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ARTICLE TYPE

Interaction of β-cyclodextrin with nile red in a single live CHO cell: An initiative towards developing a prospective strategy for excretion of adsorbed drugs from the cell membrane

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A successful endeavour has been made to develop a prospective strategy to oust the drug molecules adsorbed on the cell membranes simply by using the non-toxic β-cyclodextrin (β-CD). For the purpose, fluorescent probes of different geometries and charge characteristics, namely phenosafranin (PSF) and nile red (NR), were exploited for the in-vitro studies using different lipids as model membranes. Considering the success of the in-vitro study, the present work is extended to a live Chinese Hamster Ovary (CHO) cell using the dye NR acting as a model drug system. Steady state and time resolved confocal microscopic studies reveal that with the introduction of β-CD, some of the adsorbed dye molecules are dislodged from the CHO cell membrane to the nanocavity of β-CD resulting in the formation of dye—β-CD inclusion complex. This study promises development of a prospective strategy for removal of the adsorbed drugs from the cell membranes.

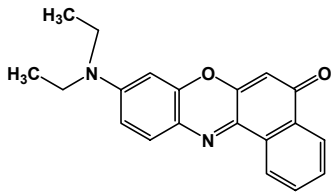
In the coming years, prime concern of biophysical and pharmaceutical research is apprehended to be finding avenues to get rid of the drug induced toxicity and the adverse side effects of the excess drugs deposited in our body, especially in the cell membranes. The severe problem can be tackled from two angles. Firstly, to look for proper and effective drug delivery systems to port the drug directly to the targeted zone so that the required dose is reduced, and secondly, to develop a strategy to remove the excess drugs adsorbed on the cell membranes. In the first context, for the last two decades accomplishments have been made in the arena of drug research in developing a wide variety of targeted drug delivery systems (DDS) composed of nanoscale materials like liposomes, micelles, dendrimers, carbon nanotubes etc.[1-5] The DDS serve as beneficial vehicles for increasing the efficacy of chemotherapeutics through improved pharmacokinetics and biodistribution, reducing the drug toxicity since the drug is only active in the target domain of the body (for example, in cancerous tissues).[1] However, not much work has been done in the second context, i.e., excretion of excess and unused drugs adsorbed in the body system to reduce or eradicate the drug toxicity or side effects. There are, of course, a few longstanding chemical methods for removing the excess drugs from the body system. For example, acidic drugs like barbiturates, salicylates, sulfonamides etc. are known to be excreted by making the urine alkaline by treating with bicarbonates and similarly, alkaline drugs like amphetamine, pethidine etc. can be removed from the body by making the urine acidic using ascorbic acid, ammonium chloride etc.[6] But, to the best of our knowledge, there is no such method for removing the adsorbed drug molecules from the cell membranes. Keeping this in mind, for the past few years, our group has been interested in developing a strategy to excrete the adsorbed drugs from the cell membrane, rather easily, by using cyclodextrins which are non toxic oligosaccharides and are well known for their ability to form inclusion complexes with variety of probes. Although CDs have been shown to remove cellular cholesterol by host-guest inclusion complexation mechanism[7], majority of the literature reports go in favour of the non-destructive character of CDs towards the cell components. On this basis, CDs are often used as DDS for controlled and safe drug delivery.[8-10] A recent study from our group has also established CDs to be safe for the serum proteins from the structural point of view.[11]

Since lipids are the principal constituents of the cell membranes, in our early in-vitro works, lipids like dimyristoyl-L-α-phosphatidylglycerol (DMPG), and dimyristoyl-L-α-phosphatidylcholine (DMPC) differing in charge characteristics and fluorescent probes of varying geometry and charge characteristics like cationic phenosafranin (PSF) and non-ionic nile red (NR) have been exploited as models of the membranes and drugs respectively.[12,13] Series of techniques like steady state fluorescence and fluorescence anisotropy, micropolarity determination, fluorescence quenching, dynamic light scattering and time resolved flurometry reveal that addition of β-CD to the probe—lipid complexes leads to the complete or partial removal of the probes from the aforesaid complexes resulting in the formation of probe—β-CD inclusion complexes.[12,13] Looking at the success of removal of the probes (PSF and NR) from the different lipid membranes, we have been inspired to extend the study further to cellular level to find if the strategy is applicable in real biological systems like a live Chinese Hamster Ovary (CHO) cell.

The fluorescent nonionic dye from the phenoxazone family, nile red (NR) (Scheme 1) has been extensively used as a staining agent in biological tissues.[14] The photophysical properties of NR have also been the subject of many studies because of the intense solvent-dependence of its fluorescence enabling the probe to be

used conveniently to study the polarity related aspects in the microheterogeneous environments including micelles, reverse micelles, cyclodextrins, zeolites, etc.[15-19]. It has also been exploited to explore the heterogeneity of membranes[20,21] and to study the formation of dendrimer-surfactant supramolecular assemblies.[22] The solvent sensitivity of the probe in terms of its fluorometric characteristics provides an easy way to assess the location of it in microheterogeneous media.[13,15-19]

Single molecule spectroscopy (SMS) provides new avenues to study various photobiological aspects in a live cell. Phenomena like fluorescence lifetime trajectory during transcription,[23] lipid rafts in live cell,[24] diffusion in different regions of cell,[25] nanoscale dynamics of membrane lipids in a live cell,[26] protein-binding dynamic[27] etc. have been studied through SMS. For our purpose in the present work, we have exploited steady-state and time-resolved confocal microscopy to study the interaction of β-cyclodextrin with nile red in a single live Chinese hamster ovary (CHO) cell. Experimental details including cell preparation, staining procedure and confocal microscopic technique have been described in the Electronic Supplementary Information (ESI).



Scheme 1: **Structure of nile red (NR)**

Fig. 1(A) shows the confocal FLIM (fluorescence lifetime imaging microscopy) image of a NR stained CHO cell. The different colors in the confocal microscopic image of NR stained CHO cell indicates different lifetimes of NR in the various parts of the cell.[28] From the image, different regions of the cell are clearly visible – nucleus, cytoplasm, membrane and a few bright spots which are ascribed to lipid droplets.[29,30] It is evident that NR is distributed almost uniformly throughout the membrane of the cell suggesting a significant binding of the dye to the constituting lipid of the membrane. This is in tune with our *in-vitro* results where NR exhibits a strong binding with both DMPG and DMPC lipids.[13] Fig. 2(A) depicts the steady state emission spectra of NR in different media like phosphate buffered saline (PBS) solution, 6 mM β-CD in PBS solution, CHO cell membrane and membrane together with β-CD solution. As already stated, the position of the emission band maximum of NR is sensitive to the polarity of the medium.[16] In aqueous buffer NR exhibits an emission maximum at around 660 nm.[13] The emission maximum of NR in the β-CD medium does not reveal a big shift in the peak position. In the CHO cell membrane, NR shows two emission maxima; one remaining in the same position as that in PBS solution, assigned to the free dye while the other much blue shifted one peaking at ~ 610 nm. This blue-shifted band arises due to the NR bound to the hydrophobic region within the CHO cell membrane. The whole set of data qualitatively agrees our in-vitro results.[13]

The appreciable blue shift in the emission maximum of NR in the cell membrane implies that the dye experiences a much less polar environment within the membrane compared to the aqueous buffer milieu. Upon addition of β-CD on the membrane bound NR, a perceptible change in the relative emission intensities at the two band positions is observed (Fig. 2). The 610 nm band is diminished drastically relative to the band peaking at 655 nm, close to the position observed in the β-CD medium.[13] This drastic modification of the emission maximum in the presence of β-CD is rationalized from the consideration of a competitive binding of the probe between the cell membrane and β-CD environment. Thus, in the presence of β-CD, the membrane bound NR molecules are dislodged from the CHO cell to get encapsulated in the nanocavity of β-CD leading to the drastic decrease in the fluorescence intensity of the 610 nm emission band. The small blue shift in the emission maximum of the final solution (membrane + β-CD) relative to the position in aqueous buffer (Fig. 2(A)) corroborates the formation of NR—β-CD inclusion complex.[13]

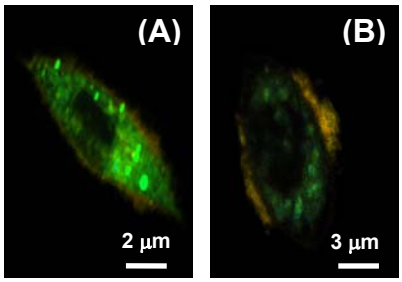


Fig. 1 Confocal FLIM images of single Chinese Hamster Ovary (CHO) cell stained by NR in absence (A) and in the presence of β-CD (B). The white line shows scale bar. Both the images are taken with same exposure time (at λ_{exc} = 470 nm) and same laser power.

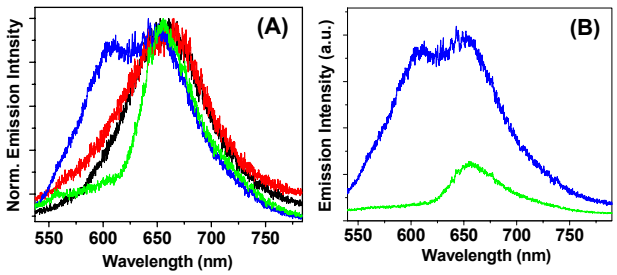


Fig. 2 (A) Normalized emission spectra of NR in buffer (black), β-CD (red), CHO cell membrane (blue) and CHO cell membrane in presence of 6 mM β-CD (green). (B) Un-nornmalized emission spectra of NR in CHO cell membrane in the absence (blue) and presence of 6 mM β-CD (green). Both the spectra are taken at same laser power (λexc = 470 nm).

Fig. 1 (B) represents the confocal FLIM image of the single CHO cell stained by NR upon addition of β-CD. The reduction in the intensity of the membrane bound dye in the presence of β-CD is also evident from a comparison of the fluorescence spectra of dye in the two environments (Fig. 2(B)). Since in Fig. 1, we have presented the two images of the single CHO cell in the same intensity scale the image after addition of β-CD appears relatively pale. The two yellow patches on both sides and away from the cell membrane depict the NR—β-CD inclusion complex. Clustering of β-CD is resulted because of the inhomogeneity of the medium as mentioned in the Experimental Section (ESI). Thus, the steady state emission study unambiguously divulge that

introduction of β-CD leads to removal of the bound probe from the cell membrane resulting in the formation of probe—β-CD complex.

Time resolved measurement serves as a tool to unearth the local environment of the dye within microheterogeneous environments. A positive indication from the steady state confocal microscopic study that NR is expelled from the CHO cell membrane upon addition of β-CD prompted us to corroborate it from the fluorescence lifetimes of the probe in the aforesaid environments. The burst integrated fluorescence lifetime (BIFL) histograms of membrane bound NR in the absence and presence of β-CD are represented in Fig. 3 and the corresponding decay parameters are given in Table 1. In buffer solution lifetime of NR

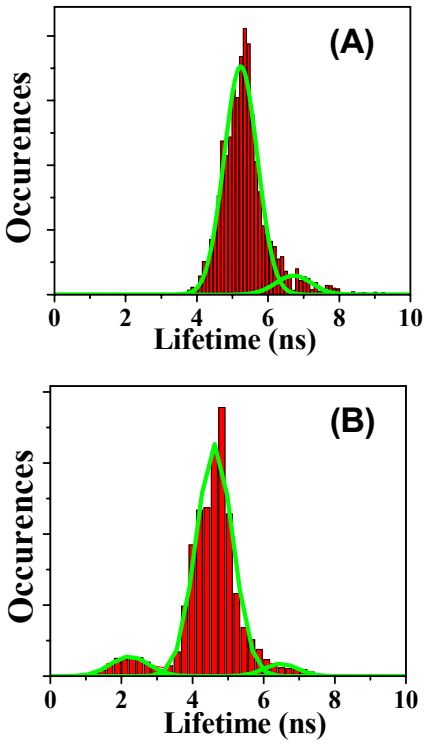


Fig. 3 Lifetime histogram of NR in CHO cell membrane in the (A) absence and (B) presence of β-CD. Excitation wavelength was 470 nm.

Table 1. Lifetime values of NR in different conditions in a CHO cell membrane

	τ_1(ns)	a_1	τ_2(ns)	a_2	τ_3(ns)	a_3
membrane	5.23	0.92	6.72	0.08		
membrane in presence of β-CD	4.62	0.88	6.48	0.04	2.23	0.08

comes out to be 0.68 ns, in well agreement with the existing literature.[13,17] In β-CD environment NR shows a bi-exponential fluorescence decay giving two lifetime values, the average being 2.40 ns. In CHO cell membrane also NR gives two lifetime components with values of 5.23 ns and 6.72 ns (Table 1). As is known, multiple lifetimes of the polarity-sensitive probes often originate because of the location of the probe in different polarity regions within the heterogeneous environments.[31] A glance on Table 1 reflects that in the presence of added β-CD the values of the two lifetime components of membrane bound NR are reduced marginally. Interestingly, we observe a third lifetime component with a much lower value of 2.23 ns (Fig. 3(B)). This lifetime value qualitatively agrees with the $\tau_{average}$ value of 2.12 (± 0.3) ns observed for NR in β-CD ($\tau_{average}$ = 2.40 ns in the present experiment and $\tau_{average}$ = 1.84 ns in the previous experiment[13]) and is assigned to the NR encapsulated in the β-CD cavity. The observation of this lifetime component is more important than the relative abundance of it since the latter depends on many factors like position of monitoring, relative binding affinities of the probe with the CHO cell membrane and the β-CD environments, absolute concentration of β-CD etc. Of course, one can reasonably presume that the extent of removal of the probe from the cell membrane upon addition of CDs should depend on the specific CD chosen for the purpose, since this controls the relative binding affinities of the probe between the two environments. In the present study, we have opted for β-CD over other CDs because of the suitable matching of the size of the probe with the cavity size of β-CD resulting in the formation of 1:1 inclusion complex.[19]

Conclusions

The present study reveals that NR bound to the membrane of a live CHO cell can be expelled by simply using the β-cyclodextrin. This is in tune with our previous studies where dyes were removed from DMPG and DMPC lipids. The degree of expulsion can be controlled as well, since it depends on the relative binding affinities of the probe with the cell membrane and the CDs. Non-toxicity of the external agent (cyclodextrins) on one hand and the high water solubility of the CDs on the other promises to make the strategy very effective since the drug molecules extracted from the cell membranes and making the inclusion complexes with the CDs can be excreted from the body easily through the normal water ejection channels. Establishment of the observation to be general for various fluorescent probes / drug molecules and a range of lipids / cell-membranes along with a variation of the extractor (like different CDs) should build a strong foundation for developing a strategy for the possible removal of adsorbed drug molecules from the live cell membranes. However, being the first attempt for the living cell system, the present exertion holds a colossal importance from the perspective of the drug research in the coming future.

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Notes and references

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1 V. V. Ranade, M. A. Hollinger, J. B. Cannon, *Drug delivery systems*, 2nd ed.; CRC, Press: Boca Raton, 2004.
2 C. K. Kim, P. Ghosh, C. Pagliuca, Z.-J. Zhu, S. Menichetti, V. M. Rotello, Entrapment of hydrophobic drugs in nanoparticle monolayers with efficient release into cancer cells, *J. Am. Chem. Soc.*, 2009, **131**, 1360-1361.
3 D. Peer, J. M. Karp, S. Hong, O. C. Farokhzad, R. Margalit, R. Langer, Nanocarriers as an emerging platform for cancer therapy, *Nat. Nanotechnol.*, 2007, **2**, 751-760.
4 A. Bianco, K. Kostarelos, M. Prato, Applications of carbon nanotubes in drug delivery, *Current opinion in Chemical Biology*, 2005**, 9**, 674-679.
5 R. Mehta, G. Lopez-Berestein, R. Hopfer, K. Mills, R. L. Juliano, Liposomal amphotericin B is toxic to fungal cells but not to mammalian cells, *Biochim. Biophys. Acta*, 1984, **770**, 230-234.
6 J. G. Buch, *Quick review of pharmacology*, 3rd Ver, 2010.pp 21.
7 R. Zidovetzki, I. Levitan, Use of cyclodextrins to manipulate plasma membrane cholesterol content: evidence, misconceptions and control strategies, *Biochim. Biophys. Acta.*, 2007, **1768(6)**, 1311-1324.
8 F. J. Otero-Espinar, J. J. Torres-Labandeira, C. Alvarez-Lorenzo, J. Blanco-Méndez, Cyclodetrins in drug delivery systems, *J. Drug Del. Sci. Tech.*, 2010, **20**, 289-301.
9 G. Tiwari, R. Tiwari, A. K. Rai, Cyclodetrins in delivery systems: Applications, *J. Pharm. Bioallied. Sci.*, 2010, **2,** 72-79.
10 F. Hirayama, K. Uekama, Cyclodextrin-based controlled drug release system, *Advanced Drug Delivery Reviews*, 1999, **36**, 125-141.
11 S. Ghosh, B. K. Paul, N. Chattopadhyay, Interaction of cyclodextrins with human and bovine serum albumin: A combined spectroscopic and computational investigation, *J. Chem. Sci.*, 2014, **126**, 931-944.
12 D. Sarkar, D. Bose, A. Mahata, D. Ghosh, N. Chattopadhyay, Differential interaction of β-cyclodextrin with lipids of varying surface charges: A spectral deciphering using a cationic phenazinium dye, *J. Phys. Chem. B*, 2010, **114**, 2261-2269.
13 B. Jana, S. Ghosh, N. Chattopadhyay, Competitive binding of nile red between lipids and β-cyclodextrin, *J. Photochem. Photobiol. B: Biology*, 2013, **126**, 1-10.
14 P. Greenspan, E. P. Mayer, S. D. Fowler, Nile red: A selective fluorescent stain for intracellular lipid droplets, *J. Cell Biol.*, 1985, **100**, 965-973.
15 J. L. Meinershagen, T. Bein, Optical sensing in nanopores. encapsulation of the solvatochromic dye nile red in zeolites, *J. Am. Chem. Soc.*, 1999, **121**, 448-449.
16 N. Sarkar, K. Das, D. N. Nath, K. Bhattacharyya, Twisted charge transfer processes of nile red in homogeneous solutions and in faujasite zeolite, *Langmuir*, 1994, **10**, 326-329.
17 A. Datta, D. Mandal, S. K. Pal, K. Bhattacharyya, Intramolecular charge transfer processes in confined systems. Nile red in reverse micelles, *J. Phys. Chem. B*, 1997, **101**, 10221-10225.
18 N. C. Maiti, M. M. G. Krishna, P. J. Britto, N. Periasamy, Fluorescence dynamics of dye probes in micelles, *J. Phys. Chem. B*, 1997, **101**, 11051-11060.
19 P. Hazra, D. Chakrabarty, A. Chakraborty, N. Sarkar, Intramolecular charge transfer and solvation dynamics of nile red in the nanocavity of cyclodextrins, *Chem. Phys. Letts.*, 2004, **388**, 150-157.
20 G. Krishnamoorthy, Probing the link between proton transport and water content in lipid membranes, *J. Phys. Chem. B*, 2001, **105**, 1484-1488.
21 O. A. Kucherak, S. Oncul, Z. Darwich, D. A. Yushchenko, Y. Arntz, P. Didier, Y. Mely, A. S. Klymchenko, Switchable nile red – based probe for cholesterol and lipid order at the outer leaflet of biomembranes, *J. Am. Chem. Soc.*, 2010, **132**, 4907-4916.
22 D. M. Watkins, Y. Sayed-Sweet, J. W. Klimash, N. J. Turro, D. A. Tomalia, Dendrimers with hydrophobic cores and the formation of supramolecular dendrimer- surfactant assemblies, *Langmuir*, 1997, **13**, 3136-3141.
23 M. Sorokina, H.-R. Koh, S. S. Patel, T. Ha, Fluorescence lifetime trajectories of a single fluorophore reveal reaction intermediates during transcription initiation, *J. Am. Chem. Soc.*, 2009, **131**, 9630-9631.
24 H. M. Kim, B. H. Jeong, J.-Y.Hyon, M. J. An, M. S. Seo,; J. H. Hong, K. J. Lee, C. H. Kim, T. Joo, S.-C. Hong, B. R. Cho, Two-photon fluorescent turn-on probe for lipid rafts in live cell and tissue, *J. Am. Chem. Soc.*, 2008, **130**, 4246-4247.
25 S. A. Kim, K. G. Heinze, P. Schwille, Fluorescence correlation spectroscopy in living cells, *Nat. Methods*, 2007, **4**, 963-973.
26 C. Eggeling, C. Ringemann, R. Medda, G. Schwarzmann, K. Sandhoff, S. Polyakova, V. N. Belov, B. Hein, C. V. Middendorff, A. Schonle, S. W. Hell, Direct observation of the nanoscale dynamics of membrane lipids in a living cell, *Nature*, 2009, **457**, 1159-1163.
27 Y. Philip, V. Kiss, G. Schreiber, Protein-binding dynamics imaged in a living cell, *Proc. Natl. Acad. Sci. U.S.A.*, 2012, **109**, 1461-1466.
28 S. Chattoraj, S. Saha, S. S. Jana, K. Bhattacharyya, Dynamics of gene silencing in a live cell: Stochastic resonance, *J. Phys. Chem. Lett.*, 2014, **5**, 1012-1016.
29 S. Ghosh, S. Chattoraj, T. Mondal, K. Bhattacharyya, Dynamics in cytoplasm, nucleus, and lipid droplet of a live CHO cell: Time-resolved confocal microscopy, *Langmuir*, 2013, **29**, 7975-7982.
30 D. K. Sasmal, S. Ghosh, A. K. Das, K. Bhattacharyya, Solvation dynamics of biological water in a single live cell under a confocal microscope, *Langmuir*, 2013, **29**, 2289-2298.
31 A. Chakrabarty, A. Mallick, B. Haldar, P. Das, N. Chattopadhyay, Binding interaction of a biological photosensitizer with serum albumins: A biophysical study, *Biomacromolecules*, 2007, **8**, 920-927.

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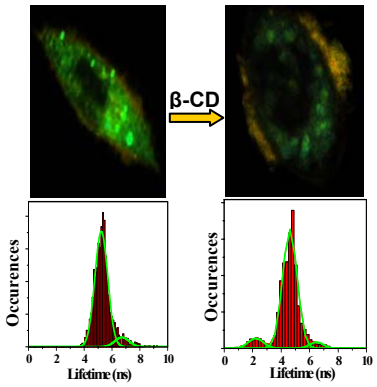

Steady state and time-resolved confocal microscopic studies in a single live Chinese Hamster Ovary (CHO) cell suggest that the biological staining dye nile red (NR) adsorbed on the cell membrane can be excreted simply by using β-cyclodextrin.