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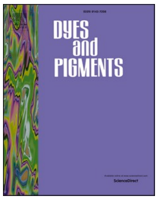

Ion sensing of sister sensors based-on calix[4]arene in aqueous medium and their bioimaging applications

Malak Alizada [a], Alev Gul [a], Mehmet Oguz [a,b], Ahmed Nuri Kursunlu [a], Mustafa Yilmaz [a,*]

[a] Department of Chemistry, University of Selcuk, Campus, 42031, Konya, Turkey
[b] Department of Advanced Material and Nanotechnology, Selcuk University, 42031, Konya, Turkey

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ABSTRACT

Here, sister calix[4]arene derivatives containing two NBD (7-nitro-benzofurazan) units in 1,3-(distal) derivatives (sensors **4&8**) were prepared and characterized by ^{1}H, ^{13}C NMR, FTIR, elemental analysis, and fluorescence spectroscopy. The ion binding properties of multi-channel sensors **4&8** were performed in the presence of various cations and anions. Both sensor compounds, **4&8**, showed "on–off" type fluorescence response towards Cu^{2+} cation and $H_2PO_4^-$&F^- anions with high selectivity in aqueous solution (CH_3CN/H_2O, 4/1, v/v). The connected spectroscopic parameters were investigated as the complex stoichiometry, binding constant etc. for a better result. Moreover, the lowest detection limit of 4 for sensing Cu^{2+} and $H_2PO_4^-$&F^- were obtained 2.4×10^{-8}, 9.1×10^{-7}, 9.7×10^{-7} mol/L respectively. the lowest detection limit of Cu^{2+} and $H_2PO_4^-$&F^- by 8 was obtained as 2.9×10^{-8}, 9.7×10^{-7}, 8.9×10^{-7} mol/L respectively. The confocal microscope was performed to investigate the sensor properties of **4&8** towards ions on living cells. Sensors **4** and **8** showed fluorescence properties on the A549 cell line, while it was observed to turn off the fluorescence intensity in cells treated with $H_2PO_4^-$, F^- and Cu^{2+} ions. Besides, compounds **4** and **8** can be used to identify $H_2PO_4^-$, F^- and Cu^{2+} ions in living cells.

1. Introduction

Recently, the design and synthesis of multifunctional chemo-sensors for the recognition of one or more ionic and neutral analytes have received considerable attention owing to their low cost, simplicity of instruments, high sensitivity and efficiency in comparison with the previous single-target chemical-sensors [1–11]. Some cations and anions can be detected by various techniques such as electrochemical methods, atomic spectroscopy, ion-selective electrodes, anodic stripping voltammetry (ASV), mass spectrometry (MS), inductively coupled plasma mass spectroscopy (ICP-MS), total reflection X-Ray fluorimetry (TXRF), voltammetry, atomic absorption spectroscopy (AAS), inductively coupled plasma atomic emission spectrometry (ICP-AES) and surface-enhanced Raman scattering and so forth [12–16]. Among different techniques for recognition both cations and anions, the fluorogenic and colorimetric analysis methods have received remarkable attention due to their easy operation, remote control, instant response time, the multiplicity of measurable parameters and so on [17–19]. Among the varied essential metal ions in human bodies, Cu^{2+} ion plays critical roles as a catalytic cofactor in diverse enzyme activities. Besides, it has multiple functions that various physiological processes like activation of dioxygen, which is essential for the survival of all living organisms. On the other hand, the abnormal levels of it can lead to some diseases such as the increase in blood pressure, vomiting, lethargy, neurotoxicity, acute hemolytic anemia and neurodegenerative [20–24].

Fluoride is biologically an important anion due to its effects on the human body. The lack or excess of fluoride in the human body can lead to severe health risks like dental caries and osteoporosis [25–27]. Similarly, phosphates necessary for the continuation of life are an indispensable constituent of two major biopolymers DNA and RNA play important roles as receptors in biological systems. They are found in the structure of some drugs such as chemotherapeutic and antiviral forms [28–30].

Calixarenes are known as one of the important classes of supramolecular compounds that have received more attention in sensor chemistry [31–37]. Calixarenes have exceptional properties owing to the flexible template, their 3D structure, easy chemical modifications and they demonstrate various structural conformation providing perfect cavities with special shapes and sizes for different ions [38–40]. They have some important properties like low cytotoxicity, biocompatibility in several bioimaging applications like cell imaging, zebrafish, etc. They are commonly used for promising materials for many sensor

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* Corresponding author.
E-mail address: myilmaz42@yahoo.com (M. Yilmaz).

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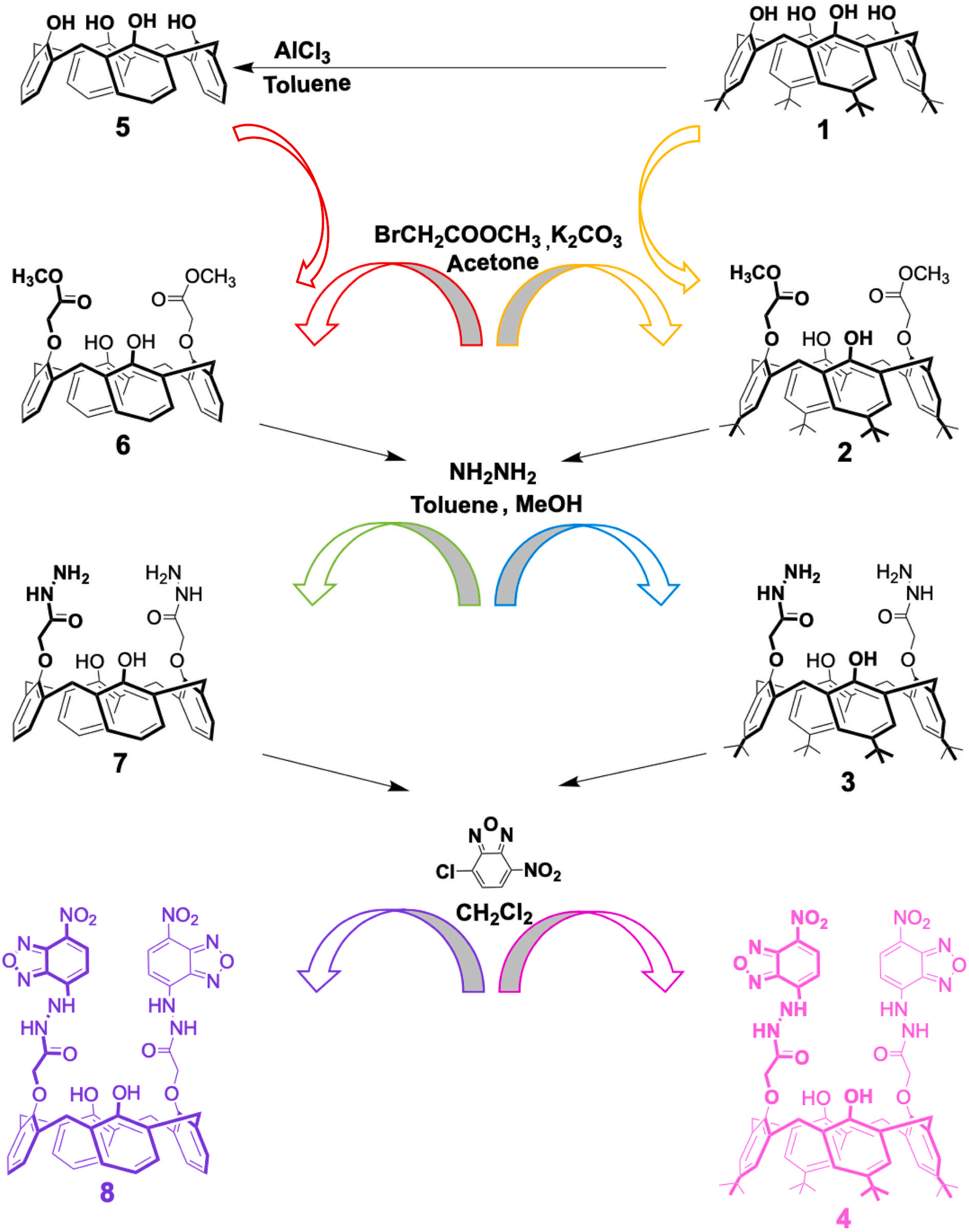

Scheme 1. The synthesis route of sensors **4** and **8**.

applications owing to their perfect sensitive and selective abilities [41–43].

Otherwise, the color-changing based on synthetic chemical probes can be observed by the naked eye in daylight or longwave light. Such as thiadiazole, NBD-chloride (4-chloro-7-nitro benzofurazan), phenolphthalein, Bodipy, rhodamines, quinolone derivatives, and acridine-based on sensors are commonly used for the recognition of metal ions in aqueous solution and living system [44–48]. Because of the biological roles along with the associated harmful effects, the synthesis of efficient multifunctional colorimetric and fluorogenic chemo-sensors for the detection of two or more cation and anion have become a highly hot topic of interest to many scientists in the worldwide [49–53]. In this work, based on the above all these ideas, we have been focusing on synthesizing two new NBD bearing calix[4]arene derivatives to

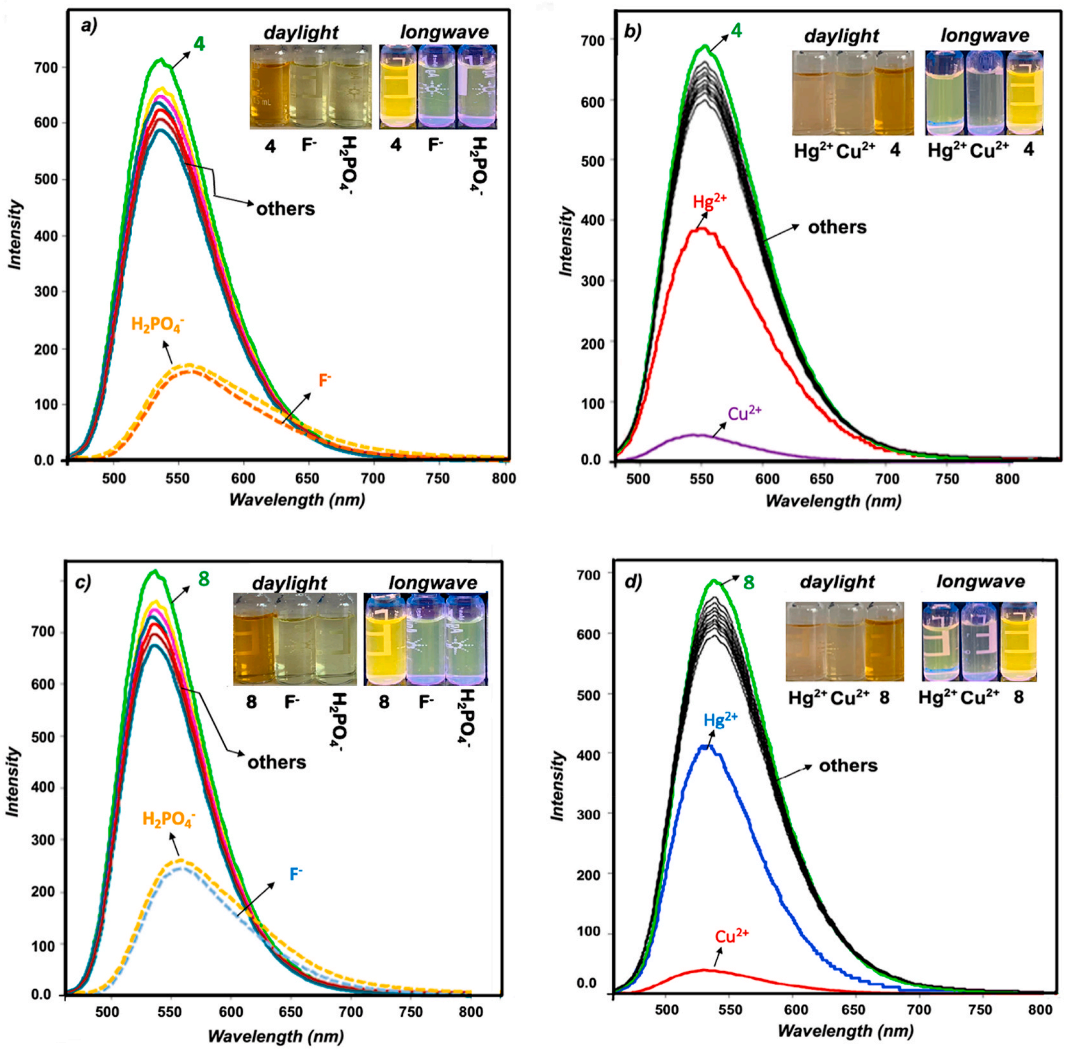

Fig. 1. **(a)** Fluorescence spectra of sensor **4** [***a***), ***b***)] & **8** [***c***), ***d***)] (5.0 μM) recorded in CH_3CN/H_2O (4/1,v/v) in the presence of various cations (Na^+, Mg^{2+}, Ca^{2+}, Fe^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Hg^{2+}, Al^{3+}, Cr^{3+}, Ag^+, Pb^{2+}) and anion (F^-, Cl^-, Br^-, I^-, $H_2PO_4^-$, HPO_4^{2-}, PO_4^{3-}, HSO_4^-, SO_4^{2-}, NO_3^-, CH_3COO^-) (20.0 equiv.) *(longwave:* 365 nm*)*.

investigate their both cations and anions detection studies and their cell imaging application in A549 cell line.

2. Experimental section

2.1. Materials, methods, and instruments

The perchlorate salts all of the metal ions (Na^+, Mg^{2+}, Ca^{2+}, Fe^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Hg^{2+}, Al^{3+}, Cr^{3+}, Ag^+, Pb^{2+}) and the tetrabutyl ammonium salts of all the anions (F^-, Cl^-, Br^-, I^-, $H_2PO_4^-$, HPO_4^{2-}, PO_4^{3-}, HSO_4^-, SO_4^{2-}, NO_3^-, CH_3COO^-) were procured from Sigma-Aldrich. Other materials and reagents used for the experiments were supplied from different commercial sources (Merck, Alfa-Aeser, Acros). All the solvents used were analytical reagent grade and were distilled and dried before use. The ^1H NMR/^{13}C NMR UV–vis and fluorescence spectra were performed with Varian NMR Spectrometer (400 MHz), Shimadzu 1700 UV–Vis spectrophotometer and Perkin Elmer LS 55 spectrometer, respectively. Also, infrared spectra were measured using a Bruker FTIR instrument and Elemental analyses were performed on a Leco CHNS-932 analyzer.

2.2. The preparing of compounds

The precursors **1**–**3** and **5**–**7** were synthesized by using known procedures [54–56]. As shown in Scheme 1, the hydrazide derivative of calix[4]arene were synthesized from the reaction between the ester derivative of calix[4]arene and hydrazine hydrate according to the literature [57]. Target calix[4]arene derivatives (**4, 8**) as shown in Scheme 1 were synthesized according to the methods given below.

2.3. Synthesis of NBD bearing calix[4]arene derivatives (4 and 8)

Precursor **3** or **7** (0.25 mmol) and 4-chloro-7-nitro benzofurazan (0.55 mmol) was dissolved in dichloromethane (50 mL), and Na_2CO_3 (1.37 mmol) was added to it. The reaction was stirred at room temperature for 6 h. The reaction was checked by TLC using ethyl acetate: petroleum ether (40:60). The solvent was evaporated under reduced pressure and the remaining solid was washed several times in diethyl ether and dried under N_2. Brick-color solid product was obtained.

Characterization of Compound 4: Mp: 161–163 °C; Yield: 85%; FTIR (ATR): 1317 cm^{-1} (NO_2) and 1525 cm^{-1} (NO_2), ^1H NMR (400 MHz, DMSO): δ (ppm) 1.13 (s, 18H, But), 1.17 (s, 18H, But), 3.81 (bs, 4H, $ArCH_2Ar$), 4.26 (bs, 4H, $ArCH_2Ar$), 4.86 (bs, 4H-OCH_2CO), 7.36 (s, 8H, Ar-H), 8.02 (bs, 2H, Ar-H_{NBD}), 8.41–8.54 (m, 2H, –CONH), 8.67 (bs,

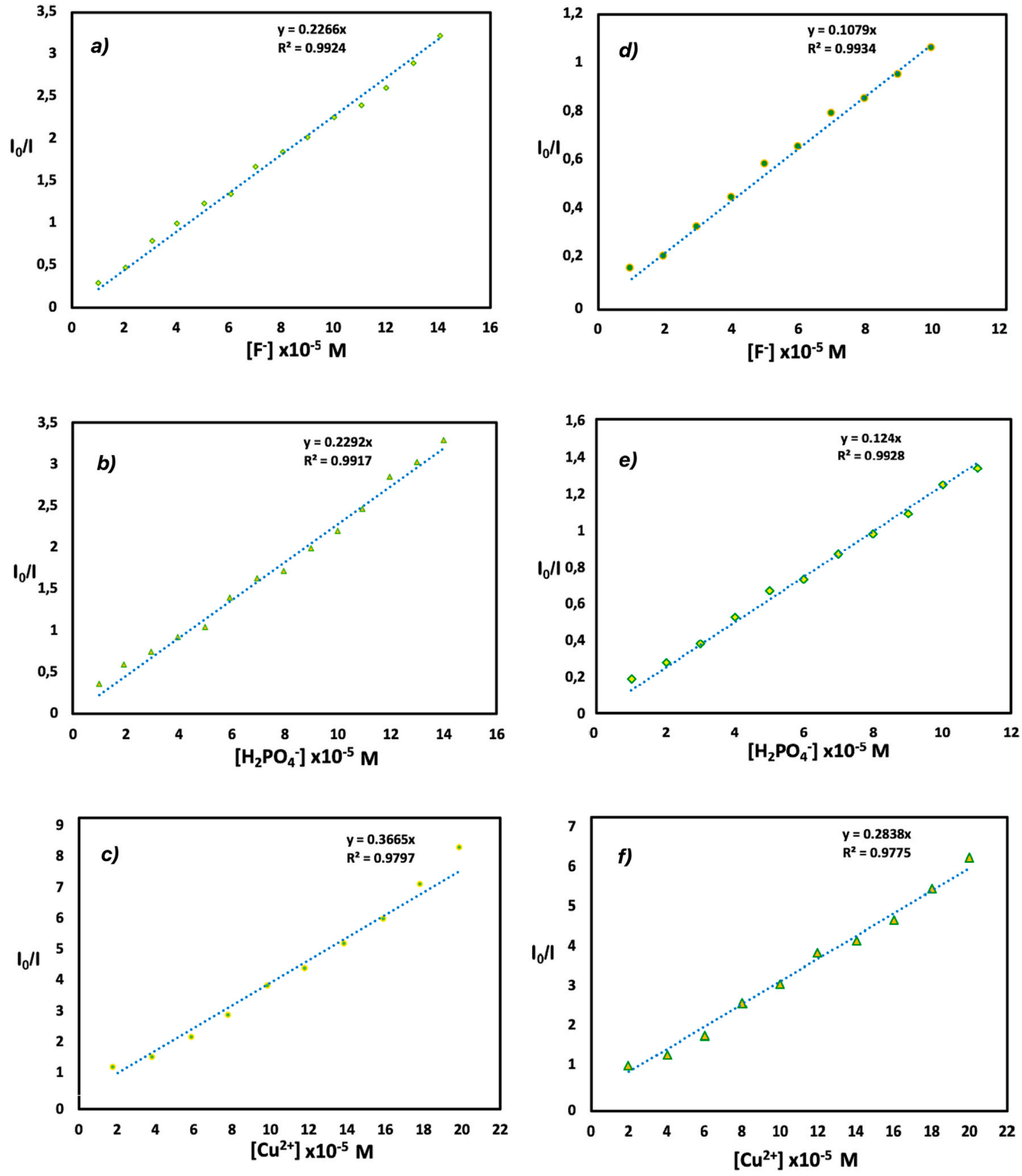

Fig. 2. The I_0/I ratios of sensor **4** [***a***), ***b***), ***c***)] & **8** [***d***), ***e***), ***f***)] (5 μM) against the various concentration graph of Cu^{2+} cation and $H_2PO_4^-$&F^- anions (1 μM, 2 μM, 3 μM, 4 μM, 5 μM, 6 μM, 7 μM 8 μM, 9 μM and 10 μM (λ_{emmax} = 560; λ_{ex} = 460 nm in CH_3CN/H_2O (4:1)).

Table 1
The binding constants R^2 values and of *anion&cation*.

Sensor	Anion&cation	K_{SV}	R^2
4	Cu^{2+}	4.7×10^5	0.9797
	F^-	1.7×10^5	0.9924
	$H_2PO_4^-$	1.9×10^5	0.9917
8	Cu^{2+}	3.1×10^5	0.9775
	F^-	0.2×10^5	0,9934
	$H_2PO_4^-$	0.18×10^5	0,9928

2H, Ar-H_{NBD}), 11.06 (s, 2H, -OH). ^{13}C NMR (100 MHz, DMSO):δ (ppm) 167,80, 150.04, 143.86, 142.28, 136.00, 133.45, 133.00, 131.01, 129.26, 127.56, 126.05, 73.58, 34.55, 34.12, 31.82, 31.33. Analytical Cal. for (%) $C_{60}H_{66}N_{10}O_{12}$; H, 5.94; C, 64.39; N, 12.51. Found: H, 6.23; C, 64.56; N, 12.77.

Characterization of Compound 8: Mp: 114–116 °C; Yield: 75%; FTIR (ATR): 1320 cm^{-1} (NO_2) and 1519 cm^{-1} (NO_2). ^1H NMR (400 MHz, DMSO): δ (ppm) 3.51 (bs, 4H, ArCH_2Ar), 4.28 (bs, 4H, ArCH_2Ar), 4.90 (s, 4H-OCH_2CO), 6.83 (s, 2H, Ar-H), 6.61 (s, 2H, Ar-H), 7.07–7.16 (m, 8H, Ar-H), 7.92 (bs, 2H, Ar-H_{NBD}), 8.31(bs, 2H, NH-CO), 8.67 (bs, 2H, Ar-H_{NBD}), 11.29 (s, 2H, -OH). ^{13}C NMR (100 MHz, DMSO):δ (ppm) 167,71, 152.62, 150.03, 143.83, 142.94, 135.95, 134.04, 131.01, 129.22, 128.00, 73.56, 55.39, 31.16. Analytical Cal. for (%) $C_{44}H_{34}N_{10}O_{12}$; H, 3.83; C, 59.06; N, 15.65. Found: H, 4.07; C, 59.24; N, 15.79.

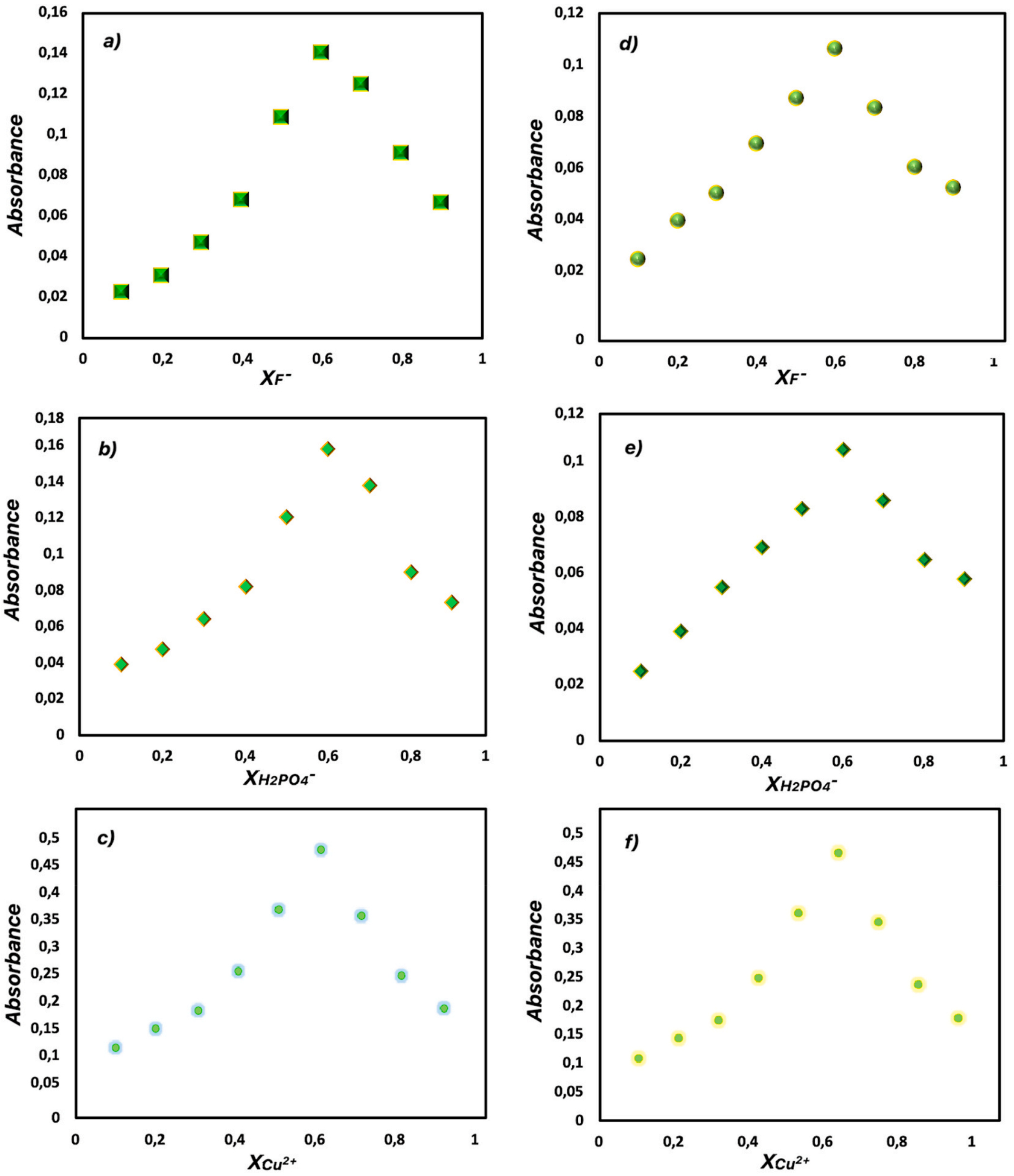

Fig. 3. Job's plot for interaction of sensor **4** [***a***), ***b***), ***c***)] & **8** [***d***), ***e***), ***f***)] (5.0 μM) with Cu^{2+} cation and $H_2PO_4^-$&F^- anions at 560 nm, respectively.

2.4. Cell culture and confocal microscope imaging

Human lung cancer (A549) cells were obtained from American Type Culture Collection, (ATCC), Rockville, MD, USA, and cultured MEM Eagle with Earle's BSS with 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin and streptomycin) at 37 °C and 5% CO_2. The medium was changed every 24 h and sub-cultured when the cells reached 80–90% growth. A549 cells were seeded on 6 well plates on the slide as 15×10^4. After 24 h for confocal imaging, the cells were treated with the medium and 50 μM of compounds **4** and **8** for 1 h. After the incubation, the medium was removed and washed three times with PBS buffer to remove compounds **4** and **8** present in the cells. Cells treated with compounds **4** and **8** were treated with 100 μM $H_2PO_4^-$, F^-, Cu^{2+} for 1 h and the medium was removed and washed 3 times with PBS buffer. Finally, the slide preparation was performed, and the image was obtained with a confocal microscope (Nikon/A1R1).

3. Results and discussion

3.1. Synthesis and characterization of NBD bearing calix[4]arene probes (4,8)

This study aims to synthesis two new NBD appended calix[4]arene derivatives to detect Cu^{2+}, F^-, and $H_2PO_4^-$ as colorimetric and fluorometric (Scheme 1). In this regard, calix[4]arene derivatives (compounds **1**, **2**, **3**, **5**, **6** and **7**) were synthesized according to previously reported methods [54–57]. Then, the NBD was reacted with calix[4]arene derivatives (**3** and **7**) in dichloromethane to synthesize compounds **4** and **8** as calix[4]arene-amide derivatives (Scheme 1). The structures of all the calixarenes were characterized by spectroscopic methods like FT-IR, ^1H NMR, ^{13}C NMR, elemental analysis (Supporting information, Figs. S1–S14). The formation of **4** was confirmed by the appearance of the characteristic nitro and amide bands at 1317&1525 cm^{-1} and 1696 cm^{-1}, respectively (Fig. S3). In ^1H NMR of **4, the peaks** at 8.02&8.67, 1.13&1.17 and 4.86 ppm assign to new two aromatic protons, two *t-butyl*

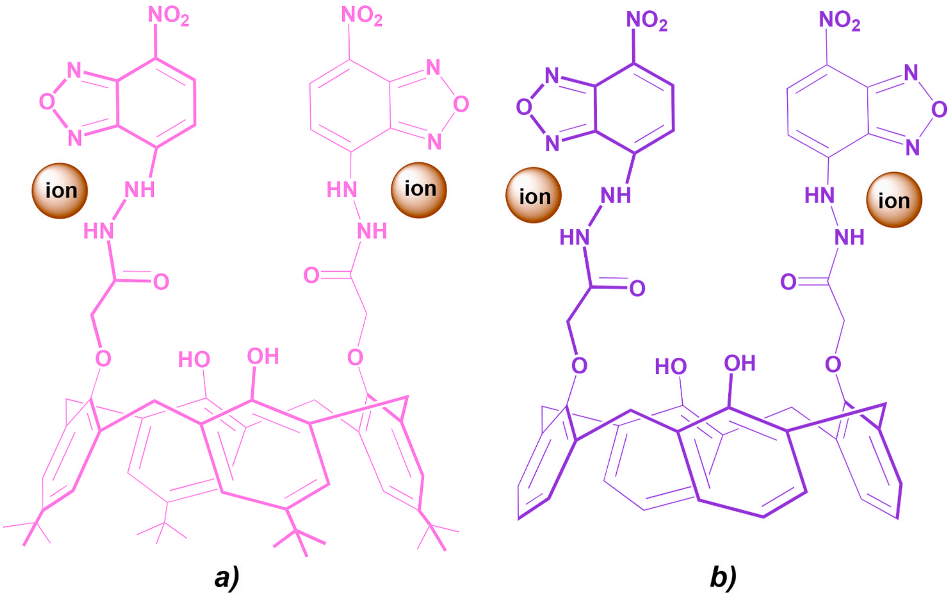

Fig. 4. Schematic representation of complex formation between sensor **4**&**8** and ions.

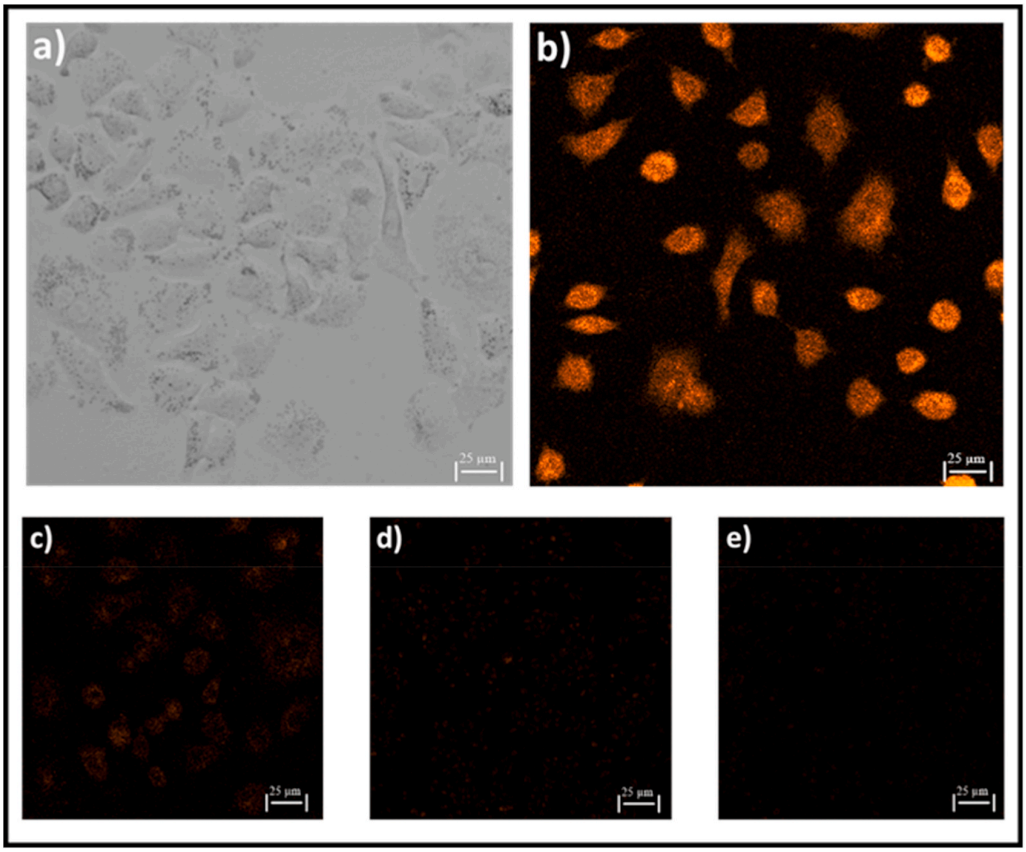

Fig. 5. Confocal microscopy images of A549 cells: **a)** bright field image of living A549 cells; **b)** cells treated with compound **4** (20 μM); **c)** A549 cells treated with **4** for 1 h, then treated with $H_2PO_4^-$ (50 μM) for 1 h; **d)** A549 cells treated with **4** for 1 h, then treated with F^- (50 μM) for 1 h; **e)** A549 cells treated with **4** for 1 h, then treated with Cu^{2+} (50 μM) for 1 h.

groups and -OCH_2 units, respectively (Fig. S9). The carbon signal of the carbonyl groups was observed at 167.7 ppm in the ^{13}C NMR spectrum (Fig. S10). In the FT-IR spectrum of **8**, the stretching bands at 1320 cm^{-1} and 1519 cm^{-1} were attributed to N=O vibration in NO_2 fragments. Moreover, the specific N-C=O stretching bands of **8** unit appeared around 1686 cm^{-1} (Fig. S6). The protons of -OCH_2 units and NBD groups appeared at 4.90 and 7.92&8.67 ppm; the carbon signals of amide groups were also observed at 167.6 ppm that all data confirmed to the structure of **8** (Figs. S13 and S14).

The cation&anion binding affinities of **4** and **8** were investigated in the presence of various cations (Na^+, Mg^{2+}, Ca^{2+}, Fe^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Hg^{2+}, Al^{3+}, Cr^{3+}, Ag^+, Pb^{2+}) and anions (F^-, Cl^-, Br^-,

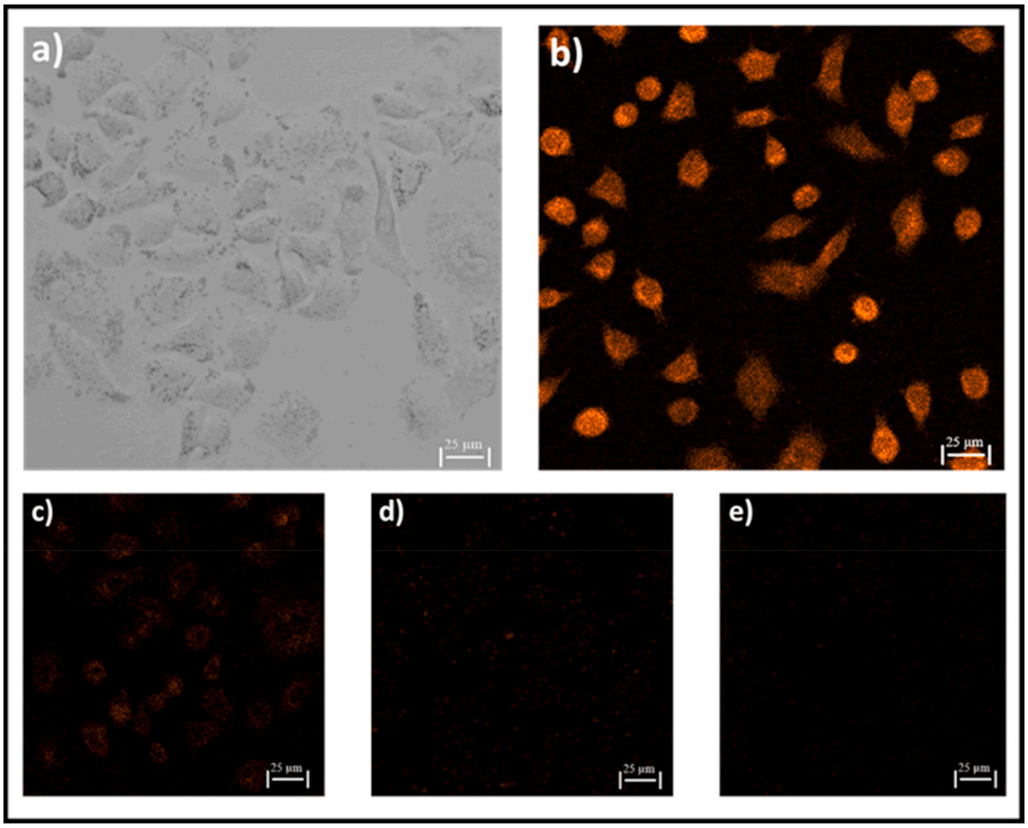

Fig. 6. Confocal microscopy images of A549 cells: **a)** bright field image of living A549 cells; **b)** cells treated with compound **8** (20 μM); **c)** A549 cells treated with **8** for 1 h, then treated with $H_2PO_4^-$ (50 μM) for 1 h; **d)** A549 cells treated with **8** for 1 h, then treated with F^- (50 μM) for 1 h; **e)** A549 cells treated with **8** for 1 h, then treated with Cu^{2+} (50 μM) for 1 h.

I^-, $H_2PO_4^-$, HPO_4^{2-}, PO_4^{3-}, HSO_4^-, SO_4^{2-}, NO_3^-, CH_3COO^-) by using fluorescence spectroscopy in CH_3CN/H_2O (4/1, v/v) solution at 5.0 μM concentration. The sister sensors, **4** and **8**, exhibited the emission maximum around 560 nm excited at 460 nm. As can be seen in Fig. 1, both sensors gave a similar result that $H_2PO_4^-$&F^- and Cu^{2+}&Hg^{2+} quenched to the intensities of **4** and **8**. The color of the solutions in both sensors changed from green to colorless under longwave light in presence of Cu^{2+}&Hg^{2+} cations. The intensities of fluorescence were significantly quenched by Cu^{2+} ion, indicating effective complexation with this cation. The strong quenching effect can be explained that the multi donor atoms on NBD fragments of sensors easily coordinated with Cu^{2+} ion and almost 20-folds quenching was observed. Similarly, $H_2PO_4^-$&F^- anions were captured by the NBD arms of sensors depending on multi inter-molecular interactions and hydrogen bonds. $H_2PO_4^-$&F^- anions caused a 4-folds quenching effect while other anions revealed an insignificant response. Moreover, the fluorescence wavelengths of sister sensors shifted slightly to blue or red in the presence of both Cu^{2+} cation and $H_2PO_4^-$&F^- anions. These changes can be attributed to an internal charge transfer between ligands and ions. According to our reference, a fluorescent sensor prepared with 4-nitrobenzofurazan, we preferred to pH study range as pH: 7 due to ideal conditions [58]. All spectroscopic measurements carried out at pH: 7.

To observe the fluorescence quenching of **4** and **8** towards Cu^{2+} cation and $H_2PO_4^-$&F^- anions, the fluorescence experiments with increasing concentrations of ions were performed, respectively (Fig. S16). As shown in Fig. 2, all graphs presented a linear curve that fluorescence intensity of sensors was gradually quenched upon the enhancing of ion concentration. When the ion sensitivity of **4** and **8** compared, I_0/I ratios show that the ion sensitivity of sensor **4** is higher for towards Cu^{2+} cation and $H_2PO_4^-$&F^- anions. Otherwise, when the cation&anion sensitivity of **4** and **8** compared, both sister sensors have a higher sensitivity for Cu^{2+} cation. This status can be explained that the complex interaction between ligand and the metal ion is stronger as to the weak intermolecular interaction between ligand and anion. Namely, the coordination between **4**&**8** and Cu^{2+} cation is more stable.

On the other hand, the binding constants of **4**&**8** toward ions were calculated by using Stern-Volmer equation as shown in Table 1. Sensor **4** has the best Ksv value for Cu^{2+} cation in parallel with the concentration studies.

To illuminate complex stoichiometry, Job plot technique was used. As known, Job plot technique is used to determine the complex stoichiometry of a binding event that the absorbance against the mole fraction is obtained with a plotted graph. When the absorbance reaches a maximum in the graph, the analyte/ligand ratio can be determined. As the graphs; the complex formations were determined as 2:1 for all ligand-anion&cation pairs (Fig. 3).

As understood from Fig. 4, it was thought that the nitrogen atoms on NBD units of **4**&**8** play an important role in capturing Cu^{2+} cation and $H_2PO_4^-$&F^- anions. So, these atoms act electron donors to Cu^{2+} while they interact with $H_2PO_4^-$&F^- anions in the hydrogen bond principle.

The experiments on the competing ions were carried out in the presence of the Cu^{2+} cation, $H_2PO_4^-$ and F^- anions (5×10^{-6} M) mixed with (Na^+, Mg^{2+}, Ca^{2+}, Fe^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Hg^{2+}, Al^{3+}, Cr^{3+}, Ag^+, Pb^{2+}) (F^-, Cl^-, Br^-, I^-, $H_2PO_4^-$, HPO_4^{2-}, PO_4^{3-}, HSO_4^-, SO_4^{2-}, NO_3^-, CH_3COO^-) at 5×10^{-6} M concentration. The results were given the fluorescence intensities of sensor **4**&**8** as a table (Table S1). Accordingly, these results confirm that sensor **4**&**8** can behave as a selective fluorescent sensor towards both Cu^{2+} cation and $H_2PO_4^-/F^-$ anions in the presence of other competing metal ions.

To confirm better the complexation between **4**&**8** and F^-, ^1H NMR experiments were performed out in the absence and presence of this anion in DMSO-d_6 (Fig. S15). The addition of 2.0 equivalents of F^- to solutions of the probes **4**&**8** resulted in significant spectral changes. Signals belong to phenolic -OH observed at 11.06 and 11.29 ppm for **4**&**8**, the amide signals at 8.49 and 8.31 ppm were disappeared.

On the other hand, it was performed the fluorescence imaging studies with confocal microscopy to control the biological applications of compounds **4** and **8** in the A549 cell line. By keeping the compounds **4** and **8** concentration stable (20 μM), a decrease was observed due to $H_2PO_4^-$, F^-, Cu^{2+} (50 μM) ions in intracellular orange fluorescence emission resulting from the complex formation with compounds **4** and **8**. As shown in Figs. 5 and 6, the control group of A549 cells (Figs. 5a-6a) was observed orange fluorescence when the lung cancer cell line was treated only with compounds **4** and **8** (Figs. 5b-6b). Fluorescence intensity decreased in cells treated with $H_2PO_4^-$, F^-, Cu^{2+} (Fig. 5c-d-e/6c-d-e). As a result, it was stated that the synthesized sensor was compatible with determining $H_2PO_4^-$, F^-, Cu^{2+} ions in cells.

4. Conclusion

In conclusion, the synthesis and spectroscopic properties of sister calix[4]arene derivatives containing two NBD having cone conformation, sensor **4** and **8**, for multi-analyte sensing were reported. Both sister sensors, **4** and **8**, showed the fluorescence quenching process for the sensitive recognition of Cu^{2+} cation and $H_2PO_4^-$&F^- anions in CH_3CN/H_2O (4/1, v/v), respectively. Interaction of sensors **4**&**8** with ions induced completely quenching of fluorescence intensity through a 1:2 (ligand:ion) binding mode. Also, the confocal microscope imaging was performed to identify $H_2PO_4^-$, F^-, Cu^{2} ions in compounds **4** and **8** in living cells. The utility of compounds **4** and **8** as a bio-analytical molecular tool was successfully illustrated by fluorescence monitoring of $H_2PO_4^-$, F^-, Cu^{2+} ions in biological processes. As a result, it was observed that the A549 cells treated with $H_2PO_4^-$, F^-, Cu^{2+} ions turn off the fluorescent intensity. The use of both sister sensors, **4** and **8** to recognize Cu^{2+} cation and $H_2PO_4^-$&F^- anions will enormously extend ion types by the various derivatives of calix[4]arene having multi-donor atoms.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dyepig.2020.108741.

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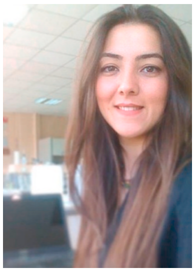
Malak Alizada. She received her master's degree from the Chemistry Department of Selcuk University, Turkey. During her studies, she worked in the field of synthesis and characterization of calixarene-based compounds, sensor chemistry and bioimaging applications. She aims to continue her education in this field.



Alev Gul. She is a PhD student in the Department of Chemistry at Selcuk University, Turkey. Her research continues on the synthesis and characterization of calixarene-based compounds, synthesis of nanoparticles, sensor chemistry, drug delivery and synthesis of anticancer agents and bioimaging applications.

Mehmet Oguz. He is a PhD student in the Department of Advanced Material and Nanotechnology at Selcuk University, Turkey. His research continues on the synthesis and characterization of calixarene-based compounds, the synthesis of nanoparticles, sensor chemistry, drug delivery and synthesis of anti-cancer compounds.

Ahmed Nuri Kursunlu was born in Karaman, Turkey. He received his MSc from the University of Selçuk, (Konya-Turkey) in 2008 before carrying out his Ph.D. studies under the supervision of Associate Prof. Dr. Ersin Güler. His research focussed on the synthesis of BODIPY-Schiff bases. Following this he spent one year in UK, first as a research fellow in the Ph.D, University of Hull with Prof. Ross W. Boyle. He graduated from Ph.D. in 2014 and is currently working as a researcher at the University of Selçuk where his work focuses on Bodipy-complex formations and applications.

Mustafa Yilmaz received his bachelor's degree in chemistry from Ataturk University (Turkey) in 1978, his Ph.D. in organic chemistry from Selcuk University Institute of Science in 1987. His research focuses on the synthesis and characterization of calixarene-based compounds and phase transfer-reaction, chiral catalysis, sensors, cation, anion recognition studies, magnetic nanoparticles and enzyme immobilization and kinetic resolution studies. He has authored over 250 articles in peer reviewed journals and 5 book chapters.