Cooperative hydrolysis of aryl esters on functionalized membrane surfaces and in micellar solutions †

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Catalytic hydrolysis of peptides, proteins, phosphates or carboxylate esters in nature is catalysed by enzymes, which are efficient, fast and selective. Most of the hydrolytic chemical catalysts published so far mimic the active site of enzymes and contain metal complexes and amino acid residues. Their synthesis can be laborious, while the hydrolytic activity is still limited compared to enzymes. We present an approach that uses fluid membranes of vesicles and micelles as a support for amphiphilic additives, which cooperatively cleave aryl ester bonds. The membrane anchored bis-Zn(II)-complex 1 is hydrolytically active and hydrolyses fluorescein diacetate (FDA) with a second order rate constant \( k_2 \) of 0.9 M\(^{-1}\)s\(^{-1}\). The hydrolytic activity is modulated by co-embedded membrane additives, bearing common amino acid side chain functional groups. With this approach, the hydrolytic activity of the system is enhanced up to 16 fold in comparison to cyclen \( k_2 \) = 14.7 M\(^{-1}\)s\(^{-1}\). DOPC and DSPC lipids form at room temperature fluid or gel phase membranes, respectively. Omitting the lipid, micellar solutions were obtained with hydrolytic activity reaching \( k_2 \) = 13.4 M\(^{-1}\)s\(^{-1}\). It is shown that cooperative hydrolysis is favoured in fluid membranes and micelles, allowing the active moieties to arrange freely. The embedding and dynamic self-assembly of membrane active components in fluid membranes and micelles provides a facile access to hydrolytically active soft interfaces.

Introduction

Carboxyesters are ubiquitously found in nature. They are involved in many biological processes and present in everyday life. Esters of drugs are better transported through membranes with respect to free drugs and cleaved inside cells by esterases. This is often used as pro-drug concept in pharmaceutical sciences. Peptides, proteins, phosphate and carboxy esters, respectively are catalytically hydrolysed by natural enzymes and many attempts to design artificial hydrolysing enzymes have been reported over time, but their efficiency is still limited when compared to the natural models. Most hydrolases contain metal ions such as zinc(II) (carboxypeptidase A, astacin) in their active centres. The stabilisation of the transition state during hydrolysis occurs by hydrogen bonding of the ester group to amino acid residues

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To investigate an alternative approach, we used functionalized membrane surfaces of vesicles and micelles for the cooperative hydrolysis of esters (Figure 1) with zinc complex 1 (Scheme 1) as a hydrolytically active metal center. This complex is known to coordinate water molecules and due to its Lewis acidity it produces hydroxide anions used for hydrolysis reactions at neutral pH. The reaction rate was found to increase dramatically once complex 1 was embedded in a lipid membrane or in micelles. Embedded in membranes,
complex 1 may also serve as a ligand for the molecular recognition of phosphate anions.\(^\text{10}\)

The effect of micellar catalysis of surfactant solutions has been studied and described in detail in the literature.\(^\text{11, 12}\) It was discovered that positively charged surfactants, such as tetraammonium salts, amines etc. embedded in micelles cleave p-nitrophenol esters in buffered solutions at pH 7 with a high efficiency.\(^\text{13-15}\) This effect is most likely caused by the high local concentration of the polar functional groups on the surface. Metallomicelles were also intensively studied for their increased hydrolytic activity with respect to homogeneous solution.\(^\text{16, 17}\)

The proposed cooperative catalytic effect by combining embedded metal complexes and membrane additives on the surface of a vesicular membrane is depicted in Figure 1. Soft interfaces are important for many biological processes and found use also in artificial systems.\(^\text{18}\) A potential advantage using a 2D functionalized membrane for hydrolysis is the non-covalent assembly of the components in a two dimensional fashion. In the fluid membrane of a vesicle, the hydrolytic active components may statistically arrange in distances optimal for hydrolysis. This concept offers the easy and facile testing of different combinations of components and their possible cooperative action. Micellar solutions of cyclen 1 and membrane additives were also examined for their hydrolytic activity. However, an advantage of the vesicular systems is that their structure is better defined and the effects of different membrane additives can be compared easier.

The membrane active amphiphilic molecules are, most likely, not randomly distributed in the membrane, but form clusters and patches. Complete phase separation or limited mobility in the membrane might inhibit the cooperative action (Figure 2).\(^\text{19}\)

Membrane additives (Scheme 2) were designed to support the hydrolysis reaction resembling functional groups that are present in the active centers of hydrolytic enzymes, e.g. imidazole, phenol, acids or amides. Micellar hydrolytic activity has been reported previously for amines, oximes and histidine.\(^\text{21, 22}\) The series was extended to cover a larger range of acidic, neutral, basic and cationic functional groups. Amphiphiles are grouped in Scheme 2 according to the presence of acidic, neutral, basic and cationic functional groups. The series was extended to cover a larger range of acidic, neutral, basic and cationic functional groups. The proposed cooperative catalytic effect by combining different combinations of components and their possible cooperative action (Figure 3).

Expected patch formation in different phospholipid membranes.

FRET in different lipids at 25°C

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measurements indicate, which species is mainly responsible for the hydrolysis. All data are summarized in Table 1 (values of the functional group).

All measurements were performed under pseudo first-order-reaction conditions and the rate constant of hydrolysis \(k_{\text{obs}}\) was derived from the slope of the increased fluorescence concentration over 20 min (s\(^{-1}\)) and from the initial FDA photometric determination is less sensitive than the measurement of fluorescence.

All reported measurements were performed in vesicular membranes prepared by sonication above their transition temperature from both DOPC and DSPC in the presence of bis-Zn-cyclen 1. To properly examine the hydrolysis, the best composition of the membrane was found to be 5 mol % of 1, 10 mol % of the respective membrane additive and 85 mol % of phospholipid. Hydrolytic activity was determined also for vesicles containing only the zinc cyclen complex 1 (5 mol %) or exclusively nonmetal ion containing membrane additives (10 mol %). The measurements indicate, which species is mainly responsible for the hydrolysis. All data are summarized in Table 1 (values sorted according to Scheme 2). Bis-Zn-cyclen 1 embedded in the membrane showed a higher activity towards hydrolysis than a non- amphiphilic solution of 1 (data not shown, see SI), which confirms previous observations for bis(4-nitrophenyl) phosphate. Non-amphiphilic models of membrane additives were also tested in hydrolysis, but found inactive (see SI). We found that 1 is much more active in rigid DSPC- than in fluid DOPC-membranes. Compounds A9, A10, A11, A13, A14 and A15, respectively exhibit high rates of hydrolysis in the absence of 1 in the membrane, often exceeding the rate of sole cyclen 1. These compounds have functional groups that have been previously reported to be hydrolytically active in micellar solutions.

### Table 1 Hydrolytic activity of vesicular systems

| Zn-cyclen 1 [5 mol%] | DOPC | DSPC |
|----------------------|------|------|
|                      | \(k_{\text{obs}}\) | \(k_{\text{obs}}\) |
| A1                   | 0.07 | 0.6  |
| A2                   | 0.9  | 2.1  |
| A3                   | 0.3  | 2.5  |
| A4                   | 2.4  | 2.2  |
| A5                   | 0.4  | 1.9  |
| A6                   | 0.4  | 2.6  |
| A7                   | 0.5  | 1.4  |
| A8                   | 0.4  | 1.9  |
| A9                   | 4.0  | 21.6 |
| A10                  | 4.4  | 6.0  |
| A11                  | 11.9 | 9.3  |
| A12                  | 0.9  | 4.2  |
| A13                  | 10.9 | 5.0  |
| A14                  | 17.2 | 5.3  |
| A15                  | 7.6  | 4.2  |
| A16                  | 1.6  | 3.3  |
| A17                  | 0.9  | 3.9  |

Only palmitic acid A1 caused a notable drop in the hydrolytic activity. The coordination of its carboxylate group to bis-Zn-cyclen 1 may provide a rational explanation for the inhibition. Amphiphiles which are expected to be neutral at the expected protonation state at pH 7.4, grouped by pK\text{a} values of the functional group.

**Results and discussion**

**Vesicular hydrolysis**

The hydrolytic activity was monitored using fluorescein diacetate (FDA, Scheme 1), a substrate for membrane esterases, often used for evaluating the viability of cell samples. FDA is a non-fluorescent diaryl ester whose fluorescence is triggered by the cleavage of its ester groups. The often used p-nitrophenol esters are more labile towards hydrolysis and their photometric determination is less sensitive than the measurement of fluorescence.

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active in fluid membranes. With exception of A9 a larger increase of the hydrolytic rate is observed in DOPC membranes than in DSPC. The effect is most pronounced for membrane additives that are only hydrolytically active in combination with complex 1, such as oxime A4 (ninefold higher activity), phenol A2 (threefold) and guanidinium A17 (threefold). A rational may be the higher dynamics of embedded amphiphiles in the membrane. All membrane additives that increase the hydrolytic activity of complex 1 have some common structural features. All compounds bear a N-O or H-O bond and are at pH 7 either neutral with a pKa value close to 7 (imidazole: 6.95, phenol: 8.05) or are protonated (amines, guanidinium). Protonated membrane additives A with more hydrogen atoms tend to accelerate the hydrolysis more: The addition of the primary octadecylamine A14 induced a more than 10 times faster hydrolysis compared to the tertiary amine A12 in DOPC.

The hydrolytic effect of micellar solutions with polar head groups is often credited to the high local concentration of positive charge on the surface, therefore creating a local high concentration of hydroxide ions, but the example of trimethylammonium salt A17 shows that this effect is only small in comparison with cations generated by protonation (amines, guanidinium). The observations indicate that the rate enhancing effect of some membrane additives may have its origin in creating hydrogen bonds, which stabilize the transition state of substrate hydrolysis reaction.

**Micellar hydrolysis**

Kinetic measurements of micellar solutions were done under the same conditions as described for the vesicular solutions. The concentration of every component was kept constant and only the lipids were omitted in the preparation step. The concentrations of amphiphiles were exceeding their cmc values (cmc for cyclen 1 is less than 0.01 mM, see SI). Fluorescence is strongly quenched in the co-micellar solutions and the hydrolysis was therefore monitored by absorption spectroscopy. Pseudo first-order-reaction rate constants of hydrolysis (k_{obs}) were calculated from the slope of the increased fluorescein absorption band (\(\lambda_{max} = 505\) nm) over 3 min (M s\(^{-1}\)) divided by the initial FDA concentration (see SI).

| Table 2 Hydrolytic activity of micellar systems |
|------------------------------------------------|
| Zn-cyclen 1 | \(k_{obs}\) | \(k_0\) |
|-----------------|-----------------|-----------------|
| with additive | \(10^3\) s\(^{-1}\) | \(10^5\) s\(^{-1}\) |
| A9 | 34.6 | n.d. |
| A11 | 30.7 | 0.5 |
| A14 | 22.4 | 1.7 |

k_{obs}: rate of system with cyclen (0.025 mM) and membrane additives (0.05 mM), k_0: micellar solutions of membrane additives. Same concentration as for vesicular measurements is used.

For micellar hydrolysis only the membrane additives A9, A11, A14 were tested, because they gave best results in vesicular systems. An enhancement of the hydrolytic activity was observed for micelles of cyclen 1 with respect to the vesicular solution. On the contrary, the membrane additives without the lipid were less active (A11, A14) or insoluble (A9). Co-micellar solutions gave high catalytic activity towards hydrolysis in all three tested system exceeding the activity of vesicles. An average 3-fold increase of activity in comparison to micellar cyclen 1 solution without membrane additive implies the existence of co-micelles and cooperative effects in the hydrolysis.

**Mechanism and kinetics**

For mechanistic studies, various concentrations of vesicular and micellar solutions were used and the observed pseudo-first-order kinetic rate constants were plotted against the overall cyclen 1 concentration in the solutions (Figure 5). The reaction mechanism with embedded cyclen 1 tends to be different for DSPC and micelles than for DOPC membranes. Two kinetic models were used: second-order kinetics are observed in the case of DSPC vesicles and micelles with k_{obs} linearly dependent on the concentration of cyclen 1 in solution. In contrast, DOPC vesicles show a Michaelis-Menten type behaviour of saturation kinetics. Apparently, a complex of substrate and catalyst is formed, which then undergoes the hydrolysis reaction. The kinetics data were non-linear fitted using equation (1) according to previously published studies.25

\[
k_{obs} = \frac{k_{cat}c_{cyclen}}{K_M+c_{cyclen}} (1)
\]

\(K_M\) describes the stability of the catalyst-substrate complex and \(k_{cat}\) the rate of its decomposition to product and catalyst.

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In a fluid DOPC membrane, Zn-cyclen 1 shows saturation kinetics even without the presence of a membrane additive, but in a rigid DSPC membrane and in micelles the hydrolysis rate increases linearly with the Zn-cyclen 1 concentration.

The kinetics of functionalized membranes with the highest hydrolytic activity consisting of cyclen 1 and membrane additives A4, A14, A9 or A11 were recorded and fitted to linear and non-linear (1) models. The derived constants are summarized in Table 2 (for data evaluation, see SI). For a better comparison of second order rate constants and reactions following a Michaelis-Menten kinetics, the initial slope was calculated dividing \(k_{cat}\) by \(K_M\) (k_2). For most of the functionalized DOPC membranes non-linear fitting gave better
results as they followed not a single kinetic models, whereas rigid DSPC systems showed mostly linear correlations. The membrane additive A9 behaves different from the other functionalized lipids and shows a linear second-order kinetic also in the DOPC. Embedded amine A14 leads to a saturation kinetic also in DSPC vesicles. Micelles of cyclen 1 and membrane additives showed the best hydrolytic activity.

Table 3  Second order rate constants

|                  | k_{cat} 10^{-3} s^{-1} | K_M 10^{-3} M^{-1} | k_2 (k_{cat}/K_M) M^{-1} s^{-1} | k_{2} M^{-1} s^{-1} |
|------------------|------------------------|-------------------|---------------------------------|------------------|
| Only cyclen 1 (5 %) | 0.3                    | 0.7               | 0.5                             | –                |
| 5 % 1 + 10 % A4   | 10.4                   | 8.4               | 1.2                             | –                |
| 5 % 1 + 10 % A9   | –                      | –                 | –                               | 1.6              |
| 5 % 1 + 10 % A11  | 40.9                   | 9.3               | 4.4                             | –                |
| 5 % 1 + 10 % A14  | 44.2                   | 3.0               | 14.7                            | –                |
| Only cyclen 1 (5 %) | –                      | –                 | –                               | 0.9              |
| 5 % 1 + 10 % A4   | –                      | –                 | –                               | 0.9              |
| 5 % 1 + 10 % A9   | –                      | –                 | –                               | 8.4              |
| 5 % 1 + 10 % A11  | –                      | –                 | –                               | 3.7              |
| 5 % 1 + 10 % A14  | 11.1                   | 2.7               | 4.1                             | –                |

Table 4  Fluorescein diesters

| Name^a | R            | logP^a |
|--------|--------------|--------|
| FDA^a  | CH_3         | 3.20   |
| F-C2^a | CH_3CH_3     | 4.22   |
| F-Ph^a | Ph           | 4.41   |
| F-iPr^a| iPr          | 4.93   |
| F-C3^a | (CH_3)CH_3   | 5.24   |
| F-tBu^a| tBu          | 5.75   |
| F-C7^a | (CH_3)CH_3   | 9.31   |
| F-C15^a| (CH_3)CH_3   | 17.47  |

^aPrepared from the acid chloride; ^bPrepared from the acid anhydride; ^cValues obtained from ACDlabs software.

To study the effect of lipophilicity and sterical hindrance of the ester group on the hydrolysis rate, a series of fluorescein esters was prepared (Table 4). All these compounds were tested under the same conditions as previously described and pseudo-first order hydrolytic rates were recorded for the three best performing membrane compositions with DSPC and DOPC lipids (Table 1, 2).

Table 4  Fluorescein diesters

Fig. 6 shows that the trends in hydrolysis for the different esters remain the same in the different vesicular systems (for data, see SI); the molecular structure of the ester does not significantly affect the mechanism. The reactivity of the different esters is not affected by the membrane fluidity (DOPC vs DSPC). The highest hydrolytic activity was reached for FDA, the least lipophilic ester (Figure 6). With increasing lipophilicity of the esters, the hydrolytic activity decreases. The lowest rates of hydrolysis were recorded for phenyl or tBu esters (F-Ph, F-tBu), which are more stable due to steric hindrance.

Comparison with hydrolytic enzymes

A direct comparison of kinetic data from the literature can be challenging due to different substrates and variations in the reaction conditions. Therefore commercially available hydrolytic enzymes were used under identical conditions to benchmark the hydrolytic rate of the functionalized vesicles. Porcine liver esterase and the lipase Candida rugosa, typical enzymes used in organic synthesis, were selected. Kinetic data were derived using the same non-linear fit as for vesicles to obtain k_{cat} and K_M. Since the enzymes are produced by extraction and no exact purity is given by the supplier, kinetic constants were calculated in weight concentration (mg ml^{-1}) for enzymes and vesicles. For vesicles the weight of cyclen complex 1, membrane additive A and the lipid was considered (see SI for data).

DOPC membranes functionalized with cyclen 1 and amine A14 reach the activity of the two purchased enzymes (Table 5). However, the catalytic activity of the enzyme active site still remains much higher compared to cyclen complex 1 and membrane additives, as the vesicles contain many hydrolytic active sites on their surface, while each enzyme has only one active centre.
Conclusions

Soft surfaces with ester hydrolysis activity were obtained by co-embedding of bis-Zn-cyclen complex 1 and amphiphilic membrane additives into a phospholipid membrane of a vesicle or producing a micellar solution. For many combinations of membrane additives and bis-Zn-cyclen complex 1 an increase of the hydrolytic activity up to 25 fold in comparison to complex 1 was observed. DOPC membranes that are fluid at the reaction temperature lead to more pronounced cooperative action of the membrane embedded amphiphiles and Michaelis-Menten saturation kinetic was observed for such membranes. Micellar solutions showed also higher activity when metal complex and additive are present. The functionalized vesicles with the highest hydrolytic activity were compared with two commercially available enzymes under identical reaction conditions. Considering the overall weight of the catalytic systems their hydrolysis activity towards fluorescein diesters is comparable.

In conclusion, we observed a significantly increased hydrolytic activity of functionalized vesicles and micelles at neutral pH towards carboxylesters, if Lewis acidic bis-Zn-cyclen complex 1 and functionalized amphiphiles are used concertedly. Their assembly has to be dynamic, as in DOPC membranes or micelles, to gain a cooperative hydrolytic effect.

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Table 5  Comparison of enzymatic and vesicular hydrolysis of FDA

|                          | k<sub>cat</sub> 10<sup>3</sup>s<sup>-1</sup> | K<sub>M</sub> 10<sup>4</sup>mg/ml | k<sub>2</sub> (k<sub>cat</sub>/K<sub>M</sub>) 10<sup>-4</sup>(mg/ml)<sup>-1</sup>s<sup>-1</sup> |
|--------------------------|-----------------------------|-----------------|----------------------------------|
| Porcine liver esterase   | 68.7                        | 0.08            | 858                              |
| Lipase Candida rugosa    | 73.2                        | 0.4             | 166                              |
| DOPC 5 % 1 + 10% A14    | 44.2                        | 0.2             | 391                              |