This manuscript was published as formal in:
Polymer Chemistry 9 (2018), 968
DOI: 10.1039/c7py01668a
https://pubs.rsc.org/en/content/articlelanding/2018/py/c7py01668a#!divAbstract

Novel cyclodextrin-based pH-sensitive supramolecular host-guest assembly for staining acidic cellular organelles

*Gabriela Pricope,[a] Elena Laura Ursu,[a] Monica Sardaru,[b] Corneliu Cojocaru,[c] Lilia Clima,[a] Narcisa Marangoci,[a] Ramona Danac,[b] Ionel Mangalagiu,[b] Bogdan C. Simionescu,[a,d] Mariana Pinteala,[a] Alexandru Rotaru[a]**

[a] *"Petru Poni" Institute of Macromolecular Chemistry, Romanian Academy, Centre of Advanced Research in Bionanoconjugates and Biopolymers, 700487 Iasi, Romania.*

[b] *Alexandru Ioan Cuza University of Iasi, Chemistry Department, Iasi 700506, Romania.*

[c] *"Petru Poni" Institute of Macromolecular Chemistry, Romanian Academy, Department of Inorganic Polymers, 700487 Iasi, Romania.*

[d] *Department of Natural and Synthetic Polymers,''Gh. Asachi'' Technical University of Iasi, Iasi 700050, Romania.*

Introduction

In the last decade fluorescence imaging has become the most powerful technique to visualize and monitor specific biological targets or processes in living systems.[1,2] Subsequently, fluorescent dyes have been extensively investigated to improve their abilities of analytical specificity and sensitivity. However, fluorescent dyes suffer from several fundamental problems including toxicity, low water solubility and poor membrane permeability when used for bio-labelling and bio-imaging.[3,4] Great efforts have been made to overcome these disadvantages. As an example, various hydrophilic groups such as sulfonate, pyridinium, glycol and carboxylate were appended to the core of dyes to increase their aqueous

solubility.[5,6] Introduction of a functional group such as ester group or carboxylic acid into dyes could also increase their solubility and membrane permeability.[7,8] Another interesting approach to solve both the solubility and the toxicity of the fluorescent dye could be the use of supramolecular assemblies. Molecules like cucurbit[8]uril[9] or cyclodextrines[10,11,12] have been reported to form host-guest inclusion complexes with drugs or fluorescent molecules increasing their solubility and solving their toxicity issues. Cyclodextrins (CDs), nontoxic oligosaccharides, are well known for their ability to form inclusion complexes with a large variety of hydrophobic molecules, increasing their solubility in water.[13,14] Although CDs have been shown to remove cellular cholesterol by host–guest inclusion complexation mechanism,[15] most literature reports are in favour of the non-destructive character of CDs towards the cell components, making CDs a perfect material for biomedical applications. Even though there are literature reports on using CDs for the improvement of florescent dyes properties as fluorescent imaging agents,[10,12,16,17] they all refer to commercial or well-known fluorescent dyes.

In this paper we report preparation of a host-guest inclusion complex between a fluorescent indolizinyl-pyridinium salt derivative and β-cyclodextrin, complex characterized by ESI-MS experiments and also investigated by molecular docking studies. The cytotoxicity of the starting compound and its inclusion complex were systemically investigated and evaluated, as well as cell membrane permeability on two cell lines. The obtained results confirmed the formation of an inclusion complex between indolizine derivative and β-cyclodextrin in 1:1 and 1:2 ratios and that the complexation reduced considerably the toxicity of free indolizine. The nontoxic inclusion complexes mixture could not only pass through the cell plasma membrane, but also specifically accumulates in cell acidic organelles. To our knowledge, this is the first demonstration of strong toxicity reduction of a fluorescent dye by the formation of cyclodextrin inclusion complex with the subsequent successful application in cell staining. The results might suggest that the proposed strategy could be potentially applied to other classes of toxic and non-soluble fluorescent dyes able to form strong host-guest inclusion complexes with cyclodextrins.

Experimental Section

Materials

All commercially available chemical reagents were purchased in their highest purity grade from Sigma-Aldrich (Germany) and used without further purification. HeLa (human cervix adenocarcinoma) cells were acquired from CLS-Cell-Lines-Services-GmbH (Germany) and NHDF (normal human dermal fibroblasts) cells were acquired from PromoCell. MTS assay (CellTiter 96 AQueous One Solution Cell Proliferation Assay) was acquired from Promega, and LysoTracker Red DND-99 was acquired from Invitrogen. Ultrapure water was used for preparing the working solutions in all the experiments. Dimethyl sulfoxide (DMSO) used for the spectrometric studies was of spectroscopic grade.

Synthesis

The synthetic route followed for the preparation of indolizinyl-pyridinium salt was reported earlier.[18] Preparation of the inclusion complex of indolizinyl-pyridinium salt and β-cyclodextrin (β-CD) was achieved by mixing equimolar amounts of components in water (i.e., heating the suspension till 110ºC for 60 minutes and then cooling down the solution under stirring for 6 h to reach the equilibrium. The clear sample solution was filtered through Phenex syringe filters (pore size: 0.45 µm) and used for analyses and experiments.

Characterisation techniques

UV/Vis spectra were obtained with a PerkinElmer Lambda 35 UV/Vis spectrophotometer using cuvettes with a sample volume of 3000 µL in DMSO or water (wavelength range 200−1000 nm). Fluorescence spectra were registered on a Fluoromax 4 (Horiba Scientific) fluorescence spectrophotometer using cuvettes with a sample volume of 3000 µL in DMSO or water (excitation at 415 nm and emission range between 430 nm and 700 nm). Mass spectrometry data were obtained using an Agilent 6520 Series Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) LC/MS. The LC system was connected directly to ionization source *via* mass spectrometer electrospray (ESI). The selected conditions were: ESI in a positive mode, drying gas debit (N2) 9L/min, gas temperature 325ºC; nebulizer pressure 25 psi, capillary voltage 4200 V; fragmentation voltage 200 V; compounds were investigated in a field of m/z 50–3000.

Cell cultures. HeLa cells (from CLS-Cell-Lines-Services-GmbH, Germany) and NHDF (from PromoCell) were cultivated in tissue culture flasks with alpha-MEM medium (Lonza) supplemented with 10% fetal bovine serum (FBS, Biochrom GmbH, Germany) and 1% Penicillin-Streptomycin-Amphotericin B mixture (10K/10K/25 µg in 100 ml, Lonza). The medium was changed with a fresh one, once every 3 or 4 days. Once confluence was reached, cells were washed with phosphate buffered saline (PBS, Invitrogen), detached with 1x Trypsin-EDTA mixture (Lonza) followed by the addition of complete growth media, centrifuged at 200 x g for 3 minutes and subcultured into new tissue culture flasks.

In vitro cell viability study (MTS assay) was measured using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega) following manufacturer protocol. HeLa cells were seeded at a density of 10^4 cells per well and NHDF cells were seeded at a density of $5x10^3$ cells per well in 96 well plates, in complete medium (alpha-MEM medium (Lonza) supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin-Amphotericin B mixture (10K/10K/25 µg in 100 ml, Lonza)). The next day, cells were treated with the indolizinyl-pyridinium salt and β-cyclodextrin inclusion complex (at concentrations equal to 34.8 µM, 52.2 µM, 69.6 µM, 86.9 µM and 17.4 µM in 100 µL of medium) and then grown for another 44 hours. Next, 20 µL of CellTiter 96® Aqueous One Solution reagent were

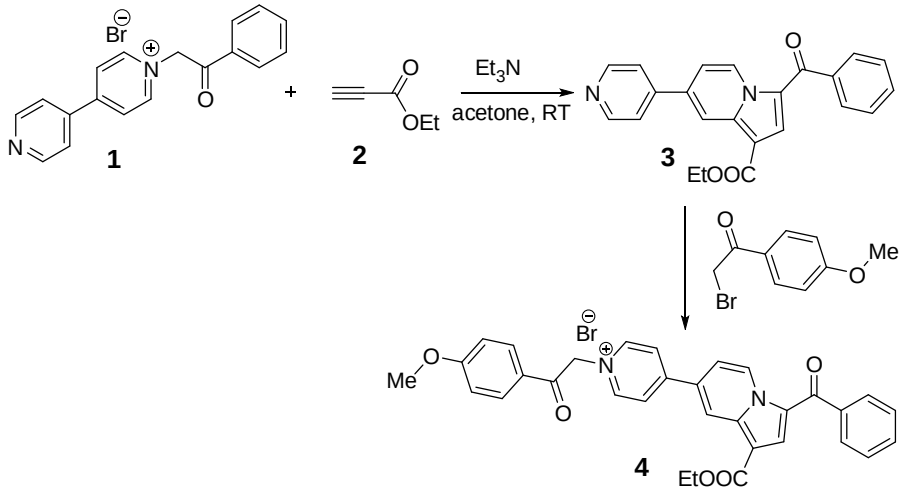
added to each well, and the plates were incubated for another 4 hours before reading the result. Absorbance at 490 nm was recorded with a plate reader (EnSight, PerkinElmer). A blank absorbance value from wells without cells but treated with MTS and with or without the inclusion complex was subtracted from the corresponding absorbance values. Cell viability was calculated and expressed as percentage relative to viability of untreated cells which served as negative control for the inclusion complex. Cells incubated with corresponding percentage of DMSO (0.5; 1; 1.5; 2; 2.5 %) served as negative control for the indolizinyl-pyridinium salt. Experiments were performed in three replicates and repeated three times. Data are presented as mean±S.D.

Cell staining with indolizinyl-pyridinium salt and β-cyclodextrin inclusion complex and Co-Staining with LysoTracker Red DND-99: HeLa and NHDF cells were incubated with different concentrations of indolizinyl-pyridinium salt and β-cyclodextrin inclusion complex (at concentrations equal to 34.8 µM, 52.2 µM, 69.6 µM, 86.9 µM and 17.4 µM) for 24 hours then imaged at 15 minutes and at 24 hours with an inverted microscope Leica DMI 3000 B with fluorescence GFP filter. Subsequently, cells stained with indolizinyl-pyridinium salt and β-cyclodextrin inclusion complex were treated with 75 nM solution of LysoTracker Red DND-99 (Invitrogen) for 30 minutes at 37°C in 5% CO_2. Cells were then washed three times in PBS and imaged in fresh cell culture medium using an inverted microscope Leica DMI 3000 B using GFP and N2.1 filters.

Results and discussion

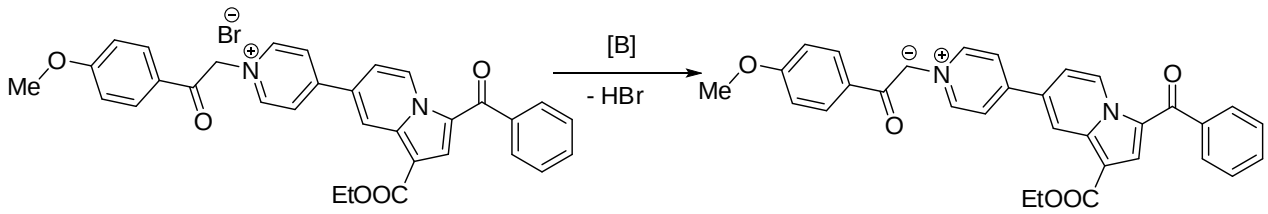
Synthesis

Fluorescent indolizinyl-pyridinium salt **4** (scheme 1) was designed as potential pH sensitive dye and synthesized in moderate yield in an adopted two step synthesis based on our methodological background in the preparation of substituted indolizines.[18,19]



Scheme 1. Synthesis of indolizinyl-pyridinium salt **4**. Compounds **1** and **3** were reported earlier.[18]

Due to the presence of the pyridinium moiety in compound **4,** it might undergo the formation of corresponding nitrogen ylide in the presence of a base according to scheme 2. This reversible property does influence its fluorescence emission spectra, making it a pH sensible fluorescent dye.



Scheme 2. General presentation of nitrogen ylide formation from compound **4**.

Applications of this type of compounds are limited due to high toxicity[20,21] and water solubility problems, compound **4** showing only a partial solubility in DMSO and dichloromethane. To achieve a better water solubility of compound **4** and to extend its applications as cell staining agent or cell pH sensitive dye, we proposed the preparation of a formulation between compound **4** and β-cyclodextrin (β-CD). To achieve this, indolizinyl-pyridinium salt **4** (5 mg, 8 µmol) was suspended in water (2.5 mL) and various amounts of β-CD corresponding to the following equivalent ratios between compound **4** and β-CD (1:1; 1:1.5; 1:2 and 1:3) were subsequently added to the prepared suspension. The thus prepared mixture was heated up to 110^{o}C to ensure the solubility of the added cyclodextrin and to facilitate the solubilisation of the compound **4** through the interaction with β-CD. The solution was left to cool down slowly to room temperature and we could observe that starting with the ratio of 1:1.5 and higher, upon cooling the reaction mixture became transparent with little to no precipitate formation noticed. This observation suggested the solubilisation of compound **4** through a possible formation of the host-guest inclusion complex. Interestingly, at 1:1 ratio the cold solution was still cloudy showing an incomplete solubilisation of **4**, leading to the conclusion that higher amounts of β-CD were needed for the stabilization of the inclusion complex. On the other hand, a higher ratio between **4** and β-CD may lead to the formation of more complex supramolecular assemblies containing the inclusion complex formed from one molecule of compound **4** and two or more β-CD units. To check this hypothesis ESI-MS experiments were performed for the reaction mixture containing different ratios between compound **4** and β-CD (1:1.5; 1:2 and 1:3), ESI-MS being a relatively straightforward and rapid technique for studying the inclusion phenomena which rapidly determine the CD:guest stoichiometry.[22,23] ESI-MS spectra (figures 1, S1-2) for 1:1.5 ratio clearly revealed several peaks corresponding to compound **4** (519 – M^{+}-Br), β-CD (1157 – M+Na^{+}), 1:1 compound **4**:β-CD complex (1654 – M^{+}-Br+CD) and 1:2 compound **4**:β-CD complex (2788.83 – M^{+}-Br+2CD).

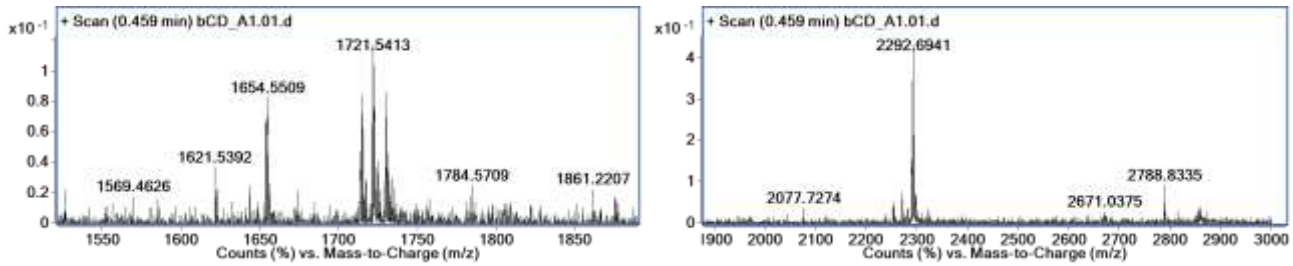


Figure 1. ESI-MS spectra of the reaction mixture of compound **4** and β-CD at 1:1.5 ratio revealing peaks corresponding to the formation of 1:1 inclusion complex (1654 – M^+-Br+CD) and 1:2 compound **4**:β-CD complex (2788 – M^+-Br+2CD).

In all investigated samples the formation of host-guest inclusion was observed. More interestingly, with increasing the stoichiometry between host and guest (3:1 ratio) molecular peaks (figures S3-4) for both 1:1 and 1:2 complexes (M^+-Br+CD and M^+-Br+2CD) were still visible. Hence, addition of cyclodextrin excess in order to assure the formation and observation of only the most stable multi-cyclodextrin adduct ions didn't result in a single species, the reaction solution (**4_CD**) presenting a mixture of 1:1 and 2:1 species. The structural foundation for the formation of these species was checked by molecular docking simulations.

Molecular docking studies

To understand the structural basis of the inclusion complexes of β-CD with compound **4** we performed a series of molecular docking (MD) studies. Generally, MD represents a computational method to evaluate the binding mode and affinity of an inclusion complex formed by two or more constituent compounds with known structures.[24] In this study, the AutoDock Vina method implemented in the YASARA Structure software package[25] was used for molecular docking simulations. Structures of β-CD and compound **4** were first drawn and optimized at PM3 level of theory using Hyperchem software[26] and then exported to YASARA program. The docking simulations indicated that the molecule of compound **4** is able to form both 1:1 and 1:2 complexes with β-CD, the selected and optimized molecular docking models being reported in figure 2.

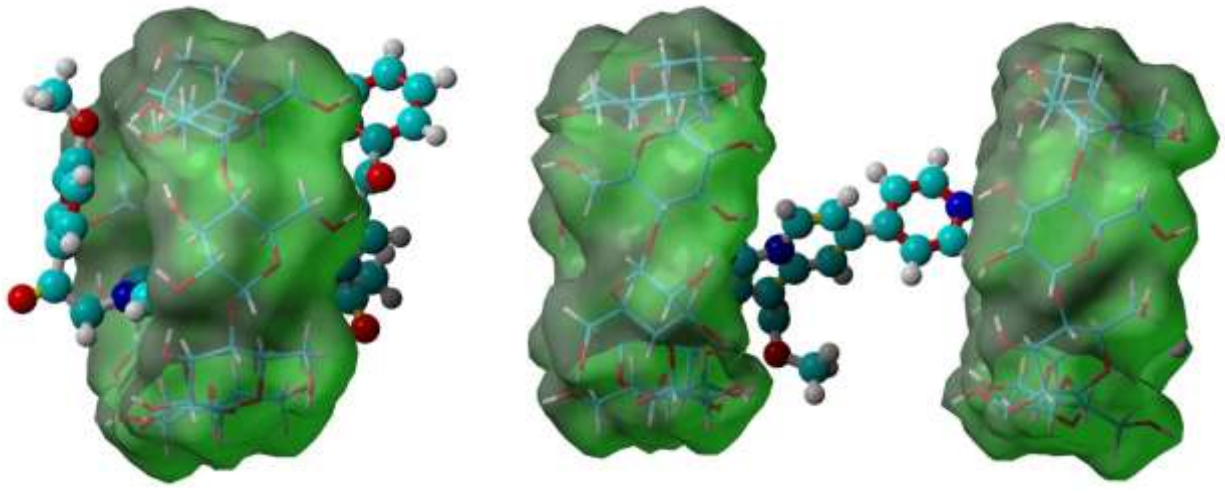


Figure 2. Molecular docking models of compound **4** in complex with β-CD showing the possibility of the 1:1 (left) and 1:2 (right) inclusion complex formation**.**

According to these results, for the **1:1** inclusion complex, the bipyridyl moiety of compound **4** is embedded in the hydrophobic cavity of β-CD. The theoretically calculated binding energy (E_b) for the optimized **1:1** inclusion complex was equal to -6.37 kcal/mol and the dissociation constant (K_d) equal to 21.4 μM. Interestingly, in case of **1:2** inclusion complex the marginal phenyl and methoxy moieties of compound **4** were deeply embedded in the hydrophobic cavity of β-CD molecules with a slightly higher value of the E_b=-7.63 kcal/mol and an surprisingly smaller K_d=2.6 μM when compared to the **1:1** inclusion complex. The obtained theoretical data suggest that both species could exist in the solution. On the other hand, even though molecular docking simulation put forward the formation of **1:2** inclusion complex due to the more favourable binding energy and the dissociation constant, the experimental ESI-MS results still support the formation of a mixture of both **1:1** and **1:2** species (**4_CD**).

Fluorescence properties of compound 4 and 4_CD

Due to the presence of the pyridinium moiety, both compounds **4** and **4_CD** can be present in solution in two different forms depending on solution pH. A stock solution of **4** in DMSO (4 mM) and water solution of **4_CD** (4 mM of compound **4** in the inclusion complex) were prepared and their emission spectra (5 μL stock solution in 2995 μL) were measured in NaOH (0.1 M, pH = 13), HCl (0.1 M, pH = 1) and 1x TAE buffer (40 mM Tris, 2 mM acetic acid and 1 mM EDTA, pH = 7.4). Figure 3 summarizes the fluorescence emission spectra of compound **4** and its inclusion complex **4_CD** at different pH values.

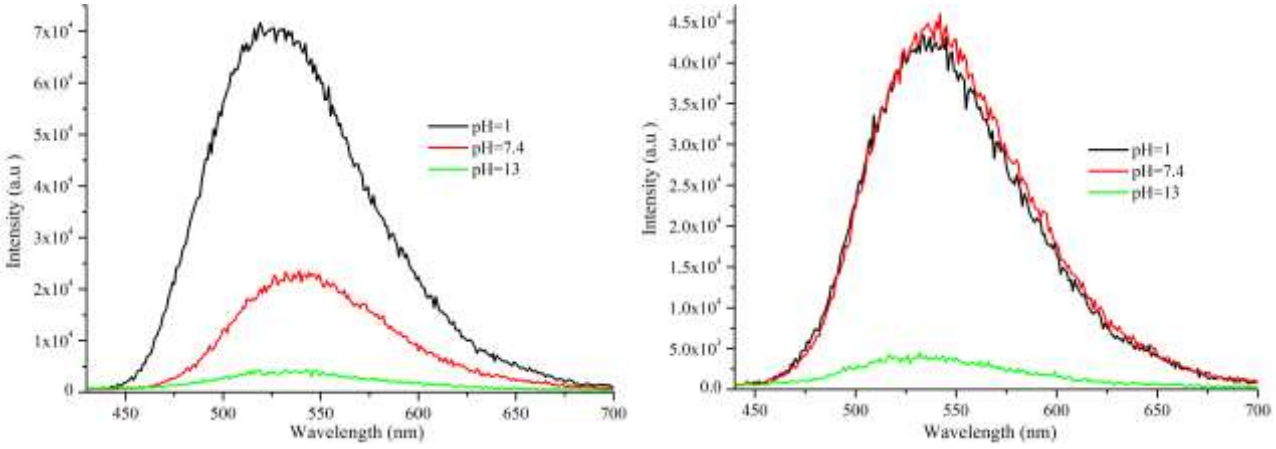


Figure 3. Fluorescence spectra of compound **4** (left) and **4_CD** (right) at pH = 1, 7.4 and 13.

The fluorescence maxima for both **4** and **4_CD** at all investigated pH values were found at 540 nm, suggesting that no shift of the spectra maxima was taking place after the formation of **4_CD**. Major differences appeared between the emission spectra of **4** and **4_CD** when comparing the intensity of the spectra at different pH values. Thus, compound **4** showed very low fluorescence intensity at pH = 13 when compared to its fluorescence at pH = 1, while at pH = 7.4 the fluorescence intensity was an average

between pH = 1 and 13 (figure 3, (left)) showing an equilibrium of different species. These data were in agreement with our previous data on the emission properties of compound **4** in organic solvents.[18] On the other hand, compound **4_CD** also showed low intensity emission at pH = 13, but surprisingly the intensity of the signal at pH = 7.4 was almost equal to the intensity for the **4_CD** solution at pH = 1 (figure 3, (right)). We speculate that these differences in the intensities of the signals at different pH values might be the results of the sterical hindrance caused to compound **4** molecule after complexation with cyclodextrin. Thus, in case of compound **4** the formation of the ylide at higher pH or formation of pyridinium salt at lower pH values is accompanied with considerable changes in molecular conformation. In the inclusion complex **4_CD**, the free molecule rotation during the pH changes is limited by the cavity/cavities of the cyclodextrin molecules within the inclusion complex.

In vitro cell viability study (MTS assay)

Prior to cell staining experiments, the cytotoxicity of the compound **4** and its inclusion complex **4_CD** was investigated against two human cancer and normal cell lines (Hela and NHDF). Cytotoxicity, measured as the inhibition of cellular lines viability, was evaluated at 48 h of incubation (figure 4). Analysing the obtained data, compound **4** alone showed very high values of toxicity at all five investigated concentrations (0 % cell viability) on the HeLa cell line (figure 4, (left)) and high (at concentrations equal to 34.8 µM, 52.2 µM, 69.6 µM and 86.9 µM) to medium toxicity (at concentration equal to 17.4 µM) on NHDF cells (figure 4, (right)).

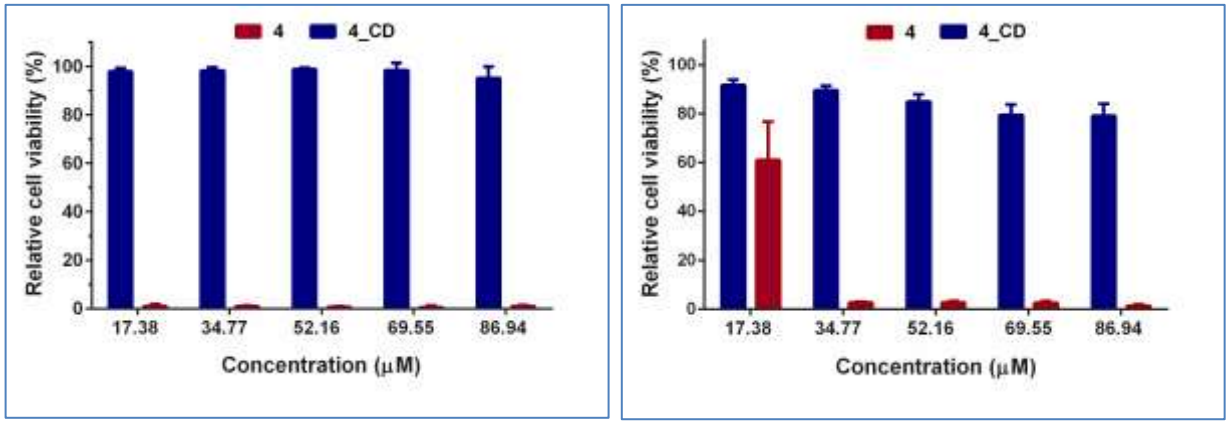


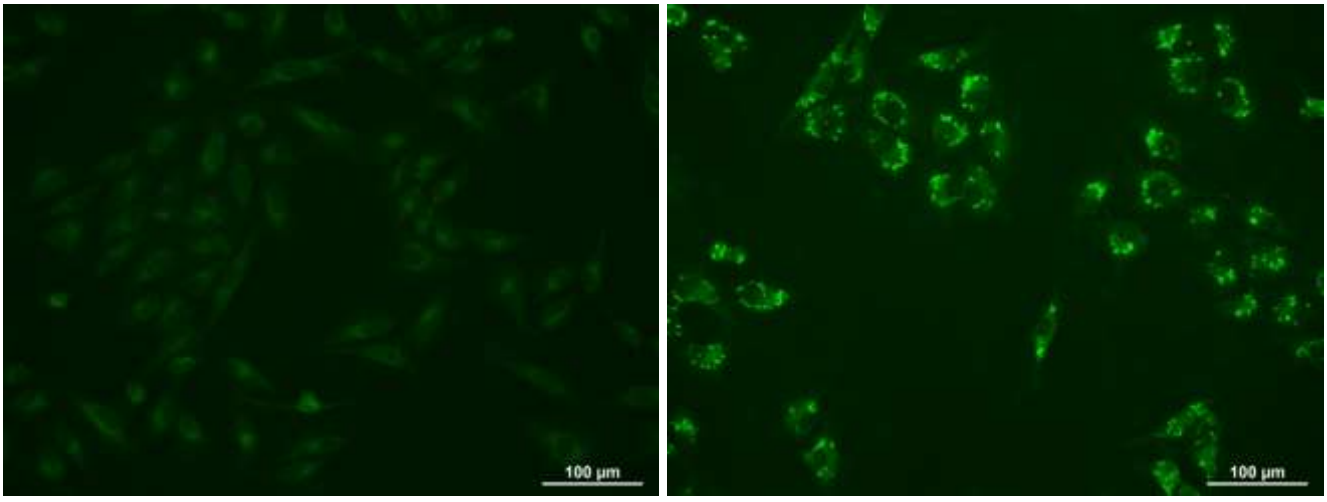
Figure 4. *In vitro* cell viability (MTS assay) results for **4** and **4_CD** on HeLa (left) and NHDF (right) cell line.

Contrarily, analysing the cell viability data of the **4_CD** solution with the same concentrations of compound **4** surprisingly high values (above 95%) of cell viability at all investigated concentrations in case of HeLa cells (figure 4, (left)) were observed and high values (above 80%) of cell viability with a slight decreasing tendency with the increase of inclusion complex concentration in case of NHDF cells (figure 4, (right)) were noticed. This astonishing effect of toxicity decrease comes in contradiction to the reported tendency of the increasing of the toxicity of a drug upon complexation with cyclodextrins. Thus,

Iacovino and co-workers have recently reported that the inclusion complexes considerably increase 5-fluorouracil ability to inhibit cell growth.[11] In particular, 5-fluorouracil complexed with β-cyclodextrin had the highest cytotoxic activity on MCF-7 cell line. Similarly, Minko and co-workers have also observed that cytotoxicity of Paclitaxel remained unchanged or slightly higher after complexation.[27] Higher cytotoxicity of complexed Paclitaxel was explained by the drug enhanced solubility which makes more soluble drug available at the cell surface for internalization. On the other hand, de Paula and co-workers reported a significant decrease in cytotoxicity of cyclodextrin complexed tetracaine as compared to plain tetracaine.[28] The decreased cytotoxicity of tetracaine upon complexation was explained by cyclodextrin blocking the mechanism of drug toxicity causing cell death. We tend to believe that in our case a similar mechanism took place when cyclodextrin was strongly shielding the cytotoxic effect of compound **4** following complexation.

Imaging of living cells using 4_CD solution

Two cell lines (Hela and NHDF) were used to characterize the staining efficiency of living cells by **4_CD**. Five solutions with different concentrations (17.4 µM, 34.8 µM, 52.2 µM, 69.6 µM and 86,9 µM) of compound **4_CD** were added to both investigated cell lines and wells were imaged simultaneously over 15 min and 24 hours with an inverted Leica DMI 3000 B microscope with fluorescence GFP filter. The comparative HeLa and NHDF cell uptake of compound **4_CD** after 15 min and 24 hours is shown in figure 5. The obtained results clearly indicated the penetration of **4_CD** through membranes of both investigated cell lines, showing a similar distribution of compound **4_CD** at 15 min incubation time point at all the investigated concentrations, primarily indicating uniform extranuclear localization (figures 5, (left) and S5-6).



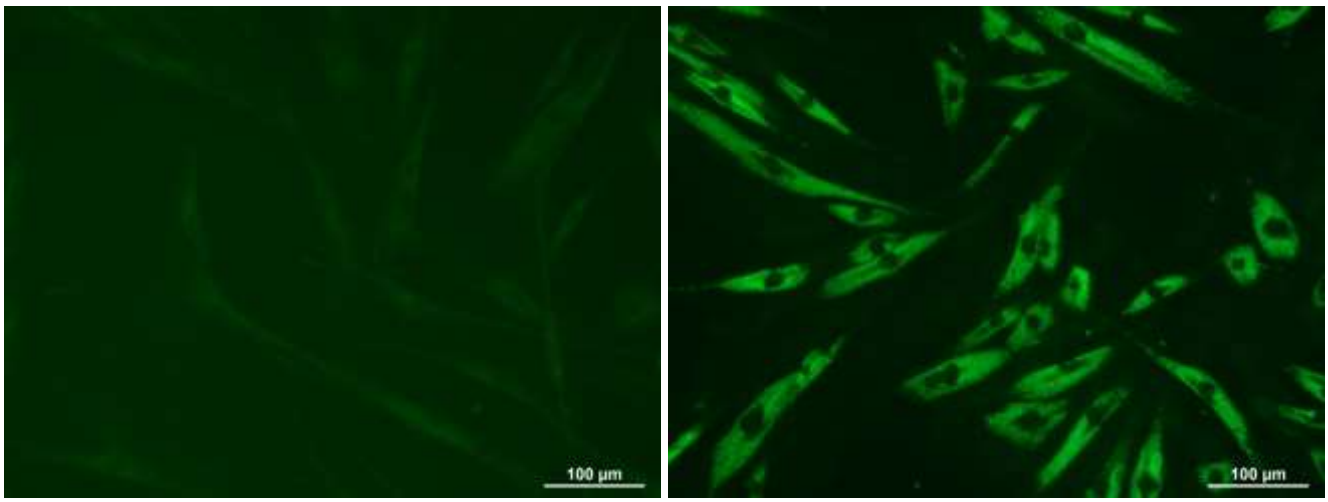


Figure 5. Compound **4_CD** uptake into HeLa (up) and NHDF (down) cell lines after 15 min (left) and 24 hours (right) incubation.

The picture changes dramatically after 24 hours of incubation. However, an extranuclear localization of the dye but with clear accumulations within the cytoplasm (figures 5, (right) and S7-8) is still observed. Additionally, an overall strong increase in the brightness of both investigated cell lines was observed when comparing images after 15 min and 24 hours at the same concentration of **4_CD**. Interestingly, in case of NHDF cells, the bright accumulations within cytoplasm are smaller and more uniform than the ones observed in HeLa cells. Large and localized accumulations of **4_CD** in HeLa cells indicated a specific interaction of the dye with particular cell components. Taking into account the nature of **4_CD** and its property to possess higher fluorescent intensity at lower pH, we suppose that accumulation of the dye takes place preferentially into the acidic organelles of the cell. To check this assumption, a co-staining experiment was performed with LysoTracker Red DND-99, a commercial agent used to stain cell acidic organelles.[29,30] Thus, HeLa cells stained with **4_CD** for 24 hours were subsequently treated with 75nM LysoTracker Red DND-99 (Invitrogen) for 30 minutes at 37°C in 5% CO_2. Cells were then washed three times in PBS and imaged in fresh cell culture medium using an inverted Leica DMI 3000 B microscope equipped with GFP and N2.1 filters (figure 6, (left) and (middle)).

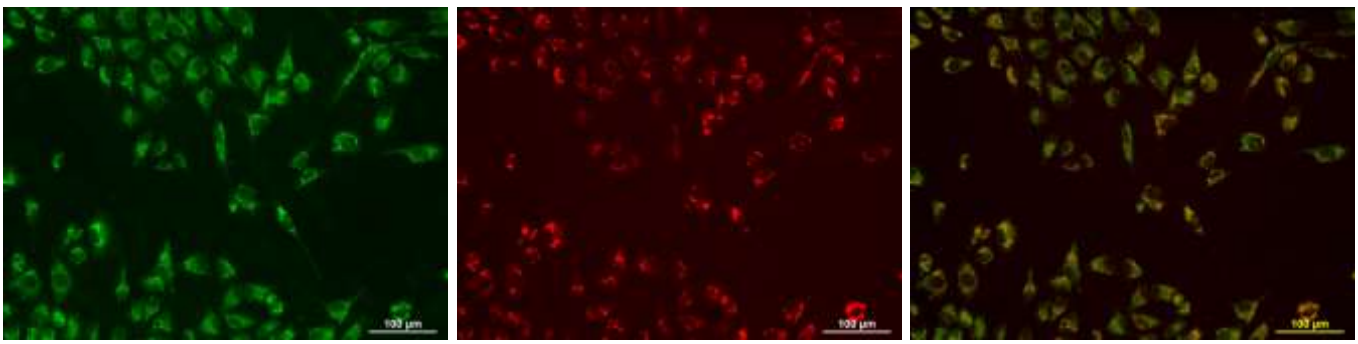


Figure 6. Cellular accumulation and distribution in HeLa cells of: left – **4_CD** after 24 h treatment (GFP filter); middle – subsequent addition of LysoTracker Red DND-99 after 30 min treatment (N2.1 filter); right – overlapped images obtained from both GFP and N2.1 filters.

Overlapping the images stained with **4_CD** and with LysoTracker Red DND-99 (figure 6, (right)) a similar accumulation of both staining agents in the same localized spots of the investigated HeLa cells was detected. The obtained results supported our suggestion on the specific accumulations into the acidic organelles of the cell. A more detailed investigation about the specificity of cell organelles staining agents revealed that classes of compounds containing pyridinium salt moieties are efficient fluorescent probes for mitochondrial staining of living cells.[31] Among the most efficient ones, OBEP,[32] M-DPT[33] and PTZ-Cy2[34] could be mentioned. The similarity of the **4_CD** structure with the reported mitochondrial staining agents suggests an in deep investigation on the possibility of the **4_CD** compound or its derivatives to specifically target mitochondria. Our future efforts will be thus focused on the synthesis and testing of the non-toxic fluorescent derivatives based on pyridyl indolizinic core, the formation of the corresponding cyclodextrin inclusion complexes and their application in staining specific cell organelles.

Conclusions

The preparation and characterization of a host-guest inclusion complex between fluorescent indolizinyl-pyridinium salt and β-cyclodextrin is described. The formation of the inclusion complex was investigated by ESI-MS experiments and molecular docking studies, proving the formation of the 1:1 and 2:1 host guest species. Several interesting features of the investigated fluorescent indolizinyl-pyridinium salt/β-cyclodextrin inclusion complexes – absence of cytotoxicity, cellular permeability, long-lived intracellular fluorescence and selective accumulation within acidic organelles – identified them as remarkable candidates for intracellular labelling of acidic organelles (lysosomes or mitochondria). Unlike tested commercially available acidic organelles marker Lysotracker Red, we did not observe a decrease in fluorescent signal in the investigated time frame (48 hours), this suggesting an excellent intracellular dye stability. Time dependent increase in fluorescent intensity and observed slow accumulation time of the inclusion complexes in acidic organelles might be useful in developing assays for the investigation and evaluation of lysosomal morphology and trafficking in intact cells. Furthermore, the structural similarity of the proposed systems with the already reported staining agents containing pyridinium quaternary salt moiety might also suggest the accumulation of the investigated systems inside the mitochondria. Taking into account that a number of reported or commercially available cell staining dyes with low toxicity and solubility have already been used in cyclodextrin formulations with good results on the improvement of their properties, our results showed that this approach may also work with new highly toxic dyes able to form host-guest inclusion complexes with cyclodextrins. We propose this methodology to be used to extend the number of fluorescent molecules currently considered for cells or cell component labelling or staining.

Conflicts of interest

There are no conflicts of interest to declare.

Acknowledgements

This work was supported by a grant of the Romanian National Authority for Scientific Research and Innovation, CNCS – UEFISCDI, project number PN-II-RU-TE-2014-4-1444. This publication is also part of a project that has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement N°667387 WIDESPREAD 2-2014 SupraChem Lab.

References

1. T. Xia, N. Li and X. Fang, *Annual Review of Physical Chemistry*, 2013, **64**, 459-480.
2. E. M. Stennett, M. A. Ciuba and M. Levitus, *Chemical Society Reviews*, 2014, **43**, 1057-1075.
3. G. K. Vegesna, S. R. Sripathi, J. Zhang, S. Zhu, W. He, F.-T. Luo, W. J. Jahng, M. Frost and H. Liu, *ACS Applied Materials & Interfaces*, 2013, **5**, 4107-4112.
4. W. Qin, D. Ding, J. Liu, W. Z. Yuan, Y. Hu, B. Liu and B. Z. Tang, *Advanced Functional Materials*, 2012, **22**, 771-779.
5. Z. X. Zhang, X. F. Guo, H. Wang and H. S. Zhang, *Analytical Chemistry*, 2015, **87**, 3989-3995.
6. X. Feng, L. Liu, S. Wang and D. Zhu, *Chemical Society Reviews*, 2010, **39**, 2411-2419.
7. S. Maruyama, K. Kikuchi, T. Hirano, Y. Urano and T. Nagano, *Journal of the American Chemical Society*, 2002, **124**, 10650-10651.
8. C. C. Woodroofe, R. Masalha, K. R. Barnes, C. J. Frederickson and S. J. Lippard, *Chemistry & Biology*, 2004, **11**, 1659-1666.
9. S. K. Konda, R. Maliki, S. McGrath, B. S. Parker, T. Robinson, A. Spurling, A. Cheong, P. Lock, P. J. Pigram, D. R. Phillips, L. Wallace, A. I. Day, J. G. Collins and S. M. Cutts, *ACS Medicinal Chemistry Letters*, 2017, **8**, 538-542.
10. S. Ghosh, S. Chattoraj and N. Chattopadhyay, *Analyst*, 2014, **139**, 5664-5668.
11. C. Di Donato, M. Lavorgna, R. Fattorusso, C. Isernia, M. Isidori, G. Malgieri, C. Piscitelli, C. Russo, L. Russo and R. Iacovino, *Molecules*, 2016, **21**, 1644.
12. M. Edetsberger, M. Knapp, E. Gaubitzer, C. Miksch, K. E. Gvichiya and G. Köhler, *Journal of Inclusion Phenomena and Macrocyclic Chemistry*, 2011, **70**, 327-331.
13. G. Grabner, S. Monti, G. Marconi, B. Mayer, C. Klein and G. Köhler, *The Journal of Physical Chemistry*, 1996, **100**, 20068-20075.
14. G. Marconi, S. Monti, B. Mayer and G. Koehler, *The Journal of Physical Chemistry*, 1995, **99**, 3943-3950.
15. R. Zidovetzki and I. Levitan, *Biochimica et Biophysica Acta*, 2007, **1768**, 1311-1324.
16. W. Xu, X. Zhu, G. Wang, C. Sun, Q. Zheng, H. Yang and N. Fu, *RSC Advances*, 2014, **4**, 52690-52693.
17. H. Yan, L. He, W. Zhao, J. Li, Y. Xiao, R. Yang and W. Tan, *Analytical Chemistry*, 2014, **86**, 11440-11450.
18. A. Rotaru, I. Druta, E. Avram, and R. Danac, *Arkivoc*, 2009, **xiii**, 287-299.
19. N.-L. Marangoci, L. Popovici, E.-L. Ursu, R. Danac, L. Clima, C. Cojocaru, A. Coroaba, A. Neamtu, I. Mangalagiu, M. Pinteala and A. Rotaru, *Tetrahedron*, 2016, **72**, 8215-8222.
20. R. Danac and I. Mangalagiu, *European Journal of Medicinal Chemistry*, 2014, **74**, 664-670.
21. V. V. A. M. Olaru, R. Danac, I.I. Mangalagiu, *Journal of Enzyme Inhibition and Medicinal Chemistry*, 2017, in press, doi: 10.1080/14756366.2017.1375483.
22. C. A. Schalley, *International Journal of Mass Spectrometry*, 2000, **194**, 11-39.

23. V. Gabelica, N. Galic and E. De Pauw, *Journal of the American Society for Mass Spectrometry*, 2002, **13**, 946-953.
24. O. Trott and A. J. Olson, *Journal of Computational Chemistry*, 2010, **31**, 455-461.
25. YASARA, Yet Another Scientific Artificial Reality Application: Molecular graphics, modeling and simulation program, http://www.yasara.org/.
26. HyperChem(TM), Release 8.0, 1115 NW 4th Street, Gainesville, Florida 32601, USA, http://www.hyper.com/.
27. M. Shah, V. Shah, A. Ghosh, Z. Zhang and T. Minko, *Journal of Pharmaceutics & Pharmacology*, 2015, **2**, 8.
28. R. A. Franco de Lima, M. B. de Jesus, C. M. Saia Cereda, G. R. Tofoli, L. F. Cabeça, I. Mazzaro, L. F. Fraceto and E. de Paula, *Journal of Drug Targeting*, 2012, **20**, 85-96.
29. O. Goldshmidt, L. Nadav, H. Aingorn, C. Irit, N. Feinstein, N. Ilan, E. Zamir, B. Geiger, I. Vlodavsky and B. Z. Katz, *Experimental Cell Research*, 2002, **281**, 50-62.
30. N. Raben, L. Shea, V. Hill and P. Plotz, *Methods in Enzymology*, 2009, **453**, 417-449.
31. H. Zhu, J. Fan, J. Du and X. Peng, *Accounts of Chemical Research*, 2016, **49**, 2115-2126.
32. S. Zhang, T. Wu, J. Fan, Z. Li, N. Jiang, J. Wang, B. Dou, S. Sun, F. Song and X. Peng, *Organic & Biomolecular Chemistry*, 2013, **11**, 555-558.
33. N. Jiang, J. Fan, T. Liu, J. Cao, B. Qiao, J. Wang, P. Gao and X. Peng, *Chemical Communications*, 2013, **49**, 10620-10622.
34. F. Liu, T. Wu, J. Cao, H. Zhang, M. Hu, S. Sun, F. Song, J. Fan, J. Wang and X. Peng, *Analyst*, 2013, **138**, 775-778.