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Terbium(III)-Cholate Functionalized Vesicles as Luminescent Indicators for the Enzymatic Conversion of Dihydroxynaphthalene Diesters

Stefan Balk,[a] Uday Maitra[b*] and Burkhard König[a*]

[a] *Faculty of Chemistry and Pharmacy, University of Regensburg, 93040 Regensburg, Germany. Fax: +49 943 1717; Tel: +49 943 4576; E-mail: burkhard.koenig@ur.de*
[b] *Department of Organic Chemistry, Indian Institute of Science, Bangalore, India. Fax: 91-80-2360-0529; Tel: 91-80-2360-1968; E-mail: maitra@orgchem.iisc.ernet.in*

Supporting Information

General methods and material

Fluorescence measurements were performed with UV-grade solvents (Baker or Merck) in 1.0 or 0.4 cm quartz cuvettes (Perkin Elmer / Hellma) and recorded on a Horiba Fluoromax4 spectrophotometer with temperature control at 20 °C. Phosphorescence measurements were recorded on a Varian 'Cary Eclipse' fluorescence spectrophotometer (total decay time: 0.020 s, delay time 0.1 ms, gate time 5.0 ms, slit 20 nm) with temperature control (20 °C) using 1 cm quartz cuvettes (Hellma). DLS measurements were performed on a Malvern Zetasizer Nano at 20 °C using 1 cm disposable polystyrene cuvettes (VWR). Starting materials were used without any further purification. Phospholipids were purchased from Avanti Polar Lipids Inc. Commercially available solvents of standard quality and water of millipor quality were used. Naphthalene- 2,3,-diyl dihexanoate (DHNdhn) synthesis is described in the Ph.D. thesis of Mr. Sandip Bhowmik "Design and Applications of Bile-salt/Lanthanide based Hydrogels", Indian Institute of Science, Bangalore, February 2013. Naphthalene- 2,3,-diyl diacetate (DHNdac) and 3-hydroxynaphthalene-2-yl- b-glucoside (DHNglu): synthesis is described in the supporting information of Chem. Commun., 2012, 48, 4624 (Sandip Bhowmik, Uday Maitra). Candida rugosa lipase was obtained from Sigma-Aldrich (2.5 Units/mg; catalogue no. 90860-5G and Batch no. 445584/1). Beta-glucosidase from almonds was obtained from Sigma-Aldrich (6.5 Units/mg; catalogue no. 49290-250 MG and Batch no. BCBD2305V). $TbCl_3$ * 6 H_2O was obtained from Sigma Aldrich (catalogue no. 212903-5G, batch no. MKBD6300V). Time dependent enzyme conversions were measured via HPLC analysis on a Agilent 1290 Series HPLC: Column: Phenomenex Luna C18, 3 µm, 150 x 2.00 mm, 100 Å; flow: 0.3 mL/min; solvent A: H_2O [0.059 Gew% TFA], solvent B: MeCN; gradient: 0-20 min: A/B 97/3, 20-30 min: A/B 2/98.

Vesicles

Preparation

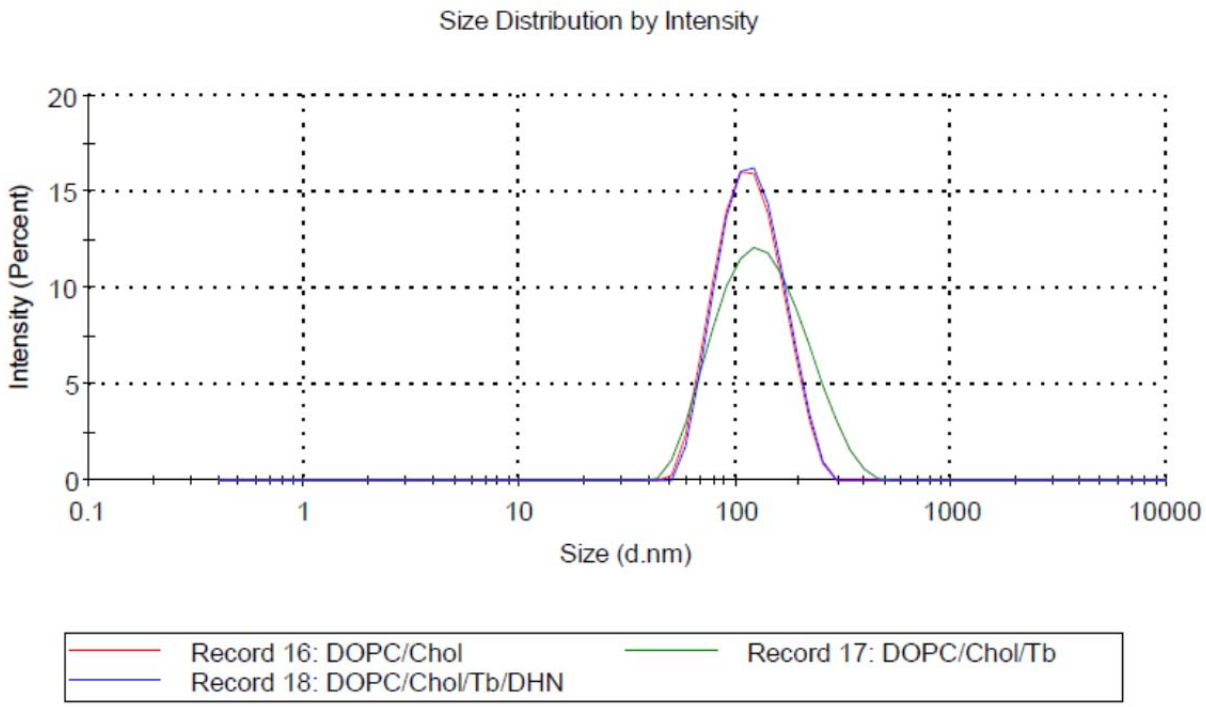
In small glass reaction vessels 1 - 5 µmol of DOPC or DSPC were dissolved in chloroform and optionally 15 mol% of dissolved sodium cholate was added and mixed. The solvent was completely removed under reduced pressure at 25 °C (DOPC) or 75˚C (DSPC) and an appropriate amount of buffer (HEPES 25 mM, pH 7.4) was added to obtain lipid concentrations of 5 mM. Vigorous shaking at 25 °C (DOPC) or 75 °C (DSPC) for 10 min yielded a turbid multi-lamellar vesicle suspension. Small uni-lamellar dispersions were obtained by extrusion through 100 nm-pore size polycarbonate membranes with a LiposoFast liposome extruder from Avestin. DOPC- cholate vesicles were equipped with 5 mol% of aqueous $TbCl_3$ * $6H_2O$ and stored for 10 min to ensure cholate - terbium-complexation. Alternative pure DOPC vesicles were post functionalized with an appropriate amount of cholate and after 10 min $TbCl_3$ * 6 H_2O was added. In order to induce a luminescent response of Tb^{3+}- gel functionalized vesicle solutions 2,3- dihydroxynaphthalene (DHN) was added.

Dynamic light scattering (DLS)

Vesicle size distributions were determined using dynamic light scattering (DLS). Vesicle **Vs1** ($C_{(DOPC)}$ = 5 mM, $C_{(Chol)}$ = 0.75 mM) was functionalized with Tb^{3+} (0.25 mM) and DHN (12.5 µM), which induced no change in the size distribution. This indicates that no aggregates beside vesicles with an average size distribution of about 110 nm are present.

Results

			Size (d.nm):	% Intensity:	St Dev (d.nm):
Z-Average (d.nm):	109,2	Peak 1:	121,7	100,0	40,71
PdI:	0,101	Peak 2:	0,000	0,0	0,000
Intercept:	0,961	Peak 3:	0,000	0,0	0,000
Result quality :	Good				



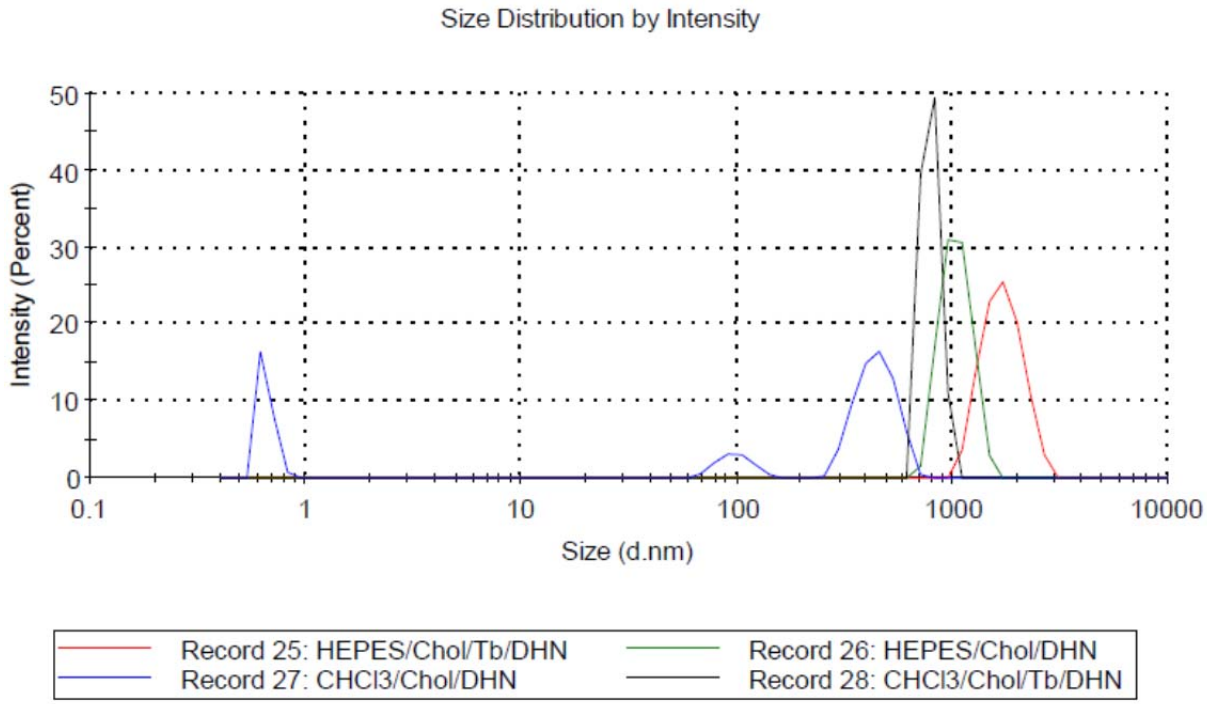
Scheme S1 Size distribution of **Vs1** ($C_{(DOPC)}$ = 5 mM, $C_{(Chol)}$ = 0.75 mM; red) before and after functionalization with Tb^{3+} (0.25 mM; blue) and additional DHN (12.5 µM; green).

For comparison, solutions of Chol (0.75 mM) in HEPES (25 mM, pH 7.4) and $CHCl_3$ have been preared in the presence of DHN (12.5 µM) before treatment with Tb^{3+} (0.25 mM). The particle size distribution of these solutions is not reproducible, has a high polydispersity index and indicates agglomeration of bile salts in aqueous and organic solvents.

Results

			Size (d.nm):	% Intensity:	St Dev (d.nm):
Z-Average (d.nm):	2024	Peak 1:	1730	100,0	363,7
PdI:	0,282	Peak 2:	0,000	0,0	0,000
Intercept:	0,898	Peak 3:	0,000	0,0	0,000

Result quality : Refer to quality report

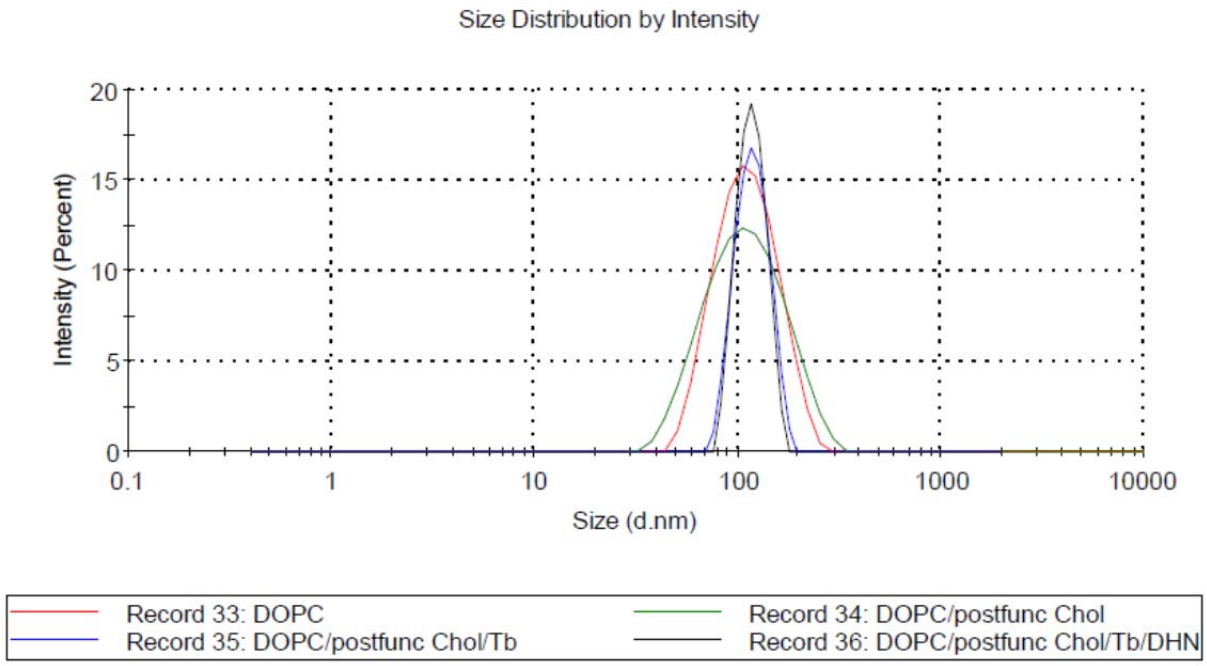


Scheme S2 Size distribution of Chol solutions ($C_{(Chol)}$ = 0.75 mM; red) with DHN (12.5 µM) in HEPES (green) and $CHCl_3$ (blue). After addition of Tb^{3+} (0.25 mM) to HEPES- Chol solution (red) and $CHCl_3$- Chol solution (black) an enlargement of aggregates is assumed.

The DOPC vesicle solution **Vs2** ($C_{(DOPC)}$ = 5 mM) was post functionalized with cholic acid (0.75 mM), Tb^{3+} (0.25 mM) and DHN (12.5 µM) giving a monodispers size distribution of vesicles.

Results

			Size (d.nm):	% Intensity:	St Dev (d.nm):
Z-Average (d.nm):	100,7	Peak 1:	116,1	100,0	39,62
PdI:	0,125	Peak 2:	0,000	0,0	0,000
Intercept:	0,947	Peak 3:	0,000	0,0	0,000
Result quality :	Good				



Scheme S3 Size distribution of **Vs2** ($C_{(DOPC)}$ = 5 mM; red) after addition of cholate (0.75 mM, green).

The aggregation of Chol (0.75 mM) in HEPES (25 mM, pH 7.4) or $CHCl_3$ after treatment with Tb^{3+} (0.25 mM) was obvious after a few minutes. In contrast, **Vs1** ($C_{(DOPC)}$ = 5 mM, $C_{(Chol)}$ = 0.75 mM) functionalized with Tb^{3+} (0.25 mM) remains a homogeneous monodispers solution.

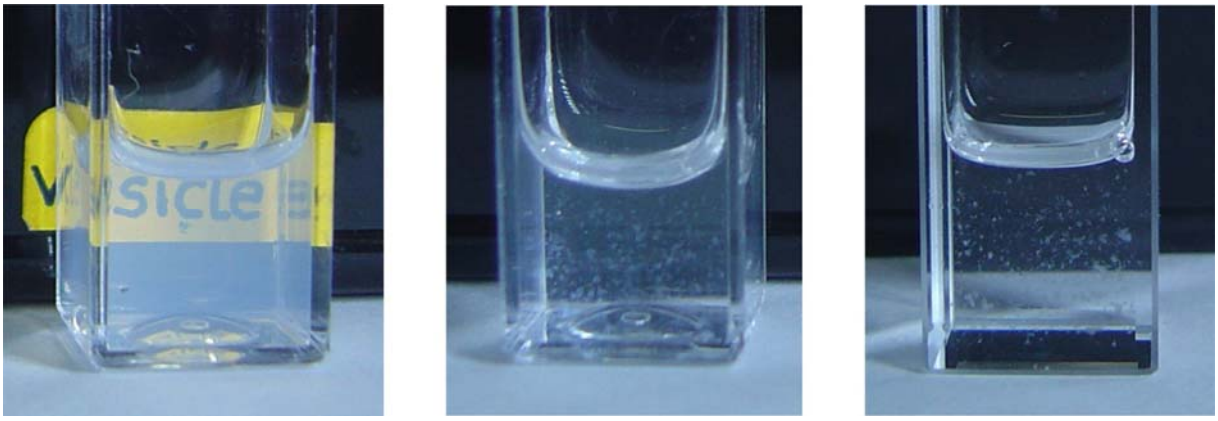
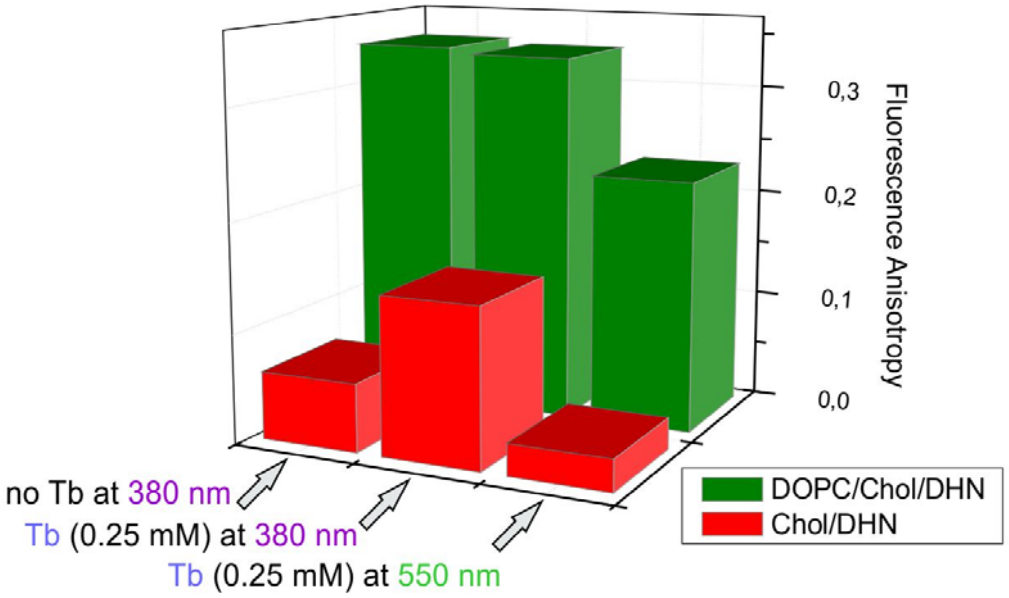


Figure S1 Solution of **Vs1** ($C_{(DOPC)}$ = 5 mM, $C_{(Chol)}$ = 0.75 mM; left) doped with Tb^{3+} (0.25 mM) yields a homogeneous monodispers solution. Chol- Tb^{3+} solutions in HEPES (25 mM, pH 7.4; middle) and $CHCl_3$ (right) indicate aggregate formation.

Fluorescence Anisotropy

The embedding of the luminescent cholic acid – terbium complexes in the vesicle membrane was confirmed by fluorescence anisotropy. The terbium complexes ($C_{(Tb^{3+})}$ = 0.25 mM) are excited by DHN (12.5 µM) at 335 nm and are bound to cholic acid doped vesicles **Vs4** ($c_{(DOPC)}$ = 5 mM, $c_{(Chol)}$ = 0.75 mM, $c_{(Tb^{3+})}$ = 0.25 mM) as they show a fluorescence anisotropy (Scheme S4, right; FA = 0.23), detected at 550 nm, which is about 8 times higher than for terbium in aqueous cholic acid solution (FA = 0.06; $C_{(Chol)}$ = 0.75 mM, $C_{(Tb^{3+})}$ = 0.25 mM, $C_{(DHN)}$ = 12.5 µM). Higher anisotropy values are detected at 380 nm for DHN (12.5 µM) with **Vs1** ($C_{(DOPC)}$ = 5 mM, $C_{(Chol)}$ = 0.75 mM) (Scheme S4, left; FA = 0.34) or in presence of 0.25 mM Tb^{3+} (Scheme S4, middle; FA = 0.33) than for aqueous cholate solutions (Scheme S4, left, $C_{(Chol)}$ = 0.75 mM, FA = 0.06 and middle $C_{(Chol)}$ = 0.75 mM, $C_{(Tb^{3+})}$ = 0.25 mM, FA = 0.03) indicate that DHN (12.5 µM) coordinates to the terbium(III)- cholate complex at the liposome membrane.



Scheme S4 Fluorescence anisotropy of DHN at 380 nm (left and middle) and Tb^{3+} at 550 nm (right) with cholate doped vesicles (green) is significant higher compared to aqueous solutions of Chol (red).

Phosphorescence measurements

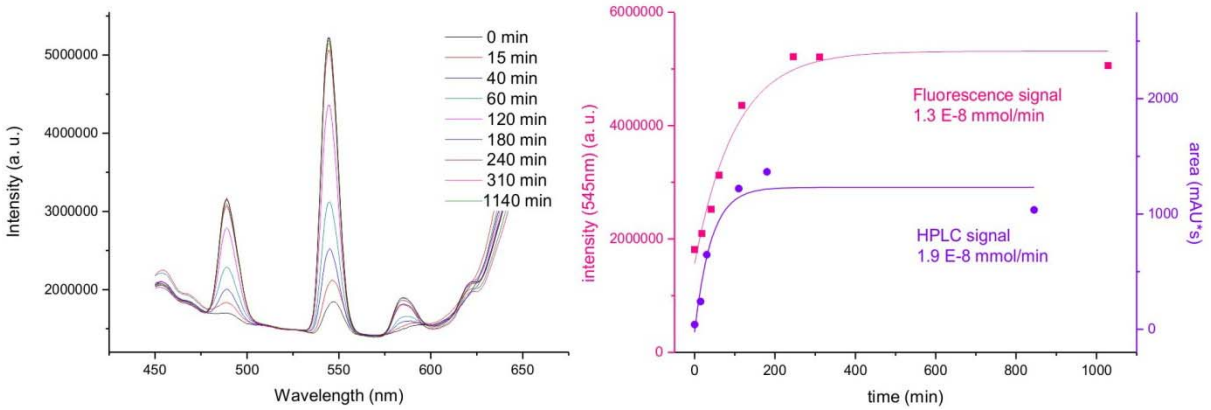
Only terbium(III), which is complexed by cholate functionalized vesicles, shows intensive, significant terbium phosphorescence at 545 nm after excitation of coordinated DHN sensitizer at 335 nm.

A significant increase of terbium phosphorescence intensity at 545 nm of **Vs4** ($c_{(DOPC)}$ = 5 mM, $c_{(Chol)}$ = 0.75 mM, $c_{(Tb^{3+})}$ = 0.25 mM) was detected during the conversion of DHNdhn (12.5 µM) by lipase (50 mg/L) for 24h. DHN is, in contrast to its ester derivative, coordinating with its dihydroxy moiety to the vesicular embedded, cholate complexed terbium, which provides an effective sensitization at 335nm (see fluorescence anisotropy Scheme S4, left).

Enzyme activity assay

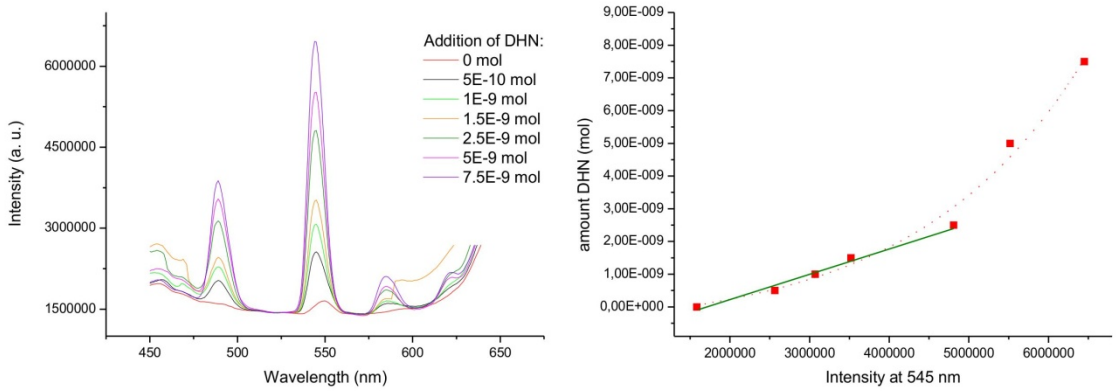
The emission intensity of **Vs4** ($c_{(DOPC)}$ = 5 mM, $c_{(Chol)}$ = 0.75 mM, $c_{(Tb^{3+})}$ = 0.25 mM) was monitored over 24 h in a cuvette (2 x 10 mm, 400 µL) at 545 nm in the presence of the respective DHN derivative ($c_{(DHN')}$ = 12.5 µM) and enzyme (50 mg/L lipase or glucosidase). (Scheme S5 and S7). Due to the higher sensitivity of the spectrometer, fluorescence emission intensity instead of phosphorescence intensity was used for the comparison with HPLC analysis of DHN.

The respective DHN derivative ($c_{(DHN')}$ = 12.5 µM) and enzyme (50 mg/L lipase or glucosidase) are incubated in a HEPES buffer solution (25 mM, pH 7.4), lacking any cholate doped vesicles or Tb^{3+}, over 24 h. Samples were analyzed by calibrated HPLC to monitor the evolving DHN amount over time.

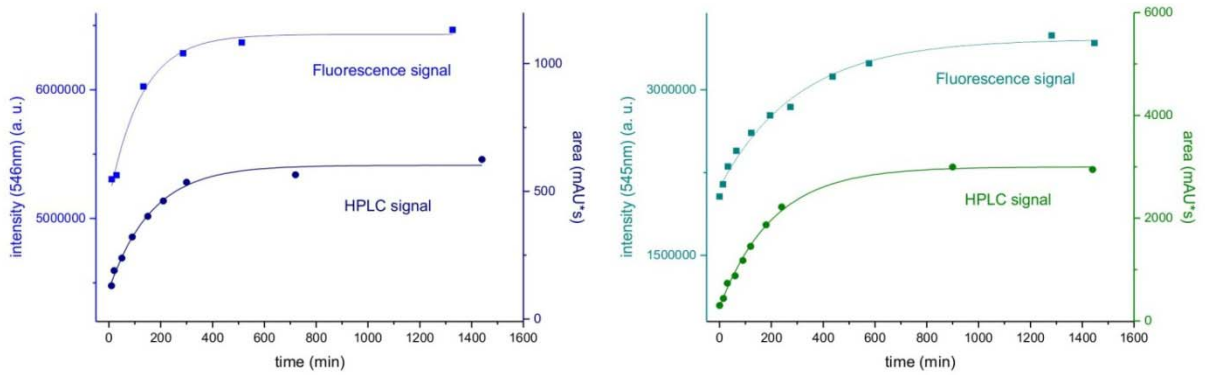


Scheme S5 Fluorescence intensity increase of **Vs4** ($c_{(DOPC)}$ = 5 mM, $c_{(Chol)}$ = 0.75 mM, $c_{(Tb^{3+})}$ = 0.25 mM) upon conversion of DHNdhn (12.5 µM) with lipase (50 mg/L) during 24h (left). DHNdhn conversion by lipase was monitored by fluorescence intensity changes of **Vs4** and HPLC analysis (right).

The initial rate constants for the lipase activity, derived from the fluorescent increase or the HPLC analysis of DHNdhn, are with 1.3 x 10^{-8} and 1.9 x 10^{-8} mmol/min, resp., comparable (Scheme S5). In order to correlate fluorescence intensity with the DHN concentration, increasing amounts of DHN were added to **Vs4** (Scheme S6). The slope of enzyme conversion (mmol/min) (Scheme S5, right) was detected during the first 60 min with an intensity increase from 1.8 x 10^{6} to 3.1 x 10^{6} cps. The value corresponds to the linear region of fluorescence intensity vs sensitizer amount (Scheme S6, right).

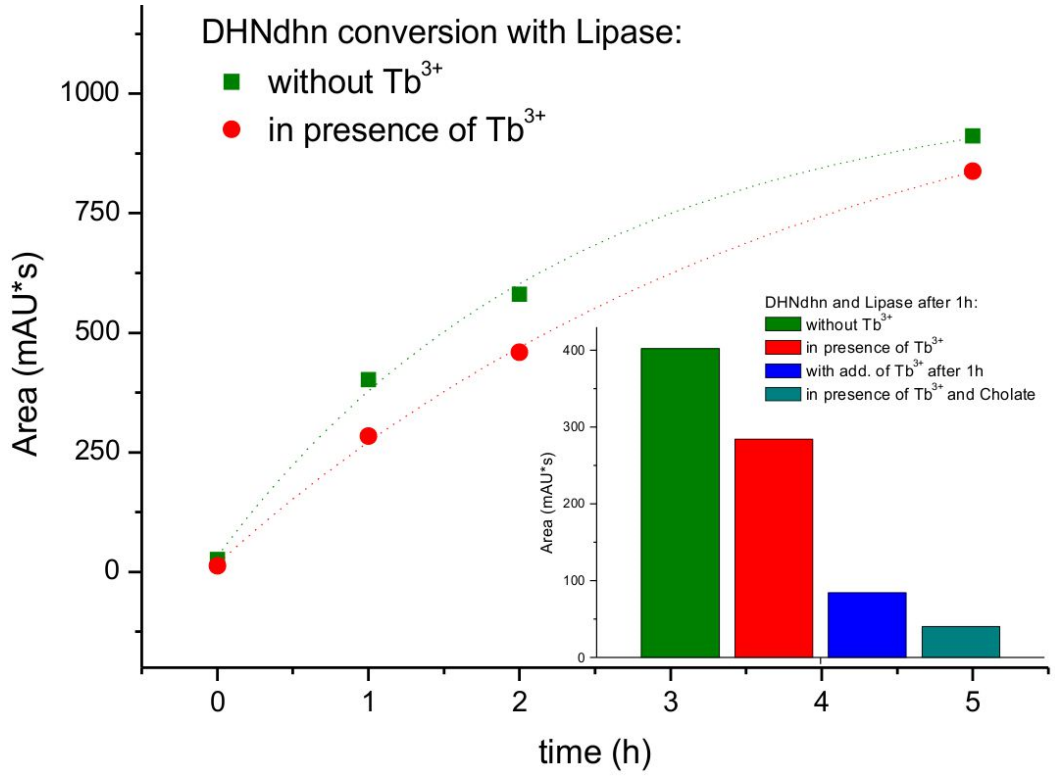


Scheme S6 Increasing fluorescence intensity of **Vs4** ($c_{(DOPC)}$ = 5 mM, $c_{(Chol)}$ = 0.75 mM, $c_{(Tb^{3+})}$ = 0.25 mM) upon addition of DHN (left). Fluorescence intensity increases linearily for small sensitizer concentrations (right), which were used to monitor enzyme activity.



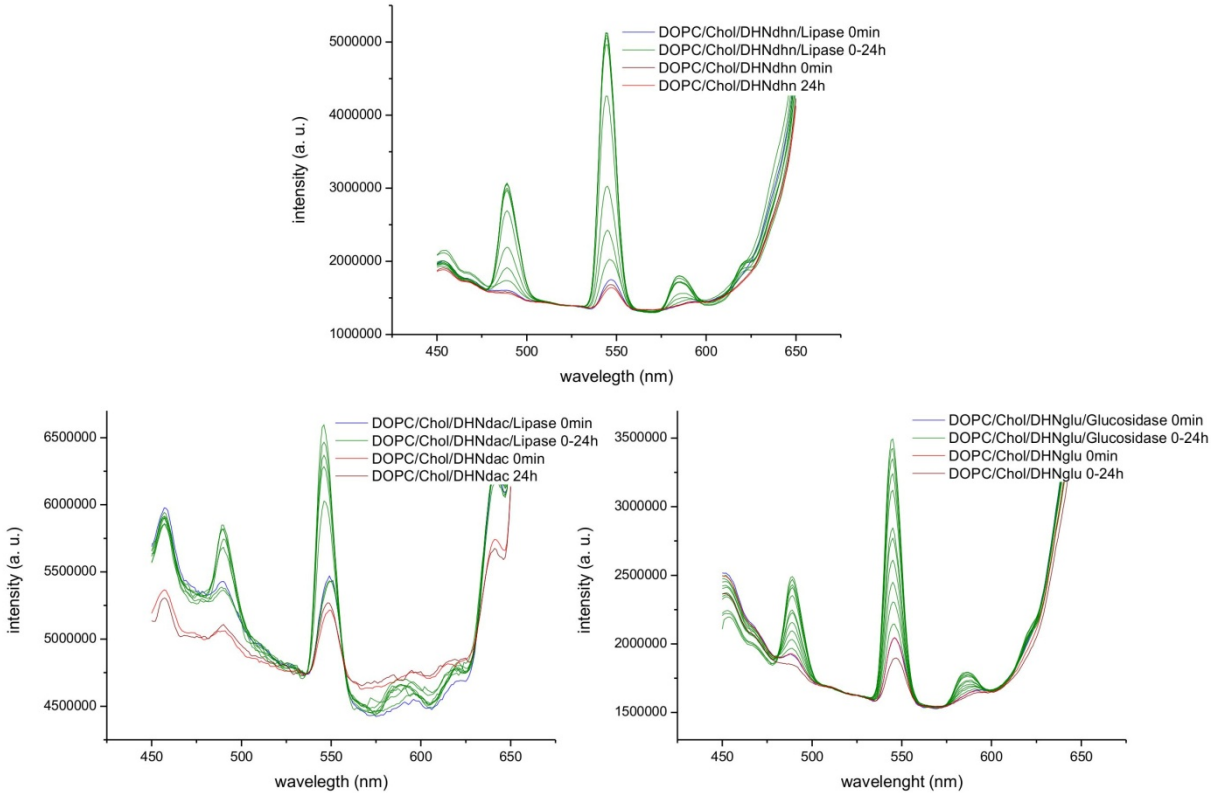
Scheme S7 Change of the terbium fluorescence intensity (left y-axis) of **Vs4** ($c_{(DOPC)}$ = 5 mM, $c_{(Chol)}$ = 0.75 mM, $c_{(Tb^{3+})}$ = 0.25 mM) during the conversion of DHNdac (12.5 µM) by lipase (50 mg/L) (left) and DHNglu (12.5 µM) by glucosidase (50 mg/L) (right). For comparison monitoring of the DHN conversion by HPLC is given on the right y-axis.

To exclude a potential inhibition of the enzyme activity in the presence of terbium ions,[1] we monitored the DHNdhn (12.5 µM) conversion with lipase (50 mg/L) by HPLC in presence and absence of Tb^{3+} (0.25 mM). The conversion of the DHNdhn is comparable in both cases, though the amount of free DHN in solution detected by HPLC is slightly decreased in the presence of Tb^{3+} (Scheme S8). However, this is not caused by inhibition of the lipase activity, but by the coordination of DHN to the lanthanide cation. The complex is not detected by HPLC. Moreover the measured amount of DHN is even lower for an identical DHNdhn lipase sample, which was treated with Tb^{3+} (0.25 mM) after enzymatic conversion before HPLC analysis or for a sample, containing Tb^{3+} (0.25 mM) and cholate (0.75 mM), showing precipitaion (Scheme S8, inset). The coordination of DHN to Tb^{3+} was confirmed by fluorescence anisotropy measurements of DHN (Scheme S4, red columns left and middle).



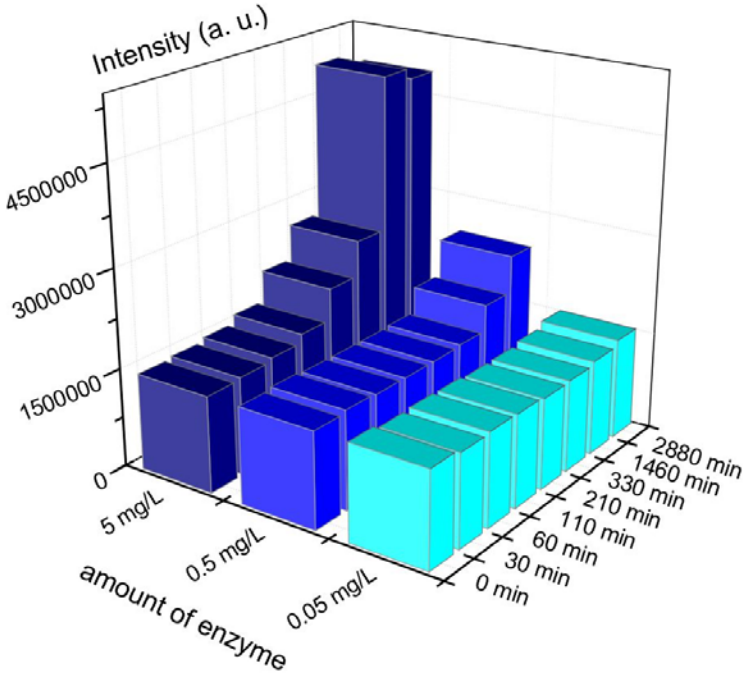
Scheme S8 HPLC analysis confirms that the enzymatic conversion of DHNdhn (12.5 µM) is not inhibited by the presence of terbium ions (0.25 mM). Changing concentrations of HPLC detectable DHN in solution in presence of terbium is a result of complex formation, which masks the DHN for HPLC analysis.

Control experiments (Scheme S9) show no significant fluorescence intensity increase of **Vs4** ($c_{(DOPC)}$ = 5 mM, $c_{(Chol)}$ = 0.75 mM, $c_{(Tb^{3+})}$ = 0.25 mM) in the presence of a DHN derivate (12.5 µM) without enzymes after 24h.



Scheme S9 Increase of emission intensity of **Vs4** over time in the presence of DHN esters in the presence and in the absence of enzymes.

A detection limit of 0.5 mg/L for lipase enzymatic activity was determined with **Vs4** ($c_{(DOPC)}$ = 5 mM, $c_{(Chol)}$ = 0.75 mM, $c_{(Tb^{3+})}$ = 0.25 mM) for the previous described assay (lipase 50 mg/L) with reduced amounts of enzyme using 24h incubation time.



Scheme S10 The limit of detection of lipase activity assay with DHNdhn and **Vs4** is 0.5 mg/L; 1 % of the enzyme standard solution (50 mg/L).

References

1. (a) W. K. Xin and X. X. Gao, *Analyst*, 1996, **121**, 687-690; (b) Y. Wu, *Biometals*, 2000, **13**, 195-201.