Liposome-Assisted Selective Polycondensation of α-Amino Acids and Peptides: the Case of Charged Liposomes

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Received March 28, 2000; Revised Manuscript Received June 7, 2000

ABSTRACT: Successive addition of *N*-carboxyanhydride (NCA) Trp to POPC liposomes showed that multiple feeding permits the formation of much longer Trp-oligomers compared to the reference system with no liposomes. Mixed POPC/DDAB liposomes were used to obtain block co-oligopeptides containing both Trp and Glu residues in the same chain. By using the lipophilic condensing agent EEDQ in the absence and presence of POPC liposomes, significant amounts of oligo-Trps were obtained only for the latter case. EEDQ-induced condensation in the presence of POPC liposomes and a library of four dipeptides, H−TrpTrp−OH, H−TrpGly−OH, H−TrpAsp−OH, and H−TrpGlu−OH, led to the selective formation of hydrophobic Trp-oligomers, since H−TrpTrp−OH is the only dipeptide out of the pool that exhibits affinity for the POPC membrane. Oligomers of H−ArgTrp−OH or H−HisTrp−OH were obtained only in significant yields in the presence of negatively charged POPC/DOPA liposomes. The case of the synthesis of H−HisTrpHisTrp−OH is shown as an example of in situ preparation of a reactive, potentially catalytically active compound.

Introduction

In a previous paper[1] we have reported on the effect of POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) liposomes on the condensation of amino acids and dipeptides. The main aim of the investigation was to find conditions for selective polycondensation, i.e., the preferential polycondensation of one amino acid (or peptide) from a pool of substrates, whereby the selection is brought about by the hydrophobic lipid membrane. The consequence of this selection would be a specific oligomer formation rather than the synthesis of all statistically possible products. This kind of study is not only relevant to the macromolecular chemistry of amino acids and peptides and to the general research on peptide interactions with lipid membranes, but also to the field of prebiotic chemistry. In this field the important question of how specific macromolecules might have been formed is still an open one.[2−4]

In the literature there are several studies on the polycondensation of amino acids with respect to the origin of life. We have cited and commented those relevant to the present work in our previous communication,[1] to which we refer, and which here is continued and extended. In the previous work, we had used POPC liposomes and two different polycondensations: (i) the condensation of tryptophan-containing dipeptides using the lipophilic condensing agent EEDQ and (ii) the oligomerization of the *N*-carboxyanhydride (NCA) of tryptophan (Trp) using the *N*,*N*′-carbonyldiimidazole (CDI) method. We showed that in the presence of EEDQ the POPC membrane is able to selectively oligomerize H−TrpTrp−OH out of a library of H−TrpX−OH dipeptides. On the other hand the CDI-induced oligomerization of Trp in the presence of POPC liposomes yields much longer oligomers (up to the 29mer) compared to the aqueous reference system (never longer than the 8mer). In this later case the growth of the hydrophobic, long oligo-Trps only takes place on the liposomes, but not in the aqueous phase, where oligomers longer than tetramer tend to precipitate.

The present paper extends this work along two main aspects: It will further develop the work of the condensation of tryptophan with the POPC liposomes and, mostly, will extend the liposome-assisted selectivity by introducing charges on the lipid membranes. In fact the resulting electrostatic interactions are very important for extending the liposome-assisted oligomerization to hydrophilic, charged amino acids and peptides. We will examine in particular the case of glutamic acid and of peptides containing histidine or arginine. The charged, mixed POPC liposomes used in these studies will either include the positively charged cosurfactant DDAB or the negatively charged lipid DOPA.

The aim is to obtain co-oligopeptides containing both hydrophobic and charged amino acid residues. In turn, this is important for the in situ production of functional and catalytically active peptides.

Results and Discussion

DCI-Induced Polycondensation of Tryptophan on POPC Liposomes. An important aspect of the experimental procedure mentioned (and not treated in detail) in our previous paper[1] is the multiple feeding, i.e., the addition of substrate in successive steps to the liposome suspension. This has an interesting analogy with the Merrifield synthesis on insoluble matrices and we wish to consider this technique in more detail. This procedure is illustrated in Scheme 1, together with all other experimental approches used in this paper.

When a Trp solution is incubated with CDI, yields of up to 95% of NCA−Trp and *N*-[imidazolyl-(1)-carbonyl]-tryptophan are obtained. Figure 1 shows the yields (% of the totally added NCA−Trp) of the oligo-Trps obtained after the first feeding (single feeding), third feeding, and sixth feeding (multiple feeding) of freshly prepared NCA−Trp aliquots to a POPC liposome suspension. It is apparent that multiple feeding can increase the yields of longer oligomers. In particular,

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10.1021/ma000540g CCC: $19.00
Published on Web 07/22/2000

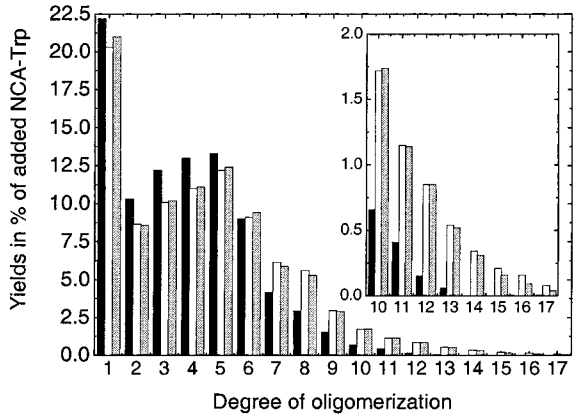
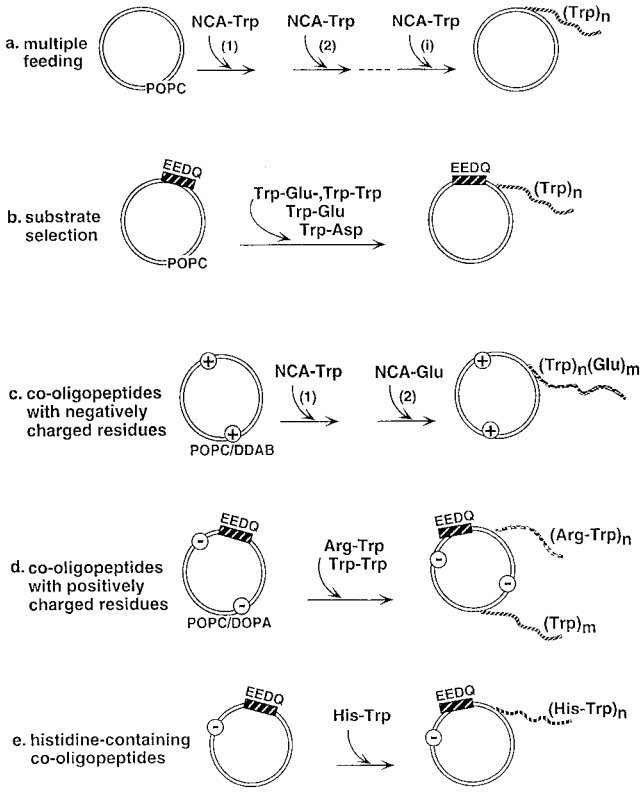


Figure 1. Effect of the number of feeding on the oligo-Trp yields is shown for a 100 nm extruded POPC liposome suspension. Single feeding: one 600 μL aliquot of a freshly prepared 40 mM NCA−Trp, 0.4 M imidazole/HCl, pH 6.80 solution (black bar). Multiple feeding: either three 200 μL aliquots (white bar) or six 100 μL aliquots (gray bar) in time intervals of 4 h added to 600 μL of a 100 nm extruded 100 mM POPC liposome suspension in 0.4 M imidazole/HCl buffer of pH 6.80 and incubated at room temperature on a shaker for another 12 h after the last feeding. The insert represents a zoom-in of the graph for the 10 to 17mer of oligo-Trp.

Scheme 1. Schematic Illustration of the Main Liposome-Assisted Condensation Reactions Carried out in This Work



oligomers with a degree of oligomerization above 13 are observed only in multiple-feeding experiments. There is however not a significant difference in product distribution for feeding steps higher than three. This is probably due to the fact that binding sites at the liposome interfaces are limited and chain elongation becomes more and more difficult with increasing feeding numbers. In the reference system without liposomes the highest oligomer produced in traces was the 8mer, independent of the number of feedings made (data not shown). In these experiments we had to use a relatively high concentration of NCA−Trp, so that an extensive investigation of the concentration of reagents could not be performed. In fact, attempts of using in the feeding a higher concentration (i.e., three feedings of 80 mM NCA−Trp) led to precipitation.

We studied the effect of lipid concentration on the yield for a constant number of feedings. The yields of the oligo-Trp's produced by multiple feeding of 20 mM NCA−Trp solutions performed in the presence of increasing lipid concentrations (1, 10, and 50 mM POPC) went through an optimum: Yields at 10 mM POPC are higher than those obtained with 50 mM POPC (data not shown). This can be interpreted assuming that a larger lipid surface may decrease the local concentration of the substrate, thus favoring the hydrolysis of NCA−Trp, which is the competing secondary reaction to the polycondensation.

Dynamic light scattering (DLS) was performed on a 100 nm extruded POPC suspension (initially 600 μL, 100 mM in POPC) before and after three feedings (each 200 μL) of a 40 mM NCA−Trp solution. The hydrodynamic radii after (before) condensation were 59.1 ± 0.6 nm (57.4 ± 0.6) at 60°, 56.3 ± 0.5 nm (55.9 ± 0.9) at 90°, and 55.3 ± 0.2 nm (54.7 ± 0.6) at 120°. The slight increase in the radius might be attributed to the oligo-Trps bound to the POPC membrane after the reaction. The low scattering angle dependency of the hydrodynamic radii for different scattering angles emphasizes that under the conditions generally used, the liposome size distribution and liposome stability did not change significantly.

An independent aspect of the NCA−Trp polycondensation, which we have not considered previously, concerns the kinetics of the reaction. Figure 2A shows the concentration change of the NCA−Trp in the presence of POPC liposomes, due to oligomerization, and of *N*-[imidazolyl-(1)-carbonyl]tryptophan which subsequently cyclizes to form an NCA−Trp. Both Trp derivatives are also partially hydrolyzed. Similar results have been reported for Gly, Ala, and Pro.[5,6] Figure 2B shows the time dependent concentration change of the oligo-Trps starting with NCA−Trp in the presence of POPC liposomes. A plateau concentration is reached after about 4 h for each oligomer, regardless of the oligomerization degree (with longer reaction times—data not shown—the plateau value tends to decrease, probably reflecting the consumption of the shorter oligopeptides at the expense of the longer ones). In Figure 2B, differences in the reaction time courses are apparent in the first phase of the curves. There is a clear lag phase for oligomers higher than the 5-mer, which is more pronounced for longer chain lengths. This is due to the fact that the longer oligomers are formed at the expenses of the shorter ones. In other words, a significant concentration of the shorter oligomers must be reached prior to the chain growth.

EEDQ-Induced Polycondensation of Dipeptides on POPC Liposomes. In addition to the CDI-induced polycondensation, the previous paper[1] presented also results on the liposome-assisted sequence-selective condensation of Trp-containing dipeptides, using the highly lipophilic condensing agent EEDQ.

The absolute yields (% of initial dipeptide) for the condensation experiments for each dipeptide alone, in the absence (Ref) and presence of liposomes (Lip) are given in Table 1. This table also reports—for the sake

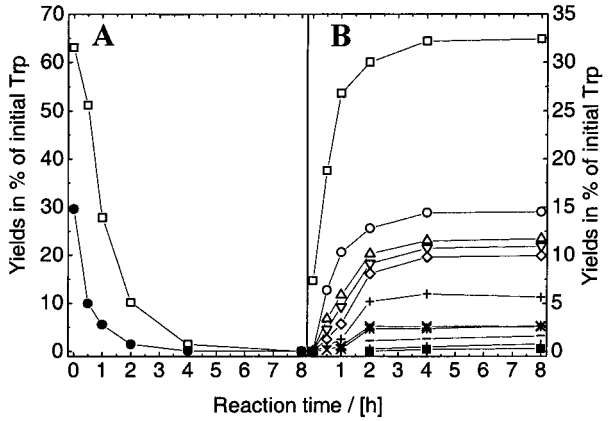


Figure 2. Kinetics of CDI-induced NCA−Trp condensation in the presence of 100 nm extruded POPC liposomes. (A) Concentration of activated tryptophan species, NCA−Trp (open squares) and *N*-[imidazolyl-(1)-carbonyl]tryptophan (filled circles) formed by incubation of Trp with CDI, decrease with time as *N*-[imidazolyl-(1)-carbonyl]tryptophan is turned into NCA−Trp and the condensation reaction of NCA−Trp proceeds. A 20 mM NCA−Trp solution in a 0.4 M imidazole/HCl buffer, pH 6.80 was mixed 1:1 (v/v) with a 100 nm extruded 20 mM POPC liposome suspension in 0.4 M imidazole/HCl buffer, pH 6.80. The first sample was taken immediately (t = 0 h). The remaining solution was further incubated at room temperature. Samples were taken at the appropriate times and analyzed by LC−MS. The major secondary reaction is the hydrolysis of the two intermediates. (B) Yields of the oligo-Trps formed with time (in % of initial Trp) under the just described conditions. 1mer (open square), 2mer (circle), 3mer (triangle up), 4mer (triangle down), 5mer (diamond), 6mer (plus), 7mer (cross), 8mer (star), 9mer (minus), 10mer (vertical line), and 11mer (filled square).

of completeness—the results obtained in the previous study,[1] as well as the association constants for the four dipeptides.

The product yields for each dipeptide in the liposome system nicely reflect the values of the association constants K_a. Of all the peptides used, H−TrpTrp−OH is the only one that exhibits high affinity to the liposome membrane, and in fact the other hydrophilic dipeptides hardly underwent oligomerization in the POPC system when used alone (see Table 1) or in the mixture of all four dipeptides. For each of the three hydrophilic dipeptides, the main product formed is their carbamate in yields of about 80% of the dipeptide reacted. About 12% of the reacted dipeptide turned into the tetrapeptide or its corresponding carbamate. It is not clear to what extent the Asp- and Glu-containing dipeptides coupled at the γ or δ carbonyl instead of the C-terminal carboxylic acid. It seems however likely that this branching does occur. When the reaction is carried out with the mixture of all four dipeptides, their products are suppressed to the benefit of the H−TrpTrp−OH oligomerization, as already preliminarly noted.[1] The theoretically possible co-oligomers within all of the four dipeptides shown in Table 1 made up only about 15% of the total peptides reacted, i.e., out of the 16 possible tetrapeptides, H−Trp_4−OH represented up about 70% of the all products. In all condensation experiments just discussed, DLS measurements and freeze-fracture electron microscopy (ff-EM) showed no significant change of size and size distribution of the liposomes at the end of the reaction.

DCI−Induced Polycondensation on Positively Charged Liposomes. The cationic surfactant DDAB is reported to form liposomes,[7−9] with no tendency for liposome fusion at concentrations of 1−10 mM. DDAB is further known to form stable mixed liposomes together with POPC or other PCs.[10] If 10 mM of NCA−Trp was incubated in the presence of DDAB containing mixed POPC liposomes at pH 7.50, the polycondensation yielded higher amounts of oligo-Trps than in the POPC system (see Figure 3). The nonamer of Trp was the longest oligomer observed in the mixed liposome system, which was shorter than for the pure POPC system where the longest oligomer of Trp was the 11mer. A possible explanation for this observation is a possible π-cation interaction[11] between the Trp residues and the DDAB ammonium group, which leads to better binding of the oligo-Trps to the mixed DDAB/POPC liposomes than to the POPC system. Longer oligomers have a higher affinity to the positively charged mixed liposomes when compared to the neutral POPC liposomes. Di-, tri-, tetra-, and pentamers bind more tightly, and this is favorable for their elongation at the beginning of the polycondensation process. However, the average length is not increased, on the contrary. This can be explained looking at the that liposomes as macromolecules with a finite number of binding sites. If it is so, as the reaction proceeds, an increased affinity of Trp-oligomers to the lipid bilayer decreases the number of binding sites for the incoming NCA−Trp.

The negatively charged residue Glu is very suitable for polycondensation reactions in the presence of positively charged liposomes. We performed block co-oligomerization experiments in the presence of POPC and positively charged DDAB/POPC liposomes by sequential feeding of NCA−Trp and NCA−Glu at pH 7.50. Schematically, this process is illustrated in Scheme 1. In these experiments, NCA−Trp was added first to either the POPC or the mixed DDAB/POPC liposomes, followed by 24 h incubation. After that, the same amount of freshly prepared NCA−Glu was added to both liposome systems, and the suspension was incubated for additional 24 h. Samples were then withdrawn and analyzed by LC−MS. The UV-traces at 280 nm are shown for the two systems in Figure 4. At this wavelength only the products containing Trp can be monitored; however, the presence of oligo-Glu residues can be detected by mass spectrometry.

It is apparent that the positively charged, mixed DDAB/POPC liposomes (Figure 4B) assisted in the formation of higher block oligomers. For example, in block oligomers containing five Trps, the decapeptide H−Glu_5Trp_5−OH was formed in 0.2% yields based on initial Trp in the DDAB/POPC system. Conversely, in the POPC system peptides no longer than H−Glu_2Trp_5−OH were detected (0.2%). It should be mentioned that the detailed analysis of Glu-oligomers involves significant analytical problems. In fact, the reversed-phase separation would have to be changed for optimal separation into ion-exchange chromatography, which is not compatible with an on-line LC−MS coupling. The existence of shorter oligo-Glus in the absence of positively charged liposomes was however indicated by MS data.

Most likely the hydrophobic and π-cation interactions have the effect of increasing the local concentration both of NCA−Trp and of its oligomer products on the charged lipid bilayer membrane interface during the first feeding reaction. As apparent from Figure 3B, the higher affinity of Trp residues to the positively charged lipid membrane leads to higher yields in the tetra- to octamer

Table 1. Polycondensation of 5 mM H−TrpX−OH, 50 mM Phosphate, 2 mM EEDQ, 1.2% ACN, and pH 5.90 in the Absence (Ref) or Presence of 100 nm Extruded 25 mM POPC Lipsomes (Lip)[a]

	% of initial dipeptide X for H−TrpX−OH							
	Gly		Asp		Glu		Trp	
products	Ref	Lip	Ref	Lip	Ref	Lip	Ref	Lip
				Peptides				
cyclo(−TrpX−)							0.4	0.4
H−(TrpX)$_2$−OH	0.3	1.9	2.4	2.3	2.1	2.0	0.3	30
H−(TrpX)$_3$−OH		0.2						2.9
H−(TrpX)$_4$−OH								0.2
				Derivatives				
EtO−CO−TrpX−OH	23	19	23	21	24	21	25	6.0
EtO−CO−(TrpX)$_2$−OH	0.3	0.9	1.3	1.2	1.2	1.4	0.1	2.1
EtO−CO−(TrpX)$_3$−OH		0.1						0.3
rest	0.7	1.3	1.5	1.8	2.0	2.8	1.3	1.3
K_a (M^{-1})	7.63 ± 0.20		0.11 ± 0.11		0.076 ± 0.032		58.26 ± 0.96	

[a] Products were incubated for 26 h on a vortex at room temperature and the products analyzed by LC−MC. The table also shows the association constant K_a measured by equilibrium dialysis between the peptides and 100 nm extruded POPC liposomes in 50 mM phosphate buffer, pH 5.90.

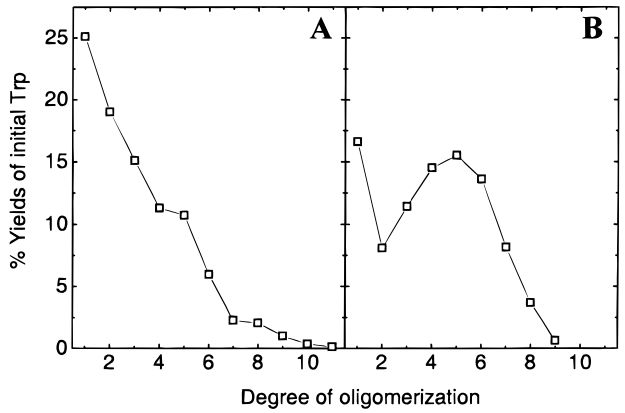


Figure 3. Condensation of NCA−Trp in the presence of positively charged liposomes (B) leading to a different product distribution than in the case of neutral lipid bilayers (A). 20 mM NCA−Trp, 0.4 M imidazole/HCl, pH 7.50, was mixed 1:1 (v/v) with either a 100 nm extruded 25 mM POPC liposome suspension in 0.4 M imidazole/HCl buffer of pH 7.50 (A) or 100 nm extruded 10 mM POPC/10 mM DDAB liposome suspension in 0.4 M imidazole/HCl buffer of pH 7.50 (B). The resulting mixture was incubated for 12 h at room temperature.

of Trp. After 24 h incubation, all initially added NCA−Trp had either oligomerized to oligo-Trps or hydrolyzed to Trp. At this point, the negatively charged NCA−Glu was added, which is expected to interact significantly with the charged POPC/DDAB liposomes, inducing a high local concentration of the monomers and, eventually, a significant binding of the product polyanionic oligomers and co-oligomers. The affinity of these products for the membrane is expectedly higher, the longer the chain. It is also to be expected that the hydrophobic and π-cation interactions for Trp residues contribute to an increased affinity. Most probably the N-terminus of the oligo-Trps formed in the first feeding, as well as that of the oligo-Glus and Glu/Trp co-oligomers produced in the second feeding, are in an environment where the local concentration of the activated amino acid exceeds that in free solution. Consequently, the probability that an oligomer will be extended at its N-terminus increases with its length, at least until it can remain tightly bound to the liposome interface.

EEDQ-Induced Polycondensation on Negatively Charged Liposomes. The liposome-assisted condensation of dipeptides can be extended to more hydrophilic

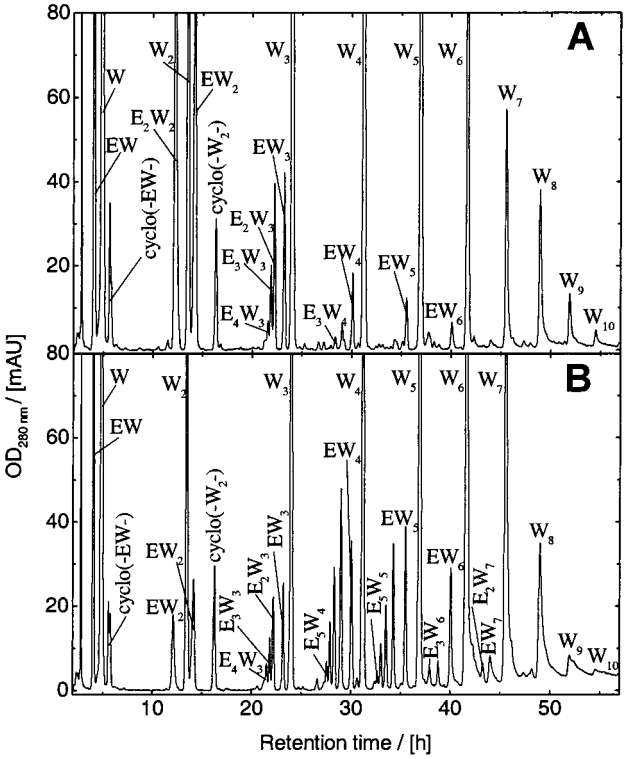


Figure 4. Block co-oligomerization of NCA−Trp and NCA−Glu in the presence of either POPC (A) or DDAB/POPC liposomes (B). 40 mM NCA−Trp in 0.4 M imidazole/HCl, pH 7.50, was mixed first 1:2 (v/v) with either 100 nm extruded 20 mM POPC liposome suspension in 0.4 M imidazole/HCl buffer of pH 7.50 (A) or 100 nm extruded mixed 10 mM DDAB/10 mM POPC liposome suspension in 0.4 M imidazole/HCl buffer of pH 7.50 (B) for 24 h at room temperature. After that period, the same volume (as for NCA−Trp) of a 40 mM NCA−Glu solution in 0.4 M imidazole/HCl, pH 7.50, was added to both systems and incubated for another 24 h. E and W are one letter codes for Glu and Trp, respectively.

dipeptides such as H−ArgTrp−OH and H−HisTrp−OH by introducing negative charges on POPC liposomes. DOPA forms stable liposomes in aqueous solutions in the absence of calcium ions and at neutral pH values.[12] It can also be used together with POPC or other phosphatidylcholines in order to form mixed, negatively charged liposomes.[13] Literature values of the pKs reported for DOPA are spread over a wide range as in

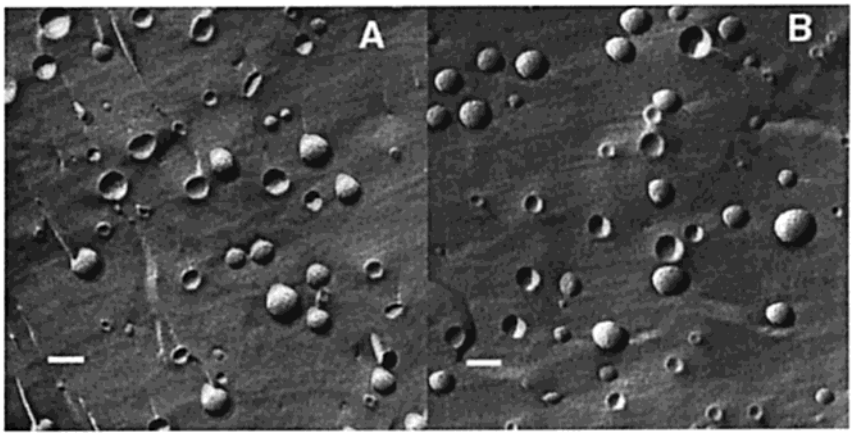


Figure 5. Freeze-fracture electron microscopy pictures obtained for 5 mM H−ArgTrp−OH, 100 nm extruded 12.5 mM DOPA/12.5 mM POPC liposomes, 50 mM phosphate, 1.2% (v/v) ACN, pH 6.40, before (A) and after incubation for 26 h with 2 mM EEDQ at room temperature (B). The length of the white bars corresponds to 100 nm.

Table 2. 5 mM H−ArgTrp−OH, 50 mM Phosphate, and 1.2% (v/v) ACN Solution Incubated with 2 mM EEDQ (Ref) and Either 100 nm Extruded 25 mM POPC (POPC) or 12.5 mM DOPA/12.5 mMPOPC Liposomes (DOPA/POPC) for 26 h at Room Temperature

	% of initial dipeptide				
	pH 5.90			pH 6.40	
products	Ref	POPC	DOPA/POPC	Ref	DOPA/POPC
		Peptides			
cyclo(−ArgTrp−)	2.3	1.7	9.6		
H−(ArgTrp)$_2$−OH	1.3	1.4	23		13
H−(ArgTrp)$_3$−OH		0.4	3.4		2.4
		Derivatives			
EtO−CO−(ArgTrp)−OH	19	9.6	3.8	8.5	3.6
EtO−CO−(ArgTrp)$_2$−OH	0.9	0.6	3.3		1.0
rest	1.9	8.1	10	0.7	2.7

the case for other phospholipids with ionizable groups.[14,15] For the present research, only the approximate apparent pK values (p$K_{1,app}$ = 3−4 and p$K_{2,app}$ = 8−9) for phosphatidic acid are of immediate interest.

First, H−ArgTrp−OH was chosen as the positively charged dipeptide to be oligomerized in the presence of negatively charged DOPA/POPC liposomes—see also Scheme 1. A mixture of 5 mM H−ArgTrp−OH, 12.5 mM DOPA, and 12.5 mM POPC was suspended in 50 mM phosphate buffer of pH 5.90, resulting in a pH 6.40 suspension. This liposome suspension was frozen 10 times in liquid nitrogen and thawed in a water bath prior to extrusion (see Experimental Section for details). The resulting 100 nm extruded DOPA/POPC liposome suspension was analyzed before and after peptide condensation with 2 mM EEDQ by DLS and ff-EM. The DLS data (60°: 51.89 ± 0.84 nm (51.82 ± 0.46), 90°: 46.52 ± 0.51 nm (46.74 ± 0.69, before condensation), and 120°: 45.13 ± 0.30 nm (45.22 ± 0.46)) and the ff-EM pictures (see Figure 5) revealed that the 1:1 composition of DOPA and POPC formed stable liposomes and showed no transformation during the condensation reaction.

Table 2 compares the product distributions of the H−ArgTrp−OH condensation at two different pH values, 5.90 and 6.40, for the aqueous reference system without liposomes, the POPC liposome system, and the negatively charged DOPA/POPC liposome system. There is a clear preference for oligopeptide formation in the presence of negatively charged DOPA/POPC liposomes. As we have seen in Table 1 for H−TrpGly−OH at pH 5.90, a weak association to the POPC liposome membrane is sufficient to produce a slightly enhanced degree of oligomerization. The same holds true for H−ArgTrp−OH with an association constant K_a of 2.29 ± 0.27 M^{-1} at pH 5.90 for POPC bilayer membranes. However, the effect is not as pronounced as for the DOPA containing liposomes, where K_a is 71.70 ± 0.66 M^{-1} at pH 5.90 and 49.7 ± 2.0 M^{-1} at pH 6.40, respectively. The DOPA/POPC system at pH 5.90 (6.40) led to yields of 580 μM (320 μM) tetrapeptide and 50 μM (40 μM) hexapeptide.

The oligopeptides HPLC chromatograms were broad and often consisted of several overlapping peaks, probably due to partial branching occurring during the polycondensation. It is clear however that the introduction of negative charge on the liposome interfaces induces a significant oligomerization of the dipeptide H−ArgTrp−OH. As already mentioned, this is probably mostly due to electrostatic interactions: in fact the guanidinium function of Arg has a pK of about 12 and is therefore completely protonated under the conditions employed here,[16] while most of the phosphatidic acid headgroups have one negative charge around neutral pH values.

Since both H−ArgTrp−OH and H−TrpTrp−OH can be oligomerized by liposome-assisted polycondensation, the question arises of whether their co-condensation is possible. The results for the DOPA/POPC liposome system and the aqueous reference system are given in Table 3. While the corresponding reference system failed to yield co-oligomers, the presence of negatively charged membranes yielded peptides in reasonable amounts (28% of the dipeptides had reacted, whereas for the aqueous reference system only 16%). In particular, in the liposome (reference) system about 13% (2%) of H−ArgTrp−OH and 15% (14%) of H−TrpTrp−OH had

Table 3. 5 mM H−TrpTrp−OH and 5 mM H−ArgTrp−OH Solution in 50 mM Phosphate Buffer, pH 6.60, Incubated for 26 h at Room Temperature with 2 mM EEDQ (in 1.2% (v/v) ACN) and Either with 100 nm Extruded 12.5 mM DOPA/12.5 mM POPC Liposomes (POPC/DOPA) or in the Absence of Liposomes (Ref)[a]

products	(in % of dipeptide reacted) DOPA/POPC:	Ref
Peptides		
cyclo(−TrpTrp−)	1.1	
cyclo(−ArgTrp−)	6.9	
H−Trp_4−OH	18	
H−Trp_6−OH	0.5	
H−$(ArgTrp)_2$−OH	7.9	
H−$ArgTrp_3$−OH	21	
H−TrpTrpArgTrp−OH	16	
H−$(ArgTrp)_2$TrpTrp−OH, H−$ArgTrp_3$ArgTrp−OH, or H−TrpTrp$(ArgTrp)_2$−OH	3.2	
H−$ArgTrp_5$−OH, H−$Trp_2ArgTrp_3$−OH, or H−Trp_4ArgTrp−OH	7.3	
Derivatives		
EtO−CO−TrpTrp−OH	6.6	82
EtO−CO−Trp_4−OH	0.5	0.3
EtO−CO−ArgTrp−OH	4.9	12
rest	6.1	5.7

[a] Analysis by DLS of the first product displayed no significant changes of the liposomal shapes and sizes during the reaction. The hydrodynamic radii before (after) the polycondensation reaction were 47.06 ± 0.59 nm (46.94 ± 0.65) at 60° and 45.72 ± 0.40 nm (46.46 ± 0.58) and 44.95 ± 0.57 nm (45.84 ± 0.38) at 120°

Table 4. 5mM H−HisTrp−OH, 50 mM Phosphate Buffer, and 1.2% (v/v) Acetonitrile Solution of Either pH 4.00, 5.00, 5.70, or 6.20 Incubated with 2 mM EEDQ in the Absence of Liposomes (Ref) or in the Presence of 100 nm Extruded 8.33 mM DOPA/16.67 mM POPC Liposomes (Lip) for 26 h at Room Temperature

	% of initial dipeptide							
	pH 4.00		pH 5.00		pH 5.70		pH 6.20	
products	Ref	Lip	Ref	Lip	Ref	Lip	Ref	Lip
Peptides								
cyclo(−HisTrp−)	0.5	5.6	0.2	8.0		4.5		2.9
H−$(HisTrp)_2$−OH	0.4	9.0	1.2	13	0.6	8.9	0.2	7.0
H−$(HisTrp)_3$−OH		0.3		0.8		0.5		
Derivatives								
EtO−CO−HisTrp−OH	11	5.2	26	8.4	20	7.2	22	11
EtO−CO−$(HisTrp)_2$−OH	0.2	1.2	0.7	2.7	0.3	1.5	0.1	1.6
rest	3.3	5.2	5.5	5.8	4.7	3.8	4.4	7.9

reacted. Of the total dipeptides reacted, 63% formed tetrapeptides and 11% hexapeptides. Of all the oligopeptides formed, 64% were co-oligomers made up of both dipeptide monomers. This means that the oligomers consisting of only one type of monomer were clearly underrepresented. Co-oligomers having -TrpTrp- residues were obtained in higher yields than these containing −ArgTrp-.

Let us consider now the case of the dipeptide H−HisTrp−OH—see also Scheme 1. The affinity constant of this compound for the POPC membrane (100 nm extruded, 25 mM POPC) was not significantly different at pH 5.90 (K_a: 0.54 ± 0.09 M^{-1}) and pH 8.00 (K_a: 0.70 ± 0.17 M^{-1}), where the side chain of His is deprotonated. Introduction of a negative charge in the membrane, by using 1:2 (mol/mol) DOPA/POPC liposomes (100 nm extruded, 25 mM total lipids), increases the affinity by about 2 orders of magnitude: at pH 4.00, K_a was 48.84 ± 0.64 M^{-1}; at pH 5.00, 42.65 ± 0.80 M^{-1}; at pH 5.70, 38.8 ± 1.0 M^{-1}; and at pH 6.20, 24.29 ± 0.68 M^{-1}. This trend most likely reflects the effect of the π-imidazole nitrogen pK_a of H−HisTrp−OH and the apparent pK_as of DOPA on the dipeptide affinity to the DOPA/POPC lipid membrane.

The EEDQ-induced condensation at all four pH values was performed for H−HisTrp−OH in the presence and absence of DOPA/POPC liposomes (see Table 4). The amount of oligomers produced was higher in the presence of bilayer membranes at all pH values. In the reference systems, the carbamate of the di- and tetrapeptide was the major product. The diketopiperazine formation seemed to be favored at lower pH values, but the opposite holds true for the secondary reaction of carbamate formation. Maximum oligopeptide formation was reached in the liposome system at pH 5.00, where about 40% of H−HisTrp−OH had reacted. At this pH the hexapeptide yield was 14 μM, whereas at pH 5.70 it was 7.1 μM, at pH 4.00 only 5.8 μM and at pH 6.20 no hexapeptide could be detected.

Chemical Functionality of in Situ Produced Peptides. The synthesis of His-containing peptides is of interest also in view of the possibility of the in situ syntheses of compounds with functionality, in particular catalytic peptides. Of particular interest would be the case in which the reactivity of a peptide is enhanced by the membrane. In this case the membrane would not only facilitate the synthesis, but also favor a particular class of chemical reactions. This would be interesting also from the prospect of the origin of molecular complexity and origin of life.

To explore this possibility, we focused on the tetrapeptide H−HisTrpHisTrp−OH, which is the product of one of the previously described reactions. For this particular work however the peptide was synthesized in higher amount by the solid-phase method. Catalysis in activated ester hydrolysis by imidazole,[17] His-containing peptides[18,31] and derivatives[32] is well-known in the literature. We investigated the possible catalysis

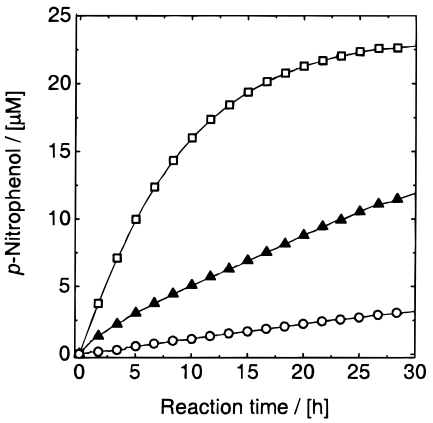


Figure 6. Release of the *p*-nitrophenol is shown for the incubation of 25 μM C16−ONp at 25 °C in a suspension of 50 nm extruded 3.33 mM DOPA/6.67 mM POPC liposomes, 50 mM borate buffer, pH 8.50, 1.2% (v/v) ACN in the absence (circles) or presence (squares) of 1 mM H−HisTrpHisTrp−OH, or 1 mM H−HisTrpHisTrp−OH in 50 mM borate buffer, 1.2% (v/v) ACN of pH 8.50, 1.2% (v/v) ACN in the absence of liposomes (triangle). The hydrolysis of C16−ONp in the absence and presence of DOPA/POPC liposome, with and without tetrapeptide, was monitored spectroscopically at 400 nm, where the released *p*-nitrophenolate has its absorption maximum.

by the tetrapeptide H−HisTrpHisTrp−OH in the hydrolysis of the long-chain activated ester, *p*-nitrophenyl palmitate (C16−ONp).[33−35] Due to the very poor solubility of C16−ONp in water, this compound binds tightly to lipid membranes in the presence of liposomes. However, it cannot be used in excess, and therefore no turnover can be studied. In Figure 6 the reactions in the liposome suspension, in the presence and absence of H−HisTrpHisTrp−OH or in the absence of liposomes, are reported. The tetrapeptide increases the rate of *p*-nitrophenolate release with respect to the two references (liposomes alone, peptide alone in buffer only). Furthermore, when the reaction is performed in the presence of liposomes and peptide, there is a further rate enhancement (2.5-fold) compared to the peptide alone.

At first sight, this looks like enhanced catalysis. However, detailed analysis of the reaction mixtures by LC−MS revealed that 1.9−2.1% of the initial H−HisTrpHisTrp−OH (19 and 21 μM peptide) had reacted with C16−ONp to yield the *N*-palmitoylpeptide in the presence of liposomes, while hardly any amide was detected in the absence of liposomes. Therefore, ca. 75−85% of C16−ONp did not hydrolyze, but underwent transamidation in the presence of liposomes.

This means that the peptide/liposome complex did not lead to major catalysis in the hydrolysis of C16−ONp, but rather to a change in selectivity of the reaction, namely from hydrolysis to transamidation. Although this is not catalysis in the proper sense, it is in fact a case for a reaction which occurred in a very specific direction because of the presence of the membrane. In conclusion, this experiment illustrates the case of a functional peptide that can be produced in situ by liposome-assisted condensation and that favors a reaction in the presence of liposomes which otherwise would hardly take place.

The results are interesting also in another aspect: A warning should be given about the data published on His-catalyzed *p*-nitrophenyl ester hydrolysis experiments, where the imidazole catalyst has been used in equimolar amounts or higher and often in the presence of great excess surfactants.[21,30,36−42] Our work demonstrates in fact, that the *p*-nitrophenolate release is not sufficient to demonstrate catalytic hydrolysis—a problem to which we did not give enough consideration in our own early work.[43]

Two messages can be elicited from these experiments: (i) the lipid membrane can induce reaction selectivity and (ii) *p*-nitrophenyl ester hydrolysis reaction mixtures with excess of surfactants or "catalysts" should be analyzed by MS in greater detail before assigning catalysis.

Concluding Remarks

The results presented above show that the liposome-assisted condensation of either NCA−amino acids or dipeptides with the hydrophobic condensing agent EEDQ can be extended to charged, hydrophilic amino acids and peptides by the use of charged lipid membranes. Accumulation, due to electrostatic and/or hydrophobic interactions, leads to higher local concentrations of the activated amino acids, peptides, and oligopeptides on the lipid bilayer. This favors the oligomerization of charged hydrophilic as well as hydrophobic amino acids and peptides. In this way, oligopeptides consisting of hydrophobic and hydrophilic amino acids such as Trp, Glu, Arg, and His were obtained. Such His-containing peptides in the presence of liposomes seem to have at least an effect on the selectivity of the C16−ONp reaction. Side reactions complicate the interpretation of the experimental results as to whether catalysis occurs.

Experimental Section

Materials. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was a gift from the Pharma Research Department of Novartis International Ltd. (Basel, Switzerland) for all EEDQ-induced condensation and related experiments. In the CDI-induced condensation experiments POPC as well as 1,2-dioleoyl-*sn*-glycero-3-phosphate monosodium salt (DOPA) were purchased from Avanti Polar Lipids (Alabaster, AL). H−ArgTrp−OH·1.5HCl·0.5H_2O (peptide content: 85%), L-Glu (>99%), H−HisTrp−OH (98%), H−TrpAsp−OH·H_2O (peptide content: 93%), H−TrpGlu−OH·0.5H_2O (peptide content: 97.4%), H−TrpGly−OH·H_2O (peptide content: 93%), H−TrpTrp−OH·2H_2O (peptide content: 91%), and Z−HisTrp−OH (>98%) were purchased from Bachem (Dubendorf, Switzerland). Fmoc−His(Trt), Fmoc−Trp(Boc), and Fmoc−Trp(Boc)−Wang resin (0.4 mmol/g) were purchased from Calbiochem-Novabiochem (Läuflingen, Switzerland). Acetic acid (AcOH) (>99.5%), boric acid (>99.5%), chloroform ($CHCl_3$) (≥99.8%), didodecyldimethylammonium bromide (DDAB) (≥98%), ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) (≥99%), 2-ethoxy-1-ethoxycarbonyl-1,2-dihydrochinoline (EEDQ) (>99%), potassium dihydrogen phosphate (KH_2PO_4), sodium dihydrogen phosphate dihydrate (NaH_2PO_4·2H_2O) (≥99%), trifluoroacetic acid (TFA) (≥99% HPLC grade), triisopropylsilane ($^iPr_3SiH$) (>99%), and imidazole (≥99.5%) were purchased from Fluka (Buchs, Switzerland). Acetonitrile (ACN) and methanol (MeOH) (HPLC grade) were purchased from Macherey-Nagel (Oensingen, Switzerland). Sodium hydrogen carbonate ($NaHCO_3$) (≥99.5%) and standard 1 M solutions of HCl (Titrisol) and NaOH (Titrisol) were purchased from Merck (Darmstadt, Germany). The deionized water was deionized a second time by a Milli-Q RG from Millipore (Volketswil, Switzerland). *N*,*N*′-Carbonyldiimidazole (CDI) and *p*-nitrophenyl palmitate (C16−ONp) were purchased from Sigma (St. Louis, MO).

The tetrapeptide H−HisTrpHisTrp−OH was synthesized using the Fmoc−Trp(Boc)−Wang resin (0.615 g, 4-fold excess),

and for each coupling step either Fmoc−His(Trt) (0.620 g, 2-fold excess) or Fmoc−Trp(Boc) (0.527 g, 2-fold excess) using a FastMoc protocol (Fmoc-strategy) on an Applied Biosystems 433A peptide synthesizer from Perkin-Elmer. According to protocol, the resin was dried at high vacuum overnight (0.4015 g). The protecting groups were removed by incubation for 2 h with a cleavage cocktail of TFA (9.5 mL), water (0.25 mL), and $^{i}Pr_3SiH$ (0.25 mL) as a scavenger. Rotatory evaporation led to precipitation. This suspension was washed three times with ice cold ether and the precipitate was separated each time by centrifugation at 3500 rpm for 5 min. The remaining precipitate was dried under high vacuum resulting in H−HisTrpHisTrp−OH (0.146 g, >95% purity (HPLC, $OD_{220\ nm}$)), a white powder, which was stored at −20 °C until further use.

Liposome Preparation. Liposomes were prepared from POPC only or, in the case of mixed liposomes, a mixture of POPC and the according amount of DOPA in $CHCl_3$ or DDAB, respectively. The lipids were first dissolved in $CHCl_3$ in a round-bottom flask. After evaporation of the solvent by rotatory evaporation under reduced pressure at 40 °C, the thin film was dried overnight at high vacuum. The dried lipid film was dispersed in buffer, or buffered peptide solution, and put on a shaker for about 15 min to help speed up the dispersion process, which led to the formation of mainly multilamellar liposomes of a considerable heterogeneity in size.[44,45] For the case of DOPA containing liposomes, sometimes concentrated solutions were used for dispersion and the pH (using a Radiometer (Copenhagen, Denmark) PHM82 pH-meter and an InLab 423 electrode from Mettler Toledo, Nänikon-Uster, Switzerland) and volume were adjusted thereafter. The liposome suspension was then frozen 10 times in liquid nitrogen (−195 °C) and thawed in a water bath at 40 °C (freeze−thaw cycles) in order to reduce the number of small unilamellar liposomes and increasing the content of multivesicular liposomes.[46,47] A significant decrease in size and lamellarity was achieved by successively passing the liposome suspension through two Nucleopore polycarbonate membranes (d: 25 mm) from Sterico AG (Dietlikon, Switzerland) with mean pore diameters of 400 nm (10 times), 200 nm (10 times), 100 nm (10 times), and in the hydrolysis experiments also 50 nm (10 times), using an extruder (The Extruder supplied by Lipex Biomembranes Inc., Vancouver, Canada).[46] Liposomes were characterized by ff-EM and DLS. The extruded liposome suspensions were normally freshly prepared, especially when containing peptides, and were never stored for longer than 2 weeks.

Determination of the Association Constant (K_a). The association properties of peptides to liposomes were studied quantitatively by the equilibrium dialysis method,[48,49] using a Dianorm equilibrium dialyzer (Dianorm, München, Germany) and a highly permeable dialysis membrane (made from neutral cellulose) with a molecular weight cutoff of 5000 Da. To start, 1.0 mL of a typically buffered, 5 mM in peptide and 100 nm extruded 25 mM lipid liposome-containing suspension and 1.0 mL of a 5 mM peptide buffered solution were put into the two chambers of each of the five Teflon cells separated by the dialysis membrane (d: 63 mm, A: 4.5 cm^2). The dialysis membranes were incubated before use for 10−15 min in boiling, deionized water (ca. 1 L), containing one spatula (ca. 1 g) of sodium hydrogen carbonate ($NaHCO_3$) and two spatulas (ca. 2 g) of ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA), washed several times with deionized water and later equilibrated overnight in the type of buffer used in the dialysis experiments that followed. The dialysis experiments were performed by rotating the cells at a constant speed of 8 rpm for 24 h at room temperature. It was shown that after this time equilibrium distribution is reached between the two chambers and DLS measurement confirmed that the size of the liposomes did not change during dialysis. The solutions were then removed from the cells, and each chamber was analyzed for peptide content using LC−MS and

$$K_a = \frac{OD_\lambda area_{Lip} - OD_\lambda area_{Aq}}{OD_\lambda area_{Aq}[Lip]_0}$$

with K_a being the association constant, OD_λarea the absorption peak area in the chromatogram of the dialysis chamber sample containing only the aqueous buffer solution (Aq) or the liposome suspension (Lip), and $[Lip]_0$ the lipid concentration in the chamber containing the liposome suspension.

Dynamic Light Scattering Measurements. A 30 μL aliquot of sample containing a typical concentration of 25 mM in lipid was diluted with 1470 μL buffer before and after the condensation experiment and transferred into a round-bottom light-scattering cuvette. The DLS was performed on a fiber-optics-based spectrometer consisting of an argon-ion-laser (Innova 200-10, $\lambda_0 = 488$ nm, Coherent), a digital autocorrelator (ALV 5000) and computer-controlled rotational stage (model 496, Newport and Controller PMC 400). A photomultiplier (ALV/PM-15) was used as the detector.[50] The intensity of the scattered laser-beam, reduced by a 9.5%-transmission filter, was measured 10 times over a period of 90 s at each angle (60, 90, and 120°). The data were evaluated using a second-order cumulant fit (Angle 1.3, ALV DLSm software) which provided a mean hydrodynamic radius of the liposomes for each angle.

CDI−Induced Amino Acid Condensation. In a typical single-feeding experiment, a concentrated amino acid solution (i.e., 60 mM in H−Trp−OH) in a 0.4 M imidazole/HCl buffer of pH 6.80 was incubated with a 2.5-fold excess of CDI at 0 °C for 2 min. The resulting NCA−amino acid solution was mixed 1:1 (v/v) with either an aqueous 40 mM lipid suspension of 100 nm extruded liposomes in 0.4 M imidazole/HCl buffer, pH 6.80, or 0.4 M imidazole/HCl buffer, pH 6.80, with no liposomes in an 1.5 mL eppendorf tube. The resulting solutions were incubated on a Vortex-Genie 2 shaker (Scientific Industries) for 12 h at room temperature. After this time neither NCA−amino acid nor *N*-[imidazolyl-(1)-carbonyl]amino acid was detected, which indicated that the reaction was complete. Then, 25 μL of the reaction mixtures with liposomes was injected immediately into the HPLC after removal from the shaker. This was done to ensure a homogeneous distribution of the oligomers formed. It was observed that samples, especially at high initial NCA−amino acid concentration and in multiple-feeding experiments of hydrophobic amino acids, led to precipitation by standing for a few hours.

In a typical multiple-feeding experiment, 200 μL of a NCA−amino acid solution was either mixed with 600 μL of a 100 nm extruded 100 mM lipid suspension or buffer alone and incubated for 12 h. Another 200 μL of a freshly prepared NCA−amino acid solution were added to the reaction mixture and incubated again for 12 h and so forth.

EEDQ-Induced Peptide Condensation. In a typical experiment, 12 μL of a EEDQ stock solution in acetonitrile was added either to 1 mL of a 5 mM H−TrpTrp−OH, 50 mM phosphate, 100 nm extruded 25 mM lipid liposome suspension of pH 5.90 (liposome system) or 1 mL of a 5 mM H−TrpTrp−OH, 50 mM phosphate, pH 5.90, (reference system) in a 1.5 mL eppendorf tube and rapidly mixed on a vortex. The resulting mixture, at 2 mM EEDQ, was incubated on a Vortex-Genie 2 shaker (Scientific Industries) for 26 h at room temperature. Then 25 μL of the resulting solution was injected into the HPLC connected to a mass spectrometry detector for product analysis. In the case of the liposome system, the solutions were characterized before and after incubation with EEDQ by ff-EM and DLS.

Liquid Chromatography Mass Spectrometry. The condensation products of the reaction samples were analyzed either by HPLC (HP1050 LC, Hewlett-Packard) connected to a diode array detector (HP1050 DAD, Hewlett-Packard) or by HPLC (HP1100 LC, Hewlett-Packard) connected to a diode array detector (HP1100 DAD, Hewlett-Packard) and a single quadrupole mass spectrometry detector (HP1100 MSD, Hewlett-Packard). A C_{18} column (ET 250/4 Nucleosil 100-5, Macherey-Nagel) was used to separate the condensation products according to their hydrophobicity (reverse-phase chromatography). Typical parameter settings for the HPLC were a 1 mL/min flow rate at room temperature, using two buffer solvents A (0.1% TFA) and B (99.9% ACN, 0.1% TFA), starting with a 2 min isocratic flow of 10% B and then driving a typical

gradient of 2.0% B/min up to 90% B. At high ACN levels, lipids such as POPC precipitate on the column and therefore the column had to be washed with about 100 mL MeOH after every 10 lipid-containing injections in order to guarantee good, reproducible separation on the column.

For the qualitative detection of peptides containing the amino acids used, UV−vis spectra from the diode array detector were compared with the absorption known from amino acid spectra. In addition to this, atmospheric pressure electrospray ionization in the positive mode (ESI^+) was used to verify the expected molecular mass of the peptide oligomers. Typical settings for HP1100 MSD were as follows: 350 °C gas temperature, 10 L/min drying gas flow, 60 psig nebulizing pressure, 3500 V capillary voltage, and 95 V fragmentor voltage. To determine the exact structure formula of the peptides and some byproducts and to verify the presence of certain amino acids in the peptides, MS/MS was applied by in-source fragmentation at a typical fragmentor voltage of 125 V.

Quantification of Trp-containing peptides was performed by measuring the optical density at 280 or 290 nm and integrating the chromatogram peaks over time, assuming that the extinction coefficient for each tryptophan−indole chromophore of the monomer did not change when incorporated into a peptide that is an oligomer of itself.[51]

Kinetics Following *p*-Nitrophenolate Release of C16−ONp. A peptide stock solution in 50 mM boric acid/borate of pH 8.50 was mixed 1:1 (v/v) with a 50 nm extruded liposome suspension in 50 mM boric acid/borate buffer of pH 8.50 and incubated for at least 10 h. Then, 3 mL of the peptide−liposome suspension (or the corresponding reference reaction samples with either buffer only, peptide but no liposomes, or liposomes only) were added into a UV quartz cuvette (3 mL/1 cm). For each system, the baseline was taken against the reference cell containing the same composition as the sample itself prior to addition of the substrate. Then, 36 μL samples of a C16−ONp stock solution in ACN were then added to each reaction sample and 36 μL ACN to the reference cells, respectively. The cells were mixed through shaking and the time-dependent absorbance was measured at 400 and 480 nm. The concentration change of *p*-nitrophenolate/*p*-nitrophenol was calculated from the difference in the optical densities at 400 and 480 nm using a molar extinction coefficient $\epsilon_{400\ nm}$ of 16 300 $cm^{-1}\ M^{-1}$ for the DOPA/POPC liposome suspensions and 18 400 $cm^{-1}\ M^{-1}$ for samples containing no liposomes.

Abbreviations

POPC: 1-palmytoyl-2-oleoyl-*sn*-glycero-3-phosphocholine. DDAB: dioctyldimethylammonium bromide. DOPA: 1,2-dioleoyl -*sn*-glycero-3-phosphate. NCA: *N*-carboxyanhydride. EEDQ: 2-ethoxy-1-ethoxycarbonyl-1,2-dihydrochinoline. CDI: *N,N′*-carbonyldiimidazole (or DCI). C16−ONp: *p*-nitrophenyl palmitate

Acknowledgment. The authors thank Prof. Dr. Paul Rys and Andreas Dutly (Chemical Engineering Laboratory, ETH Zurich, Switzerland), Prof. Dr. D. Hilvert and Richard Quaderer (Laboratory of Organic Chemistry, ETH Zurich, Switzerland) for their kind instrumental support, Michaela Wessicken (Laboratory for Electron Microscopy I, ETH Zürich) for making the electron microscopy pictures, and Anna Suter from Novartis (Basel, Switzerland) for the POPC used in the present work.

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MA000540G