou, R. Verpoorte, and I.N. Papadoyannis, *J. Sep. Sci.*, 2004, **27**, 1181–1188
58 J.E Leklem, in Handbook of vitamins, ed. Machlin LJ, Marcel Dekker, New York, 1991, 341–392.
59 H.E. Sauberfich. Methods in Vitamin B-6 Nutrition: Analysis and Status Assessment, ed. J.E Leklem, R.D. Reynolds, Plenum Press, New York, 1981, 203–239.
60 P. Edwards, *Clin. Chem.*, 1989, **35/2**, 241–245.
61 T. Toukairin-Oda, E. Sakamoto, N. Hirose, M. Mori, T. Itoh, H. Tsuge, *J. Nutr. Sci. Vitaminol.*, 1989, **35**, 171-180.
62 T.E. Hefferan, B.M. Chrisley, J.A. Driskell, *J. Chromatogr. B: Biomed. Sci. Appl.,* 1986, **374**, 155-161.
63 T.R. Guilarte, B. Shane, P.A. McIntyre, *J. Nutr.*, 1981, **111**, 1869-1875.
64 M. Koneswaran and R. Narayanaswamy, *Sens. Actuators, B: Chem.*, 2015, **210**, 811–816.
65 F. Zare, M. Ghaedi and A. Daneshfar, *RSC Adv.*, 2015, **5**, 70064–70072.



Luminescent DPPC vesicles,decorated by new amphiphilic tetracarboxylate derivatives of p-tert-butylthiacalix[4]arene with Tb(III) were used for the selective detection of pyridoxine hydrochloride.