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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 phenosafarin (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. 1,3 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, 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-phosphatidylethanolamine (DMPC) differing in charge characteristics and fluorescent probes of varying geometry and charge characteristics like cationic phenosafarin (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 fluorometry 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. It has also been exploited to explore the heterogeneity of membranes to study the formation of dendrimer-surfactant supramolecular assemblies. 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.

Single molecule spectroscopy (SMS) provides new avenues to study various photobiological aspects in a live cell. Phenomena like fluorescence lifetime trajectory during transcription, lipid rafts in live cell, diffusion in different regions of cell, nanoscale dynamics of membrane lipids in a live cell, protein-binding dynamic 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)](image)

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. 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. 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 DMPC and DMPC lipids. 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. In aqueous buffer NR exhibits an emission maximum at around 660 nm. 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.

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. 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.
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 comes out to be 0.68 ns, in well agreement with the existing literature. 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. 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). This lifetime value qualitatively agrees with the \( \tau_{\text{average}} \) value of 2.12 (± 0.3) ns observed for NR in β-CD (\( \tau_{\text{average}} = 2.40 \) ns in the present experiment and \( \tau_{\text{average}} = 1.84 \) ns in the previous experiment) 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.

**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

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.