The fluorescent probe NR12S detects changes in plasma membrane cholesterol levels

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- **The NR12S fluorescent membrane probe can be used to semi-quantify cholesterol levels in the plasma membranes of living cells**

Introduction

Cholesterol plays an important role in eukaryotic cell membrane organisation and function, and while distributed variably between cellular membranes it is enriched in the plasma membrane (PM)[1]. Several methods exist for measuring the total amount of cholesterol in cells, for example the enzyme coupled Amplex® red cholesterol assay. Here we describe a method for investigating changes in plasma membrane cholesterol levels using the lipophilic fluorescent probe NR12S[2] on the BMG LABTECH microplate reader FLUOstar® Omega. We have illustrated the efficacy of the NR12S-assay by depleting cells of cholesterol using a combination of treatments: incubation with methyl-ß-cyclodextrin (MßCD) and incubation in delipidated medium containing lovastatin. Methyl-ß-cyclodextrin (MßCD) has been widely used to remove cholesterol from cell plasma membranes[3] whereas lovastatin is an inhibitor of cholesterol biosynthesis[4].

Assay Principle

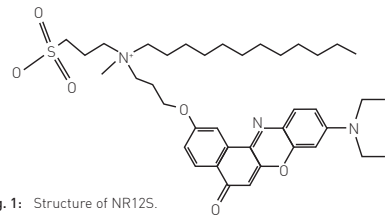

Fig. 1: Structure of NR12S.

NR12S is a Nile Red-based compound that can be used as a fluorescent membrane probe to measure lipid order changes in the cell plasma membrane. The fluorescence emission spectra of the compound changes in response to lipid order, shifting towards shorter wavelengths when incorporated into a liquid ordered (increased cholesterol) phase compared to a liquid disordered phase[5]. The zwitterionic head group and long alkyl chain mean that the compound selectively stains the outer leaflet of model and cell PM[2]. The NR12S probe changes its emission ratio (FIR560nm/630nm) as a function of cholesterol[2].

Materials & Methods

- 96 well flat bottomed black plates (Greiner)
- FLUOstar Omega microplate reader (BMG LABTECH)
- NR12S (Dr A Klymchenko, Universitié de Strasbourg)
- RPMI 1640 (phenol red-free, R7509) (Sigma Aldrich)

Untreated or lipid depleted human haematological cell lines (Daudi, Ramos and HSB-2) ($6x10^6$ per treatment) were washed twice in RPMI and re-suspended at $2x10^6$ cells/ml. Lower levels of plasma membrane cholesterol are expected in lipid deprived cells compared to control cells. For staining, an equal volume of 0.04µM NR12S, diluted just before use in RPMI, was added to the cells which were incubated in the dark at room temperature for 7 minutes. The cells were then washed twice in RPMI and re-suspended in 600µl RPMI. Aliquots (100µl) were added to 96 well black plates and fluorescence intensities were measured.

Instrument settings

Measurement Type:	Fluorescence intensity (Top)
Flashes per well:	10
Reading Mode:	Endpoint

Multichromatic

Excitation wavelength:	520nm
Emission wavelength 1:	560nm
Emission wavelength 2:	630nm
Gain:	2800
Positioning delay:	0.2 seconds

Results & Discussion

Methyl-ß-cyclodextrin (MßCD) was used to remove cholesterol from the PM of three different cell lines. Figure 2 shows that exposure of cells to increasing concentrations of MßCD for 1h caused a decrease in the NR12S fluorescence intensity ratio 560nm/630nm indicating a decrease in the amount of PM cholesterol.

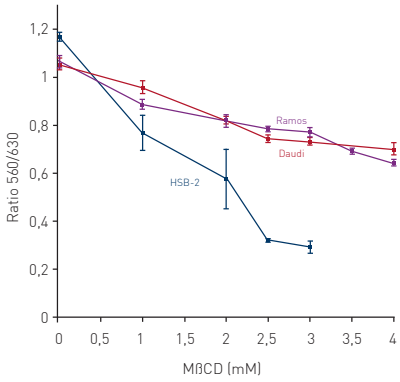

Fig. 2: Ratio of the emission intensities at 560 and 630nm of NR12S in Daudi (■), Ramos (■) and HSB-2 (■)cells incubated with MßCD for 1 hour.

Rev. 02/2017
Keywords: **cholesterol, fluidity, membrane**

Having established that removal of cholesterol from the PM decreased the 560nm/630nm fluorescence intensity ratio we investigated the effects of different treatments on Daudi cell membrane cholesterol levels. Conditions were optimised to deplete cells of cholesterol while maintaining their viability and proliferative capacity.
Cells were incubated with 1mM MβCD or with medium only for 1h before culturing them in medium with 10% FCS (R10) or in delipidated medium (dR10) with lovastatin, for 24h. Figure 3A shows a 10 % decrease in cells treated with MβCD and subsequently kept in full medium with 10 % FCS. Treatment with MβCD and incubation in lovastatin decreased plasma membrane cholesterol by 30%.

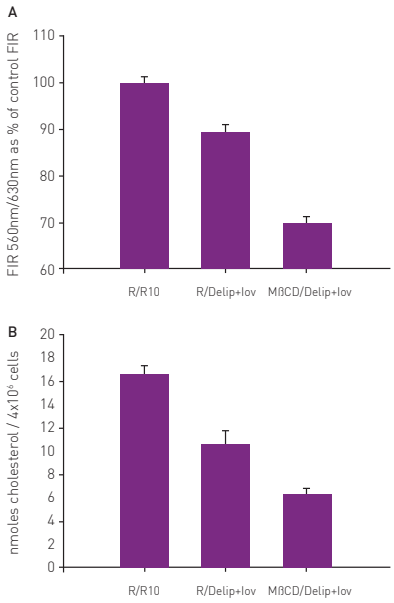

Fig. 3: **(A)** Plasma membrane cholesterol in lipid deprived Daudi cells The ratios of the emission intensities of NR12S at 560 and 630 nm are presented as a percentage of the ratio obtained for cells exposed to RPMI 1640 and incubated in R10. **(B)** nmoles cholesterol present in lipid deprived cells as determined by ESI MS.

Total cell cholesterol levels determined using electrospray ionisation (ESI) mass spectrometry (MS) (Fig. 3B) largely agree with results obtained using the NR12S assay to measure PM cholesterol. Daudi cells initially treated with MβCD and grown in dR10 with lovastatin contain less cholesterol than those exposed only to dR10 containing lovastatin. The cholesterol levels measured by ESI MS show a larger decrease because total cellular cholesterol is being measured.

Figure 4 shows the effects of returning lipid deprived cells to full medium after two, six and twenty four hours. After 6h there was a clear increase in the plasma membrane cholesterol of lipid deprived cells and this did not alter significantly after a further 18h in full medium.

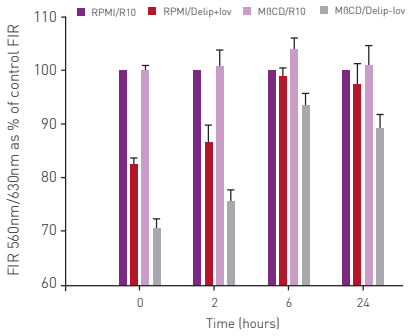

Fig. 4: **Repletion of cholesterol levels in lipid deprived Daudi cells.** The ratios of the emission intensities of NR12S at 560 and 630 nm are presented as a percentage of the ratio obtained for cells exposed to RPMI and incubated in R10 at the corresponding time point. FIR = Fluorescence intensity ratio

Conclusion

The fluorescent NR12S probe indirectly monitors changes in the plasma membrane cholesterol levels of living cells, thus expanding on data obtained by ESI-MS, which allows quantitation of total cholesterol in cell lysates. The sensitivity of the filter-based fluorescence measurement by the FLUOstar Omega ensures reliable detection of cholesterol-induced changes at the two emission wavelengths and is furthermore supported by accelerated ratio calculation using the MARS analysis software.

References

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