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Dynamic combinatorial chemistry at the phospholipid bilayer interface

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Abstract

Background: Molecular recognition at the environment provided by the phospholipid bilayer interface plays an important role in biology and is subject of intense investigation. Dynamic combinatorial chemistry is a powerful approach for exploring molecular recognition, but has thus far not been adapted for use in this special microenvironment.

Results: Thioester exchange was found to be a suitable reversible reaction to achieve rapid equilibration of dynamic combinatorial libraries at the egg phosphatidyl choline bilayer interface. Competing thioester hydrolysis can be minimised by judicial choice of the structure of the thioesters and the experimental conditions. Comparison of the library compositions in bulk solution with those in the presence of egg PC revealed that the latter show a bias towards the formation of library members rich in membrane-bound building blocks. This leads to a shift away from macrocyclic towards linear library members.

Conclusions: The methodology to perform dynamic combinatorial chemistry at the phospholipid bilayer interface has been developed. The spatial confinement of building blocks to the membrane interface can shift the ring-chain equilibrium in favour of chain-like compounds. These results imply that interfaces may be used as a platform to direct systems to the formation of (informational) polymers under conditions where small macrocycles would dominate in the absence of interfacial confinement.

Background

Dynamic combinatorial chemistry [1-3] is a growing field in the general area of systems chemistry [4-11] and revolves around equilibrium mixtures of molecules or supramolecules that can exchange the building blocks from which they are constituted. The resulting dynamic combinatorial libraries (DCLs) are inherently responsive to influences that alter the relative thermodynamic stabilities of the library members. For example, addition of a template (a guest molecule or a biomolecule) to a DCL will result in a stabilization of those library members that bind to the template, inducing a shift in the product distribution, ideally in favour of the best binders and at the expense of the other unwanted library members. This responsiveness makes dynamic combinatorial chemistry an important tool for the discovery of new synthetic receptors [12-21] and ligands for biomolecules [22-25]. Moreover, the technique has potential for the development of catalysts by using a transition-state analogue as a template [26,27]. Molecular recognition in DCLs can also occur between or within library members, enabling the discovery of replicating and/or self-assembling systems [28-38], catenanes [39-41] and the exploration of folding of macromolecules [42-44].

While the vast majority of the work on dynamic combinatorial chemistry is confined to homogeneous solutions and in a few cases, two-phase systems [14,15,45], its application to the chemistry at interfaces is largely unexplored, apart from one example of a dynamic combinatorial approach to bilayer membrane transport [21]. Nonetheless, molecular recognition at interfaces is of extreme importance in many different disciplines, ranging from nanotechnology to cell biology. Furthermore, molecular recognition at interfaces can differ markedly from the corresponding process in bulk solution, as a result of a different microenvironment and confinement in two dimensions [46-48]. This prompted us to develop dynamic combinatorial methodology to allow the use of

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this technique at interfaces. We have focussed our efforts on the phospholipid bilayers that mimic the cell membrane and now report the successful adaptation of reversible thioester chemistry [33,44,49-54] for making DCLs at the lipid bilayer interface. We have developed analytical protocols that enable the monitoring of product distributions of libraries of molecules that are bound to the lipid bilayer. Our results show that the kinetics of thioester exchange are comparable in bulk solution and at the bilayer interface, despite the different microenvironments of these two systems. We also show that the partitioning and two-dimensional organisation of building blocks at the membrane surface can bias the product distributions of the libraries from small mainly cyclic products in bulk solution toward larger linear products at the bilayer.

Results and discussion

Many different reversible covalent chemistries have now been developed that allow for the construction of DCLs in aqueous solution. The most popular of these are hydrazone and disulfide chemistry [1]. However, both processes have equilibration times that are typically in the range of 2-5 days and sometimes even several weeks [55]. This makes these chemistries less applicable to bilayer systems, many of which have limited physical stabilities. For example, unilamellar vesicles prepared from phospholipids are often only stable for approximately 24 hours. Thus, alternative reversible chemistries are required that allow equilibrium to be reached within this timeframe and under relatively dilute conditions. Literature reports suggest that the equilibration of thioesters in bulk aqueous solution is relatively fast [33,44,49-54], which led us to investigate the potential of this reaction in bilayer vesicle systems. As a model system we chose unilamellar vesicles made from egg phosphatidyl choline.

We have designed a series of simple building blocks, featuring an aromatic core to facilitate analysis of the resulting libraries by HPLC using UV/vis detection (Figure 1). The thioesters were attached either directly to the benzene ring (**1a**) or connected through a methylene spacer (**1b**). We have compared the reactivity of both constructs in bulk aqueous solution.

Thioester chemistry in bulk aqueous solution

The rates of thioester exchange and competing hydrolysis were studied by mixing thioester **1a** or **1b** with *N*-acetyl-L-cysteine **2** (Figure 1; pK_a 9.52 [56], which is comparable to the pK_a of exchange product **4**, which is

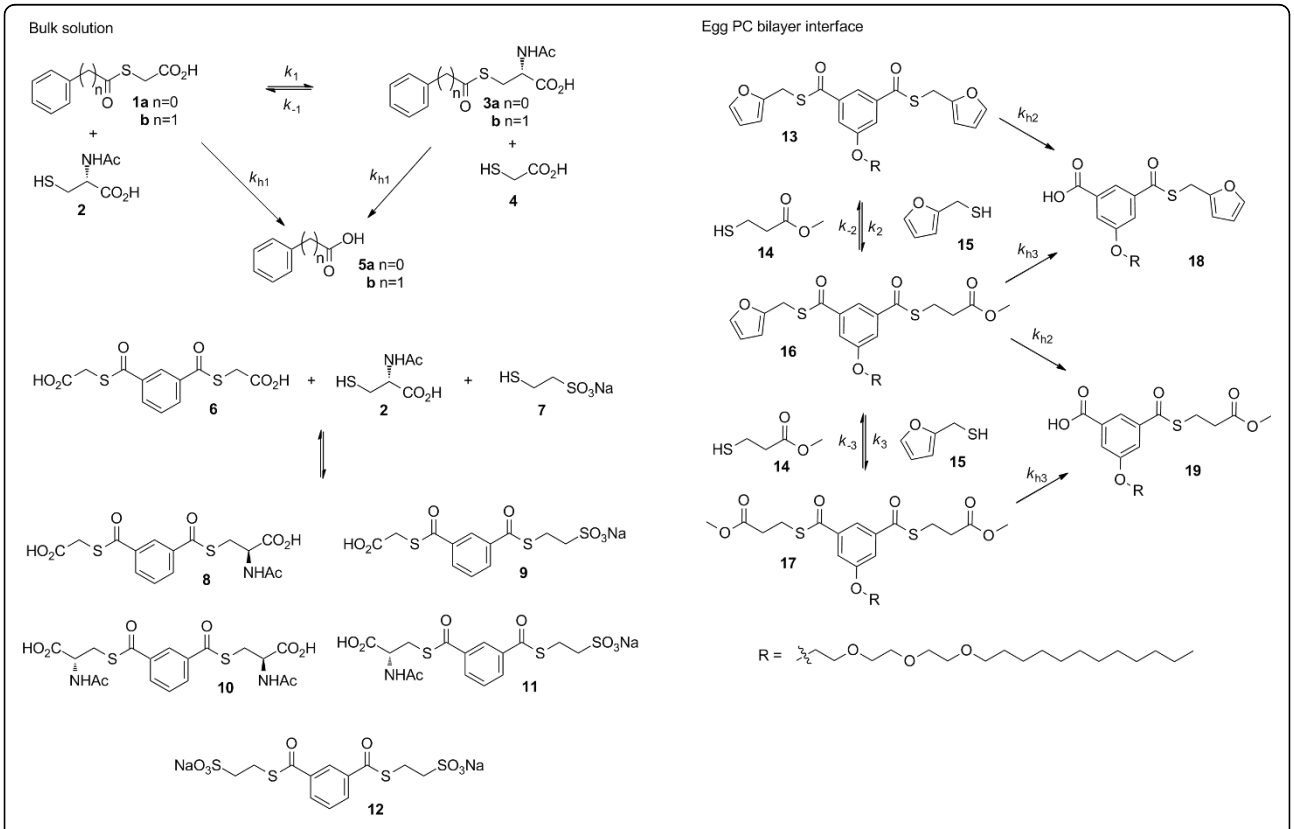

Figure 1 DCLs used for analysing the kinetics of thioester exchange and hydrolysis in bulk solution and at the lipid bilayer interface.

10.06 [57]) in equimolar amounts and monitoring the formation of the thioester exchange product (**3**) and the hydrolysis product (**5**) using HPLC analysis. The amounts of the two products were determined from the peak areas in the HPLC traces, assuming that all thioesters have the same molar absorptivity. This data was plotted as a function of time and fitted using Dynafit software [58]. As both temperature and pH are known to have a strong influence on the kinetics of transthioesterification and hydrolysis of thioesters, these experiments were performed at three different temperatures (20, 30 and 40°C) and two different pHs (7.0 and 8.0). A comparison of the data shows that rates of thioester exchange are comparable for DCLs containing **1a** (Figure 2a and 2c) or **1b** (Figure 2b and 2d) when subjected to the same conditions, whereas hydrolysis of **1b** proceeds significantly faster than that of benzoic acid-derived thioester **1a**. This observation is consistent with reports that aliphatic thioesters are prone to hydrolysis, particularly at higher pH [51,59,60]. These plots also demonstrate that thioester exchange proceeds considerably faster with increased pH. While DCLs at pH 7.0 equilibrate in approximately 35 hours, at pH 8.0 equilibrium is reached within 5 to 7 hours, depending on the structure of the building block.

The rate constants for thioester exchange obtained by fitting the data are in the range of 10^{-2} $M^{-1}s^{-1}$ (Table 1), which is about one order of magnitude lower than the corresponding rate constants determined for thioesters with aliphatic moieties at the acyl carbon [61,62]. Transthioesterification rates are comparable for **1a** and **1b** whereas hydrolysis is approximately 20 times faster in **1b**. Surprisingly, hydrolysis becomes less competitive upon raising the pH from 7.0 to 8.0 as the rate of transthioesterification is increased 6-8-fold while the rate of hydrolysis merely doubles (Table 1). The temperature-dependence of the two competing processes, on the other hand, is comparable, so working at a higher temperature is a feasible method of achieving faster equilibration.

The experiments with the model thioesters **1a** and **1b** established that it is preferable to utilise building blocks with the acyl carbon directly attached to the aromatic core; including an aliphatic spacer leads to significant competition by hydrolysis. Therefore, in further experiments we focused on the former design.

Dynamic combinatorial chemistry relies on thermodynamic equilibrium being reached. In order to prove that this is indeed the case, we have performed experiments in which the same DCL is approached from two different starting points. For this purpose bis(thioester) **6** was synthesised (Figure 3) and a DCL containing this building block and the two thiols **2** and **7** was set up and equilibrated (Figure 1). The other starting point consisted of two libraries, one containing **6** and **2**, and the second containing **6** and **7**. Both of these libraries were allowed to equilibrate separately, then mixed and allowed to re-equilibrate. Comparable product distributions were obtained in both experiments (Figure 4), indicating that thermodynamic equilibrium is reached using the conditions established in the kinetic studies above.

The above results indicate that thioester exchange proceeds smoothly in bulk solution in the required timescale. Thus, the scene was set to perform similar experiments at the lipid bilayer interface.

Thioester chemistry at the phospholipid bilayer interface

In order to study thioester chemistry at the phospholipid bilayer interface a derivative of **6** was prepared that was equipped with an alkyl chain for incorporation in lipid vesicles and a triethylene glycol spacer to facilitate access of thiols to the thioester functionality of the head group (**13** in Figure 1). The synthetic route to this compound is shown in Figure 3. To set up phospholipid-based DCLs, large unilamellar vesicles

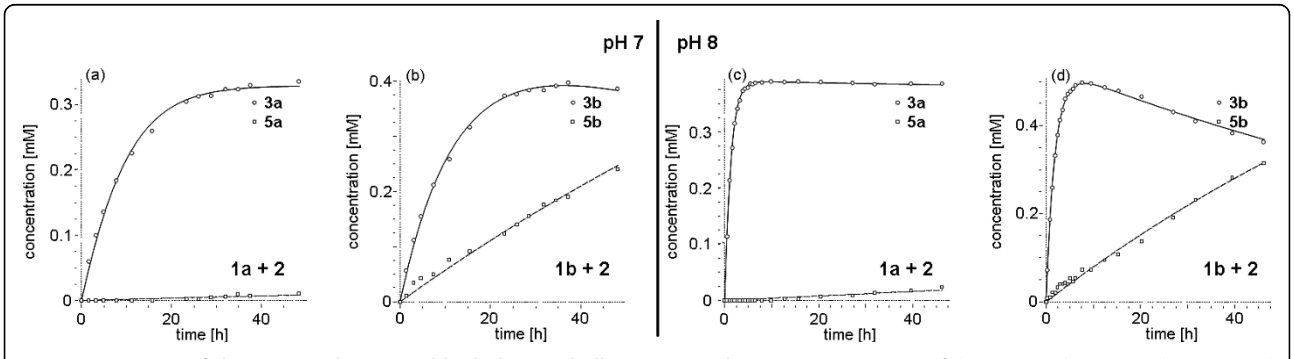

Figure 2 Kinetics of thioester exchange and hydrolysis in bulk aqueous solution. Concentrations of thioester exchange products (○) and hydrolysis products (□) as a function of time in DCLs made from thioester **1a** or **1b** and thiol **2** (1.0 mM each) at pH 7.0 (a,b) and pH 8.0 (c,d) at 40°C. Solid lines represent the fit of the data.

Table 1 Rate constants defined in Figure 1 for thioester exchange and hydrolysis at different temperatures and pHs

starting material	pH	T [°C]	k_1 [$M^{-1}s^{-1}$]	k_{-1} [$M^{-1}s^{-1}$]	k_{h1} [s^{-1}]	k_1/k_{-1}	K[a]
1a	7.0	40	$0.868 \times 10^{-2} \pm 1.8 \times 10^{-4}$	$3.51 \times 10^{-2} \pm 1.3 \times 10^{-3}$	$5 \times 10^{-5} \pm 2 \times 10^{-5}$	0.25	0.29
	8.0	40	$7.58 \times 10^{-2} \pm 6.8 \times 10^{-3}$	$18.5 \times 10^{-2} \pm 2.3 \times 10^{-2}$	$1.1 \times 10^{-4} \pm 1 \times 10^{-5}$	0.41	0.43
		30	$2.93 \times 10^{-2} \pm 3 \times 10^{-4}$	$6.42 \times 10^{-2} \pm 1.1 \times 10^{-3}$	$6 \times 10^{-5} \pm 1 \times 10^{-5}$	0.46	0.46
		20	$1.22 \times 10^{-2} \pm 1 \times 10^{-4}$	$2.92 \times 10^{-2} \pm 4 \times 10^{-4}$	2×10^{-5} 1×10^{-5}	0.42	0.44
1b	7.0	40	$1.08 \times 10^{-2} \pm 2 \times 10^{-4}$	$1.00 \times 10^{-2} \pm 8 \times 10^{-4}$	$1.64 \times 10^{-3} \pm 3 \times 10^{-5}$	1.08	1.10
	8.0	40	$8.13 \times 10^{-2} \pm 1.4 \times 10^{-3}$	$6.12 \times 10^{-2} \pm 2.1 \times 10^{-3}$	$2.29 \times 10^{-3} \pm 3 \times 10^{-5}$	1.33	1.28
		30	$4.09 \times 10^{-2} \pm 1.2 \times 10^{-3}$	$4.01 \times 10^{-2} \pm 2.1 \times 10^{-3}$	$8.0 \times 10^{-4} \pm 4 \times 10^{-5}$	1.02	1.08
		20	$1.54 \times 10^{-2} \pm 2 \times 10^{-4}$	$1.69 \times 10^{-2} \pm 6 \times 10^{-4}$	$3.2 \times 10^{-4} \pm 2 \times 10^{-5}$	0.91	0.92

[a] Determined from the concentrations of thiol and thioesters observed in the equilibrated libraries.

(200 nm diameter) were prepared in phosphate buffer pH 8.0 by mixing egg phosphatidylcholine (egg PC) with 10 mol% **13**, followed by extrusion through a polycarbonate membrane. Different thiols were then added to the resulting solutions.

Where finding suitable analytical conditions for studying DCLs in bulk solution was straightforward, identification of products in DCLs in the presence of phospholipids was considerably more challenging. Even though, lacking a chromophore, lipids do not interfere with the UV/Vis detection of compounds, they complicate the MS analysis significantly. In initial attempts we found that egg PC is not eluted completely from the column under the conditions used for analysis and leaches slowly with every gradient chromatography performed. The resulting broad MS signal of the phospholipids concealed those of any other species present in the DCLs, hampering the identification of thioester exchange products. After exploring several different approaches, the best method for removing the egg PC from the column was found to be washing with THF containing triethylamine and trifluoroacetic acid at regular intervals, usually after five to ten chromatography runs had been performed. In combination with using very low injection volumes for LC-MS analysis, these measures proved suitable for achieving MS traces that allowed the assignment of library members in a DCL containing egg PC.

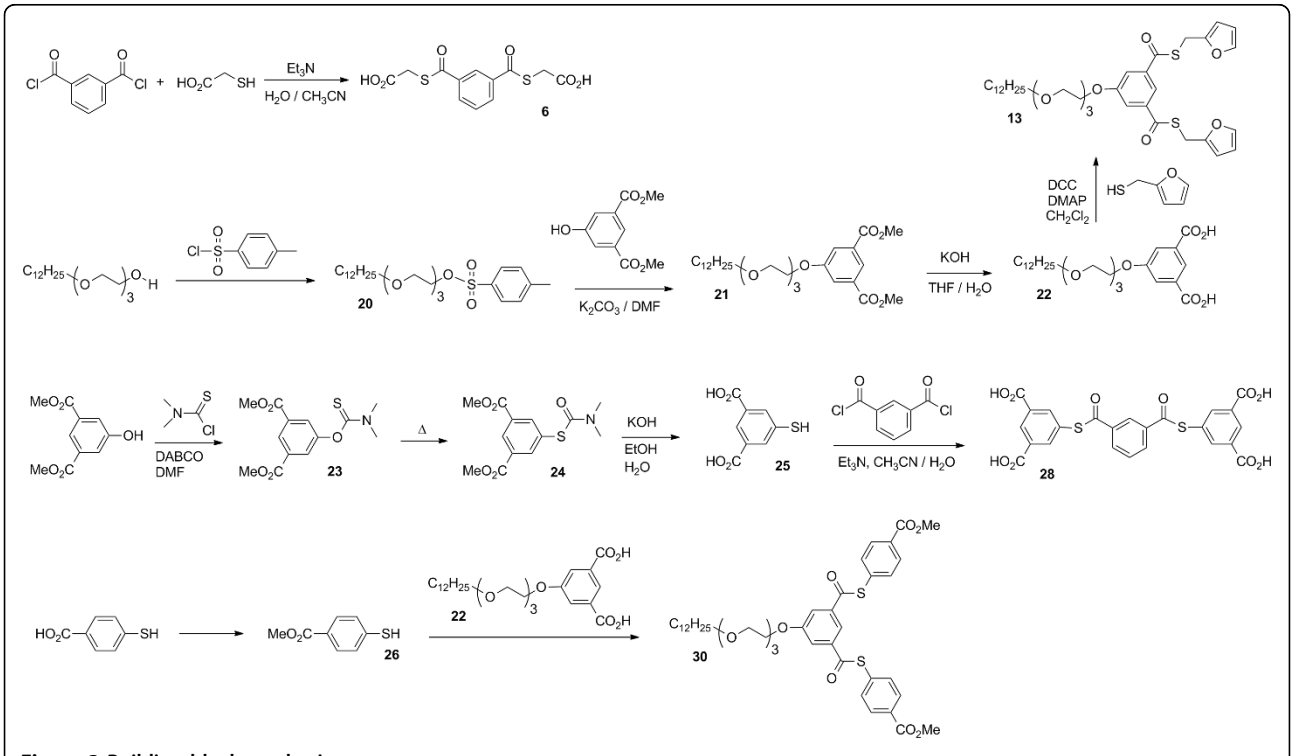

Figure 3 Building block synthesis.

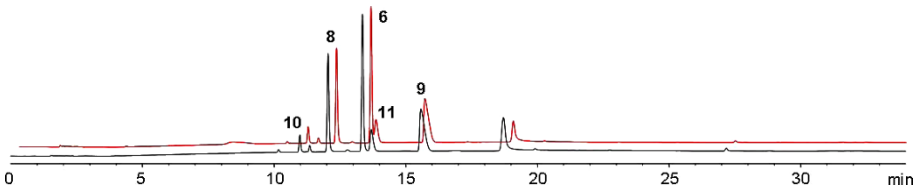

Figure 4 Evidence for equilibration in bulk solution. HPLC analysis of two DCLs reaching the same distribution from two different starting points. Black: 1.0 mM **6**, 1.0 mM **2** and 1.0 mM **7** mixed and equilibrated. Red: 1.0 mM **6** and 2.0 mM **2** equilibrated separately from 1.0 mM **6** and 2.0 mM **7**, then mixed and re-equilibrated.

The kinetic analysis of thioester exchange for a mixture of bis(thioester) **13** with thiol **14** (Figure 1; pK_a 9.33 [61]) at the lipid bilayer was carried out using the optimised conditions for this type of thioester building block of pH 8.0 and a temperature of 40°C. As **13** has two thioester moieties, the accumulation of two thioester exchange products and two hydrolysis products was recorded over time and the data fitted using Dynafit software (Figure 1 and Figure 5). The extinction coefficients of thioester starting material and thioester exchange products were assumed to be the same. Although furan exhibits a low level of absorbance at 260 nm, it is negligible in comparison with that of the aromatic ring system. Thioester exchange at the lipid bilayer proceeds somewhat slower than in solution under the same conditions, presumably due to a combination of structural differences of the reacting molecules and a lower polarity of the microenvironment of the lipid bilayer [61-63] in comparison with bulk solution. However, equilibrium is reached within 24 hours and Table 2 shows that the rate constants are in the same order of magnitude at the lipid bilayer and in solution (cf. second row in Table 1), making this system suitable for studying dynamic combinatorial chemistry at the lipid bilayer.

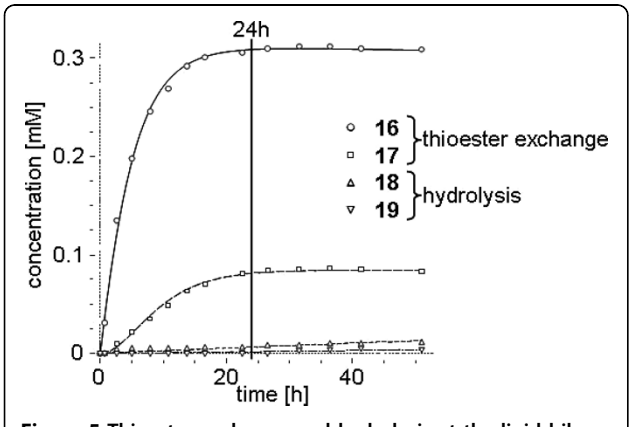

Figure 5 Thioester exchange and hydrolysis at the lipid bilayer interface. Formation of thioester exchange products **16** (○) and **17** (□) and hydrolysis products **18** (Δ) and **19** (∇) in a DCL made from **13** and **14** (1.0 mM each) at the lipid bilayer at pH 8.0 at 40°C. Solid lines represent the fit of the data.

In order to show that equilibrium is also reached at the lipid bilayer, experiments were carried out analogous to those performed in solution, but now starting from thioester **13**. The two thiols used in this case are **4** (pK_a 10.06 [57]) and **7** (pK_a 9.53 [57]), and the two HPLC traces of the DCLs obtained from different starting points are again in excellent agreement (Figure 6). Libraries were allowed to equilibrate for 24 hours. Interestingly, the equilibrium distribution strongly favours the less polar starting material, with only small amounts of the mono-exchange products present, despite the large excess of both thiols **4** and **7**. These global concentrations are, however, unlikely to be reflected by the local concentrations of the thiols in the vicinity of the lipid bilayer interface where thioester exchange is taking place. The microenvironment created by the lipids is considerably less polar than bulk solution [63-65], thus disfavouring the recruitment of more polar species such as negatively charged **4** and **7** to the lipid bilayer interface, presumably due to costly desolvation.

We also probed whether the thiol building blocks are able to cross the bilayer membrane and/or the membrane-bound thioesters are able to undergo flip-flop. Experiments were performed in which thiols were added after vesicle formation (single-sided addition) or where vesicles were prepared in the presence of thiol (double-sided addition). If the membrane permeation of the thiols through the bilayer and thioester flip-flop are both slow on the timescale of the experiment, single-sided and double-sided addition would result in different DCL compositions. For single-sided addition only the fraction of thioester building block present in the outer leaflet of the lipid bilayer would be exposed to the thiols and therefore be able to participate in transthioesterification. The HPLC analysis of two DCLs where thiols were added prior to, or after vesicle formation show comparable product distributions (see Additional file 1, Figure S1), indicating that diffusion of thiols across the lipid bilayer and/or flip-flop of the uncharged thioester **13** are fast on the timescale of the experiment.

Table 2 Rate constants as defined in Figure 1 for thioester exchange and hydrolysis at the lipid bilayer interface in a DCL made from 13 and 14 at pH 8.0 and 40°C

starting material	k_2 [$M^{-1}s^{-1}$]	k_{-2} [$M^{-1}s^{-1}$]	k_3 [$M^{-1}s^{-1}$]	k_{-3} [$M^{-1}s^{-1}$]	k_{h2} [s^{-1}]	k_{h3} [s^{-1}]
13	1.65×10^{-2} $\pm 0.2 \times 10^{-3}$	3.31×10^{-2} $\pm 0.8 \times 10^{-3}$	1.32×10^{-2} $\pm 0.5 \times 10^{-3}$	5.07×10^{-2} $\pm 2.7 \times 10^{-3}$	0.008×10^{-2} $\pm 0.9 \times 10^{-5}$	0.004×10^{-2} $\pm 0.6 \times 10^{-4}$

Comparing DCL distributions at the bilayer interface with those in bulk solution

With reaction conditions established for solution-phase as well as vesicle interface-based thioester exchange chemistry, we proceeded to increase the complexity of the DCLs by using dithiols and comparing the resulting product distribution in solution with that at the lipid bilayer. We explored the use of both aromatic and aliphatic dithiols. Larsson *et al.*[49] pointed out that in thioester DCLs that contain both types of thiols the equilibrium strongly favours aliphatic thioester and free aromatic thiols due to the significant differences in pK_as between aliphatic and aromatic thiols. Given this poor compatibility between these two classes of thiols, DCLs containing building blocks **6** and **13** were mixed with aliphatic dithiol **27** (Figure 7). In order to also explore aromatic thiols we have prepared thioesters **28** and **30** (see Figure 3 for the synthetic routes), which were mixed with aromatic dithiol **29** (Figure 7).

The HPLC traces of the libraries with aliphatic thiols are shown in Figure 8, with Figure 8a showing the DCL in bulk water with **6** and **27**, and Figure 8b showing the DCL made from **13** and **27** in the presence of egg PC. The product distributions in both libraries are completely different. In solution the cyclic heterodimer dominates and a small amount of the cyclic tetramer is also observed. At the lipid bilayer only a very small amount of a cyclic species is formed and the mixture is dominated by starting material **13** and to a lesser extent linear thioesters.

Similarly, the libraries containing aromatic thiols also show significant differences in the species that are formed in bulk water and at the lipid bilayer (Figure 9). In bulk water no compounds can be detected that contain more than one unit of thioester building block **28**. Significant amounts of disulfide and hydrolysis side products are formed. At the lipid bilayer many different linear species are present containing multiple copies of thioester **30** (the membrane-bound analogue of **28**), with **13** being the major species. Note that the relative HPLC peak areas do not reflect relative concentrations when the library members exhibit different molar absorptivities.

Although it was pointed out in the previous section that less polar thiol building blocks may favour the thioester exchange species rather than the starting material, the poor water-solubility of more hydrophobic building blocks prohibited their use in this study.

The differences in product distribution in bulk water and at the lipid bilayer observed for both aliphatic and aromatic thiol-thioester systems studied here can be attributed to differences in local concentration. Libraries at the lipid bilayer interface are biased towards species that are rich in the membrane-bound amphiphilic building block **13** which is probably caused by a local concentrating effect of the lipid vesicles.

Conclusions

Our results show that it is possible to use dynamic combinatorial chemistry based on reversible thioester chemistry at the phospholipid bilayer interface and achieve equilibration within 24 hours at pH 7.0 or 8.0 at millimolar building block concentrations. Conditions for LC-MS analysis of the libraries of molecules bound to the egg PC membranes have been developed. Kinetic

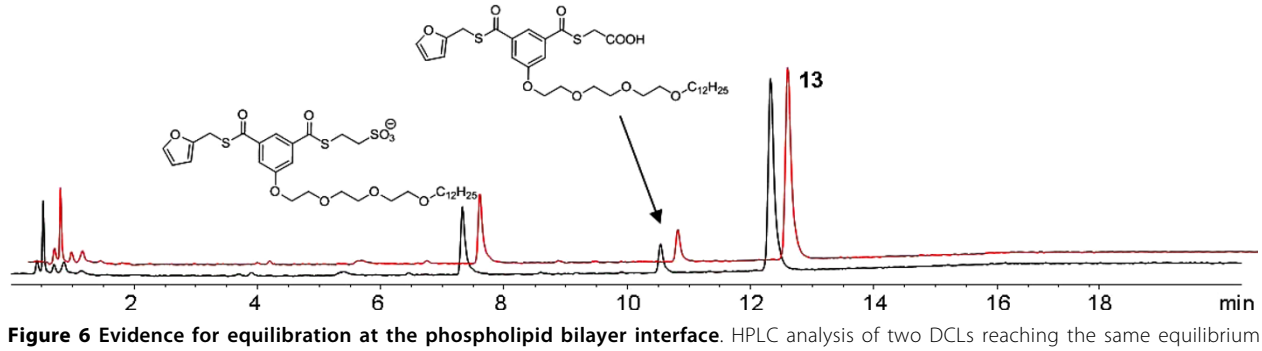

Figure 6 Evidence for equilibration at the phospholipid bilayer interface. HPLC analysis of two DCLs reaching the same equilibrium distribution from two different starting points. Black: 10 mM egg PC, 1.0 mM **13**, 10 mM **4** and 10 mM **7** mixed and equilibrated. Red: 10 mM egg PC, 1.0 mM **13** and 20 mM **4** equilibrated separately from 10 mM egg PC, 1.0 mM **13** and 20 mM **7**, then mixed and equilibrated.

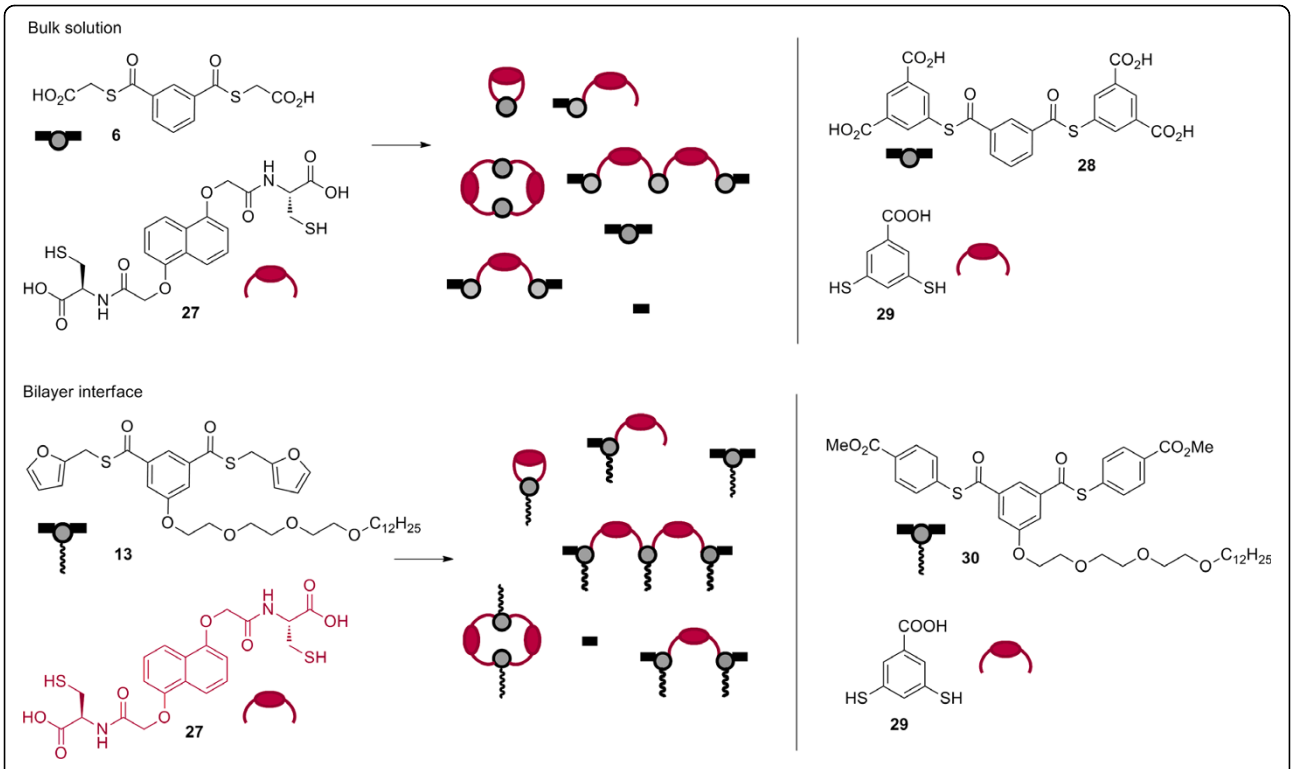

Figure 7 Thioester DCLs featuring aliphatic (left) and aromatic (right) dithiols in bulk aqueous solution (top) and at the phospholipid bilayer interface (bottom).

investigations revealed that thioester hydrolysis is not competitive with thioester exchange, provided that thioesters are used that are derived from aromatic carboxylic acids. Perhaps counterintuitively, hydrolysis is less competitive at the higher pH, because the rate of hydrolysis increases less rapidly with increasing pH than the rate of exchange.

DCLs at the membrane interface facilitate access to library members featuring multiple copies of membrane-bound building blocks. Thus, at the membrane interface the composition of the DCLs is biased towards larger linear species, while smaller macrocyclic species are more abundant in solution-phase libraries. We attribute this difference to the confinement of the building blocks to two dimensions at the bilayer interface. This confinement leads to a high local concentration of the membrane-bound building blocks, shifting the ring-chain equilibrium in favour of chain-like compounds. These

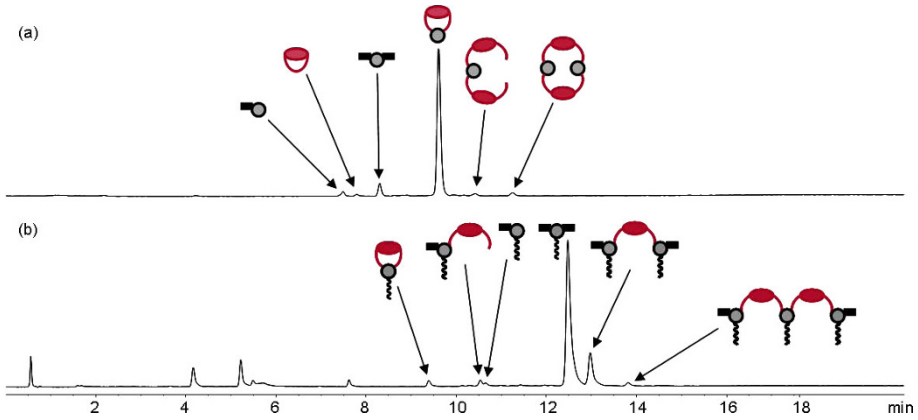

Figure 8 Comparing the compositions of DCLs with aliphatic dithiols in bulk aqueous solution with that at the lipid bilayer interface. HPLC analyses of DCLs containing (a) 1.0 mM **6** and 1.0 mM **27** or (b) 10 mM egg PC, 1.0 mM **13** and 1.0 mM **27**.

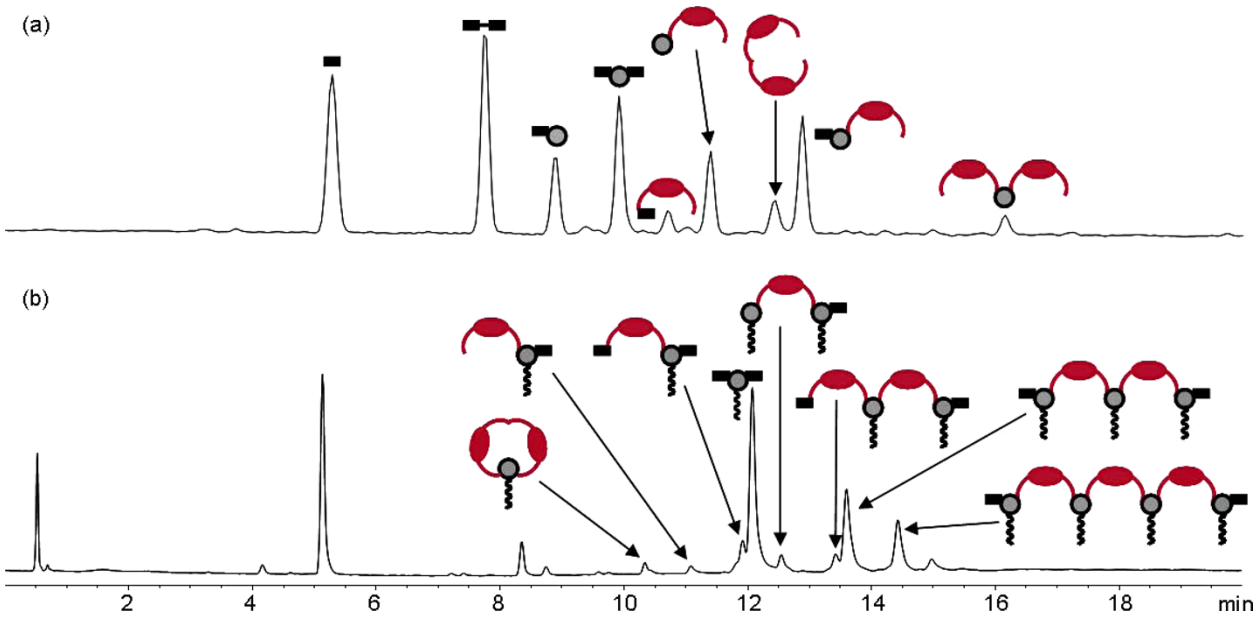

Figure 9 Comparing the compositions of DCLs with aromatic dithiols in bulk aqueous solution with that at the lipid bilayer interface. HPLC analyses of DCLs containing (a) 1.0 mM **28** and 1.0 mM **29** or (b) 10 mM egg PC, 1.0 mM **30** and 1.0 mM **29**.

results imply that interfaces may be used as a platform to direct systems to the formation of (informational) polymers under conditions where small macrocycles would dominate in the absence of interfacial confinement.

Experimental

Materials

Chemicals were purchased from Aldrich, Acros, Alfa Aesar, Avocado Organics, Fluka, or Lancaster Synthesis and used without further purification. Solvents used in synthesis were distilled prior to use and anhydrous solvents were distilled from a drying agent under argon. LC-MS grade solvents (acetonitrile, water, formic acid and trifluoroacetic acid) were obtained from Romil and used without further purification. Thin layer chromatography was carried out on glass or aluminium plates coated with silica gel 60 F254 (Merck). Column chromatography was performed on silica gel (0.040-0.063 mm) purchased from Breckland Scientific Systems. Silica gel (0.015-0.040 mm) from Merck was used for dry column vacuum chromatography. Dithiols **27** [66] and **29** [67] have been prepared following literature procedures.

2-(Benzoylthio)acetic acid 1a

Benzoic acid (10 mmol, 1.22 g) was dissolved in anhydrous CH_2Cl_2 (20 mL). Oxalylchloride (12 mmol, 1.02 mL) was added and the solution was stirred at room temperature overnight under a N_2 atmosphere. The solvent was evaporated in vacuo and the resulting acid chloride was used for the next step without further purification.

To a stirred solution of mercaptoacetic acid (1.87 mmol, 0.13 mL) and triethylamine (3.82 mmol, 0.53 mL) in a 1:1 mixture of water and acetonitrile (10 mL), the acid chloride (2.01 mmol, 0.31 g) was added. After 4 hours the mixture was washed three times with hexane. The aqueous phase was acidified and extracted three times with CH_2Cl_2. The combined organic phases were dried over sodium sulphate, filtered and the solvent was evaporated. Column chromatography (silica, DCM/acetone/formic acid 96:4:0.5) yielded 1.37 g (71% over two steps) of a white solid. Characterisation matched that described in ref. [68].

2-(2-Phenylacetylthio)acetic acid 1b

Prepared analogous to the procedure for the synthesis of **1a**. Yield: 1.32 g (63% over two steps) of an oil that crystallized upon scratching. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ = 7.37 - 7.27 (m, 5 H, a-c), 3.89 (s, 2 H, e), 3.70 (s, 2 H, d). ^{13}C-NMR ($CDCl_3$, 400 MHz): δ = 195.8, 173.9, 132.7, 129.7, 128.8, 127.7, 49.9, 31.3. Exact mass calculated: 209.0278, found: 209.0277 [M-H$^+$]. Melting point: 90 - 92°C.

S,S-Diacetic acid benzene-1,3-bis(carbothioate) 6

Mercaptoacetic acid (2.02 mmol, 0.14 mL) and triethylamine (3.97 mmol, 0.55 mL) were dissolved in a 1:1 mixture of water and acetonitrile (10 mL). After addition of isophthaloyl dichloride (0.99 mmol, 0.20 g) the solution

was stirred at room temperature for 5 hours. The mixture was washed with hexane three times and then lyophilised. The residue was redissolved in water and the product precipitated by acidifying with 3N HCl. Recrystallisation from methanol yielded a white powder (33 mg, 11%).

^{1}H-NMR (CD_3OD, 400 MHz): δ = 8.49 (t, 1 H, ^{4}J = 1.5, d), 8.23 (dd, 2 H, ^{4}J = 1.7, ^{3}J = 7.8, e), 7.69 (t, 1 H, J = 7.8, f), 3.94 (s, 4 H, b). ^{13}C-NMR (CD_3OD, 400 MHz): δ = 190.9, 171.9, 138.3, 133.1, 131.0, 126.5, 32.3. Exact mass calculated: 336.9817, found: 336.9825 [M+Na$^+$]. Melting point: 199 - 201°C.

2-(2-(2-(Dodecyloxy)ethoxy)ethoxy)ethyl 4-methylbenzenesulfonate 20

Anhydrous pyridine (10.01 mmol, 0.81 mL) was added to a solution of triethyleneglycol monododecyl ether (2.01 mmol, 0.69 mL) in anhydrous CH_2Cl_2 (20 mL) stirring at 0°C under a N_2 atmosphere. After 10 minutes *p*-toluenesulfonyl chloride (4.98 mmol, 0.95 g) was added. The solution was allowed to warm up slowly to room temperature and left to stir for a further 2 days before washing it with 3 N HCl, saturated sodium bicarbonate solution and brine. The organic layer was dried over sodium sulfate and filtered. After the solvent had been evaporated the residue was purified using dry column vacuum chromatography (0-35% EtOAc in hexane (v/v) in 2.5% increments) to yield a clear oil (0.69 g, 73%).

^{1}H-NMR ($CDCl_3$, 400 MHz): δ = 7.77 (d, 2 H, J = 8.3, c), 7.31 (d, 2 H, J = 8.4, b), 4.13 (t, 2 H, J = 4.7, d), 3.66 (t, 2 H, J = 4.7, e) 3.59-3.51 (m, 8H), 3.41 (t, 2 H, J = 6.9, g), 2.42 (s, 3 H, a), 1.56 (m, 2 H, h), 1.29-1.23 (m, 18 H, i), 0.85 (t, 3 H, j). ^{13}C-NMR ($CDCl_3$, 400 MHz): δ = 144.6, 133.0, 129.7, 127.9, 71.4, 70.7, 70.6, 70.4, 69.9, 69.1., 68.6, 31.8, 29.6 - 29.4, 29.4, 29.2, 26.0, 22.6, 21.5, 14.0. Exact mass calculated: 473.2937, found: 473.2949 [M+H$^+$].

Dimethyl 5-(2-(2-(2-(dodecyloxy)ethoxy)ethoxy)ethoxy) isophthalate 21

2-(2-(2-(Dodecyloxy)ethoxy)ethoxy)ethyl 4-methylbenzenesulfonate (**20**; 4.99 mmol, 2.36 g) and dimethyl-5-hydroxyisophthalic acid (5.00 mmol, 1.05 g) were dissolved in anhydrous DMF (100 mL) and potassium carbonate (19.97 mmol, 2.76 g) was added. The mixture was stirred at 100°C for 4 hours. After cooling down, CH_2Cl_2 (100 mL) was added and the mixture was stirred for 10 minutes at room temperature, then washed with 3 N HCl and brine, dried over sodium sulfate and filtered. The solvent was evaporated to yield 2.23 g (87%) of a clear oil.

^{1}H-NMR ($CDCl_3$, 400 MHz): δ = 8.27 (t, 1 H, J = 1.5, b), 7.77 (d, 2 H, J = 1.5, c), 4.21 (t, 2 H, J = 4.8, d), 3.93 (s, 6 H, a), 3.89 (t, 2 H, J = 4.8, e), 3.75 - 3.56 (m, 8 H, f), 3.44 (t, 2 H, J = 6.9, g), 1.56 (m, 2 H, h), 1.31 - 1.25 (m, 18 H, i), 0.87 (t, 3 H, j). ^{13}C-NMR ($CDCl_3$, 400 MHz): δ = 166.1, 158.9, 131.8, 123.1, 120.0, 71.6, 70.9, 70.7, 70.7, 70.1, 69.5, 68.1, 52.4, 31.9, 29.7 - 29.6, 29.5, 29.3, 26.1, 22.7, 14.1. Exact mass calculated: 511.3271, found: 511.3292 [M+H$^+$].

5-(2-(2-(2-(Dodecyloxy)ethoxy)ethoxy)ethoxy)isophthalic acid 22

Dimethyl 5-(2-(2-(2-(dodecyloxy)ethoxy)ethoxy)ethoxy) isophthalate (**21**; 4.36 mmol, 2.20 g) was dissolved in THF (60 mL). 1 M KOH (20 mL) was added and the solution was stirred for 15 hours. The THF was evaporated and the remaining aqueous solution was acidified and extracted with CH_2Cl_2 three times. The organic phase was washed with 3 N HCl and brine, dried over sodium sulfate. Evaporation of solvent gave 2.06 g (98%) of an off-white solid.

^{1}H-NMR ($CDCl_3$, 400 MHz): δ = 8.02 (t, 1 H, J = 1.2, a), 7.58 (d, 2 H, J = 1.1, b), 4.15 (t, 2 H, J = 4.2, c), 3.92 (t, 2 H, J = 4.0, d), 3.86 - 3.63 (m, 8 H, e), 3.45 (t, 2 H, J = 6.8, f), 1.56 (m, 2 H, g), 1.30 - 1.21 (m, 18 H, h), 0.86 (t, 3 H, i). ^{13}C-NMR ($CDCl_3$, 400 MHz): δ = 169.2, 158.5, 130.9, 123.8, 120.1, 71.6, 70.7, 70.5,70.4, 69.9, 69.6, 67.7, 31.9, 29.7 - 29.6, 29.5, 29.5, 29.3, 26.0, 22.7, 14.1. Exact mass calculated: 483.2958, found: 483.2968 [M+H$^+$]. Melting point: 80 - 82°C.

S,S-Bis(furan-2-ylmethyl) 5-(2-(2-(2-(dodecyloxy)ethoxy) ethoxy)ethoxy)benzene-1,3-bis(carbothioate) 13

5-(2-(2-(2-(Dodecyloxy)ethoxy)ethoxy)ethoxy)isophthalic acid (**22**; 0.40 mmol, 193 mg), 2-furanmethanethiol (0.99 mmol, 0.1 mL) and DMAP (0.10 mmol, 12 mg) were dissolved in anhydrous CH_2Cl_2 (10 mL) and cooled to 4°C on an ice bath. Dicyclohexylcarbodiimide (1.00 mmol, 206 mg) was added and the solution was stirred over night, allowing it to warm up to room temperature slowly. The mixture was extracted with 3 N HCl and brine. The organic layer was dried over sodium sulfate and filtered. After solvent evaporation the residue was purified using dry column vacuum chromatography (0-25% EtOAc in hexane (v/v) in 2% increments) to yield a clear oil (226 mg, 83%).

^{1}H-NMR ($CDCl_3$, 400 MHz): δ = 8.13 (t, 1 H, J = 1.5, e), 7.68 (d, 2 H, J = 1.5, f), 7.35 (d, 2 H, a), 6.32 - 6.29 (m, 4 H, b, c), 4.36 (s, 4 H, d), 4.21 (t, 2 H, J = 4.8, g), 3.88 (t, 2 H, J = 4.8, h), 3.74 - 3.56 (m, 8 H, i), 3.44 (t, 2 H, j), 1.56 (m, 2 H, k), 1.31 - 1.25 (m, 18 H, l), 0.88 (t, 3 H, m). ^{13}C-NMR ($CDCl_3$, 400 MHz): δ = 189.7, 159.3, 150.0, 142.4, 138.3, 118.7, 117.8, 110.7, 108.3, 71.6, 71.0, 70.7, 70.7, 70.0, 69.5, 68.2, 31.9, 29.7, 29.6, 29.5, 29.3, 26.1, 22.7, 14.1. Exact mass calculated: 675.3025, found: 675.3043 [M+H$^+$].

Dimethyl 5-(dimethylcarbamothioyloxy)isophthalate 23

Dimethyl-5-hydroxyisophthalic acid (9.99 mmol, 2.10 g) was dissolved in anhydrous DMF (10 mL) and cooled to 4°C. DABCO (19.97 mmol, 2.24 g) was added in portions, followed by dropwise addition of a solution of dimethylthiocarbamoyl chloride (19.98 mmol, 2.47 g) in anhydrous DMF (10 mL). The resulting suspension was allowed to warm up to room temperature slowly. After 24 hours the reaction mixture was poured into water (100 mL) and the precipitate was filtered off and washed with water. Recrystallisation from methanol yielded a white powder (2.32 g, 78%).

^{1}H-NMR ($CDCl_3$, 400 MHz): δ = 8.57 (t, 1 H, J = 1.5, b), 7.92 (d, 2 H, J = 1.5, c), 3.93 (s, 6 H, a), 3.45 (s, 3 H, d), 3.36 (s, 3 H, d). ^{13}C-NMR ($CDCl_3$, 400 MHz): δ = 187.0, 165.4, 153.9, 131.6, 128.4, 128.0, 52.5, 43.4, 38.8. Exact mass calculated: 298.0749, found: 297.0749 [M+H$^+$]. Melting point: 105 - 106°C.

Dimethyl 5-(dimethylcarbamoylthio)isophthalate 24

Dimethyl 5-(dimethylcarbamothioyloxy)isophthalate (**23**; 2.99 mmol, 890 mg) was heated at 215°C under a N_2 atmosphere for 1 hour. The mixture was cooled to about 70°C and ethanol (20 mL) was added. The pale brown crystals that appeared upon cooling down to room temperature were filtered off to give the product (802 mg, 90%).

^{1}H-NMR ($CDCl_3$, 400 MHz): δ = 8.69 (t, 1 H, J = 1.5, b), 8.34 (d, 2 H, J = 1.5, c), 3.94 (s, 6 H, a), 3.10 (s, 3 H, d), 3.04 (s, 3 H, d). ^{13}C-NMR ($CDCl_3$, 400 MHz): δ = 165.5, 140.7, 131.2, 130.5, 52.5, 37.0. Exact mass calculated: 298.0749, found: 297.0746 [M+H$^+$]. Melting point: 122 - 124°C.

5-Mercaptoisophthalic acid 25

A 1.2 M solution of potassium hydroxide in a 1:1 mixture of ethanol and water (20 mL) was degassed by purging with N_2 for at least 2 hours. Dimethyl 5-(dimethylcarbamoyl-thio)isophthalate (**24**; 2.02 mmol, 0.60 g) was added and the mixture was stirred at 80°C for 30 minutes under nitrogen, and then for another 1.5 hours, allowing it to cool down to room temperature slowly. Addition of concentrated HCl (10 mL) led to the precipitation of the product which was filtered off, washed with dilute HCl (0.3%) and dried under vacuum (yield: 0.33 g, 83%).

^{1}H-NMR (CD_3OD, 400 MHz): δ = 8.36 (t, 1 H, J = 1.5, a), 8.11 (d, 2 H, J = 1.5, b). ^{13}C-NMR (CD_3OD, 400 MHz): δ = 168.2, 135.4, 134.5, 133.3, 128.4. Exact mass calculated: 196.9911, found: 196.9914[M-H$^+$]. Melting point: 280 - 282°C.

S,S-(5-Mercaptoisophthalic acid) benzene-1,3-bis (carbothioate) 28

5-Mercaptoisophthalic acid (**25**; 2.02 mmol, 0.40 g) and triethylamine (11.97 mmol, 1.66 mL) were dissolved in a 1:1 mixture of water and acetonitrile (50 mL). Isophthaloyl dichloride (0.99 mmol, 0.20 g) was added and the solution was stirred at room temperature for 4 hours. The mixture was extracted with hexane three times and after evaporation of the solvent, the residue was redissolved in water. Fractional precipitation by careful addition of 3 N HCl gave a white powder which was recrystallised from methanol to yield the product (47 mg, 9%).

^{1}H-NMR (DMSO-d_6, 400 MHz): δ = 8.55 (t, 2 H, J = 1.6, a), 8.46 (t, 1 H, J = 1.8, c), 8.34 dd, 2 H, J = 7.8, J = 1.8, d), 8.28 (m, 4 H, b), 7.75 (t, 1 H, J = 7.8, e). ^{13}C-NMR (DMSO-d_6, 400 MHz): δ = 187.9, 165.7, 139.2, 136.3, 132.6, 131.2, 131.1, 130.7, 130.0, 127.9, 127.7, 125.4. Exact mass calculated: 548.9926, found: 548.9929 [M+Na$^+$]. Melting point: 263 - 265°C.

Methyl 4-mercaptobenzoate 26

4-Mercaptobenzoic acid (8.56 mmol, 1.32 g) was dissolved in methanol (40 mL). After addition of concentrated sulfuric acid (1 mL), the solution was refluxed for 24 hours. The solvent was evaporated and the residue was purified using dry column vacuum chromatography (0-25% EtOAc in hexane (v/v) containing 0.1% formic acid, in 2% increments) to yield a white solid (1.43 g, 99%).

^{1}H-NMR ($CDCl_3$, 400 MHz): δ = 7.89 (dt, 2 H, J = 8.5, J = 2.0, b), 7.29 (dt, 2 H, J = 8.5, J = 2.0, c), 3.90 (s, 3 H, a), 3.60 (s, 1 H, d). ^{13}C-NMR ($CDCl_3$, 400 MHz): δ = 166.6, 138.3, 130.2, 128.1, 127.2, 52.1. Exact mass calculated: 191.0140, found: 191.0137 [M+Na$^+$]. Melting point: 57 - 58°C.

S,S-Bis(methyl-4 benzoate) 5-(2-(2-(2-(dodecyloxy)ethoxy) ethoxy)ethoxy)benzene-1,3-bis(carbothioate) 30

5-(2-(2-(2-(Dodecyloxy)ethoxy)ethoxy)ethoxy)isophthalic acid (**22**, 0.40 mmol, 193 mg), methyl 4-mercaptobenzoate (**26**; 1.00 mmol, 168 mg) and DMAP (0.10 mmol, 12 mg) were dissolved in anhydrous CH_2Cl_2 (10 mL) and cooled to 4°C on an ice bath. Dicyclohexylcarbodiimide (1.00 mmol, 206 mg) was added and the solution was stirred overnight, allowing it to warm up to room temperature slowly. The mixture was extracted with 3 N HCl and brine, and the organic layer was dried over sodium sulfate and filtered. After solvent evaporation the residue was purified using dry column vacuum chromatography (0-25% EtOAc in hexane (v/v) in 2% increments) to yield a clear oil (180 mg, 35%).

^{1}H-NMR ($CDCl_3$, 400 MHz): δ = 8.26 (t, 1 H, J = 1.4, d), 8.13 (dt, 2 H, J = 8.5, J = 1.9, b), 7.77 (d, 2 H, J = 1.5, e), 7.62 (dt, 2 H, J = 8.5, J = 1.9, c), 4.25 (t, 2 H, J = 4.6, f), 3.95 (s, 6 H, a), 3.92 (t, 2 H, J = 4.6, g), 3.76 - 3.56 (m, 8 H, h), 3.44 (t, 2 H, i), 1.60 - 1.55 (m, 2 H, j), 1.32 - 1.24 (m, 18 H, k), 0.87 (t, 3 H, l). ^{13}C-NMR

($CDCl_3$, 400 MHz): δ = 188.0, 166.3, 159.6, 138.2, 134.6, 132.5, 131.2, 130.2, 118.9, 118.3, 71.5, 70.9, 70.7, 70.7, 70.0, 69.5, 68.3, 52.3, 31.9, 29.6, 29.5, 29.3, 26.1, 22.7, 14.1. Exact mass calculated: 783.3237, found: 783.3263 $[M+H^+]$.

Preparation of vesicles

A solution of 25 mg egg PC in chloroform (1 mL) was placed in a pyrex glass test tube and, where appropriate, a stock solution of one of the amphiphilic thioester building blocks in chloroform was added to give the desired ratio of lipid to thioester. The chloroform was evaporated under a stream of nitrogen to leave a lipid film on the wall of the test tube, which was dried under vacuum over night. Then, 100 mM phosphate buffer (1.65 mL) was added to give a concentration of 20 mM egg PC and the solution was vortexed for 30 seconds or until the lipid film was completely dispersed. The mixture was allowed to rest, vortexed again for 30 seconds and allowed to rest for 20 minutes. Five freeze-thaw cycles were followed by extrusion through polycarbonate membranes, ten times through 400 nm pore size and ten times through 200 nm pore size. The resulting vesicle solution was allowed to rest for one hour before libraries were set up.

Preparation of DCLs

DCLs in bulk aqueous solution were prepared by dissolving building blocks in 100 mM phosphate buffer to the appropriate concentrations (library volume: 0.5 mL) and adjusting the pH to 8 with 1 M KOH. These stock solutions were mixed to give the final concentrations given in the text.

The libraries were then stirred at 40°C for 5 hours, unless otherwise stated, and analysed by HPLC and LC-MS.

DCLs at the bilayer interface were prepared by dissolving thiol building blocks in 100 mM phosphate buffer to the appropriate concentrations and adjusting the pH with 1 M KOH. These stock solutions were then mixed with the freshly prepared vesicle solutions. In some cases, when the thiols to be used were not soluble in water, a highly concentrated stock solution in 2-propanol was prepared and a few microlitres of this were injected into the vesicle solution to give the appropriate final concentration. Libraries were equilibrated at 40°C for 24 hours and analysed by HPLC and LC-MS.

Table 3 HPLC gradient A

Time (mins)	Acetonitrile (0.1% FA)	Water (0.1% FA)
0	5	95
10	95	5
13	95	5

Table 4 HPLC gradient B

Time (mins)	Acetonitrile (0.1% FA)	Water (0.1% FA)
0	5	95
25	95	5
28	95	5

HPLC analysis

HPLC analysis was performed on an Agilent HP 1100 system fitted with an online degasser, quaternary pump, autosampler, heated column compartment and diode array detector set to a wavelength of 260 nm.

DCLs in bulk aqueous solution: Separation of library members in the kinetic studies and libraries shown in Figure 8a was achieved by injection of 5 μl of library solution onto a Zorbax Eclipse XBD-C8 (150 × 4.6 mm, 5 μm) column and elution with a gradient of acetonitrile (0.1% formic acid (FA)) and water (0.1% FA) at a flow rate of 2 mL/min. HPLC gradient A was used (Table 3).

Libraries shown in Figures 4 and 9a were analysed by injecting 5 μl of library solution onto a Waters Symmetry C8 column (150 × 4.6 mm, 3.5 μm) and eluting with a gradient of acetonitrile (0.1% FA) and water (0.1% FA) at a flow rate of 1 mL/min. HPLC gradient B was used (Table 4).

DCLs at the bilayer interface: Separation of library members was achieved by injection of 5 μl of library solution onto a Waters Symmetry C6 (150 × 4.6 mm, 5 μm) column and elution with a gradient of acetonitrile (0.1% formic acid) and water (0.1% formic acid) at a flow rate of 2 mL/min. HPLC gradient C was used (Table 5).

LC-MS analysis

LC-MS analysis was performed using an Agilent HP 1100 system coupled to an Agilent XCT iontrap MSD mass spectrometer. For DCLs in bulk water an injection volume of 2 μL was used, while for DCLs at the bilayer interface an injection volume of 0.5 μL was used. Mass spectra were acquired in standard-enhanced scan mode using a drying temperature of 335°C, a nebuliser pressure of 20.0 psi, drying gas flow of 6.0 L/min and an ICC target of 50,000 ions. The instrument was tuned for the target mass of 1200. Agilent Chemstation software (Rev A.10.02) and Bruker Daltonik LC/MSD Trap software 5.2 (Build 374) was used to operate the LC-MS and analyse the data produced.

Table 5 HPLC gradient C

Time (mins)	Acetonitrile (0.1% FA)	Water (0.1% FA)
0	5	95
20	95	5
25	95	5

Additional material

Additional file 1: PDF. Supporting information. HPLC data for single-sided and double-sided addition of thiols to DCLs at the bilayer interface and LC-MS data for the DCLs shown in Figures 8 and 9.

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Authors' contributions
HYAY prepared dithiol **27** with the advice of JKMS. FMM performed all other experiments and the data analysis. FMM and SO interpreted the results, developed the conclusions and wrote the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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