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"Investigation on the origin of biological enzymatic catalysis"

"Studio sull'origine della catalisi enzimatica in sistemi biologici"

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Riassunto

Nel contesto dell'origine della vita, l'avvento dell'evoluzione Darwiniana fu reso possibile grazie all'insorgere di meccanismi molecolari che consentivano di trasmettere, ereditariamente, le variazioni di fitness dei primi semplici organismi.

Un utile modello teorico-sperimentale che permette di studiare tale scenario evolutivo primordiale consiste in una popolazione di protocellule in competizione tra loro. Ciascuna protocellula è costituita da un polimero genetico in grado di replicarsi all'interno di una vescicola lipidica anch'essa in grado di replicarsi. In questo modello, il polimero genetico incapsulato nella protocellula determina la *fitness* del sistema, ad esempio perché codifica per un catalizzatore che a sua volta genera un metabolita utile alla vita e alla riproduzione della protocellula.

Le vescicole lipidiche (liposomi) sono i migliori modelli sperimentali di protocellule, grazie alle loro proprietà di auto-assemblamento e incapsulamento di soluti al loro interno (Luisi, Walde et al. 1999). In particolare, è un fatto ben accettato che vescicole lipidiche costituite da acidi grassi a catena lunga rappresentino realisticamente le protocellule più antiche (Deamer, Dworkin et al. 2002).

La seril-istidina (Ser-His) è uno dei più semplici peptidi catalitici noti. E' stato dimostrato che in particolari condizioni sperimentali, facilita la sintesi di altri peptidi e di RNA (Li, Zhao et al. 2000; Gorlero, Wieczorek et al. 2009; Wieczorek, Dorr et al. 2012).

Lo scopo di questa tesi è quello di sviluppare modelli sperimentali che permettano di studiare i meccanismi alla base dell'evoluzione Darwiniana in tempi primitivi. In particolare, verranno investigate the proprietà di semplici protocellule costituite da acidi grassi, contenenti semplici catalizzatori di natura peptidica.

Innanzitutto verrà mostrato come la Ser-His sia in grado di catalizzare la sintesi di un polimero di tipo "peptide nucleic acid" (PNA) (Gorlero, Wieczorek et al. 2009). Il PNA è un polimero la cui catena principale è costituita da un polipeptide achirale, derivatizzato con gruppi

laterali tipici degli acidi nucleici (basi aromatiche). Il PNA può formare accoppiamenti di tipo Watson-Crick con acidi nucleici naturali, ed è stato proposto come un possibile precursore prebiotico degli acidi nucleici (RNA, DNA), o come polimero complementare ad essi, almeno in alcune fasi del mondo prebiotico (Egholm, Buchardt et al. 1992; Nielsen, Egholm et al. 1994; Nielsen 2007).

Successivamente, si costruirà un modello più complesso mediante la compartimentalizzazione di piccoli catalizzatori come Ser-His e Ser-His-Gly all'interno di vescicole di acidi grassi, dimostrando come si possa indurre la competizione tra popolazioni di protocellule.

Infine, verrà illustrato come un altro semplice dipeptide come la cisteil-cisteina (Cys-Cys) possa facilitare la divisione di protocellule indotta fotochimicamente.

Più in generale, il lavoro presentato in questa tesi si concentra sul problema della catalisi in condizioni prebiotiche, e i dati verranno discussi in termini di rilevanza di tali meccanismi nell'origine della vita.

Abstract

The advent of Darwinian evolution required the emergence of molecular mechanisms for the heritable variation of fitness.

One model for such a system involves populations of competing protocells, each consisting of a replicating genetic polymer encapsulated within a replicating membrane vesicle. In this model, the encapsulated genetic polymer imparts enhanced fitness to its protocell by, for example, coding for a catalyst that generates a useful metabolite.

Vesicles are the best known model for testing properties of the self-assembled protocell membranes, and a model of protocell encapsulation. (Luisi, Walde et al. 1999)

It is generally agreed that at the earliest stage of the prebiotic bilayer membrane formation membranes consisted of simple, long chain carboxylic acids. (Deamer, Dworkin et al. 2002)

Ser-His is one of the simplest peptide catalysts, under specific conditions facilitating synthesis of peptides and RNA. (Li, Zhao et al. 2000; Gorlero, Wieczorek et al. 2009; Wieczorek, Dorr et al. 2012)

The aim of this work consists in the development of experimental models to study the primitive mechanisms of Darwinian evolution. In particular, I will investigate the properties of simple fatty acid protocells that contain small catalytic peptides.

First I have shown how Ser-His can catalyze synthesis of peptide nucleic acid polymer. (Gorlero, Wieczorek et al. 2009)

Peptide nucleic acid is peptide bond polymer of notably achiral backbone, capable of standard Watson-Crick based pairing with natural nucleic acids. It has been proposed as a likely prebiotic precursors, or at some stages complementary genetic polymer, to natural RNA and DNA. (Egholm, Buchardt et al. 1992; Nielsen, Egholm et al. 1994; Nielsen 2007)

The work presented in this thesis addresses the problem of prebiotic catalysis on the molecular polymer and the protocell level.

In the studies of catalytic activity of small dipeptides, I showed how prebiotic catalytic dipeptide Ser-His can catalyze synthesis of prebiotically plausible genetic polymer PNA, and how two simple catalysts, Ser-His and Ser-His-Gly can induce competition between populations of protocells. I have also showed that another simple prebiotic dipeptide, Cys-Cys, can facilitate photochemically induced division of protocell vesicles.

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List of abbreviations

AcPheLeuNH$_2$ – N-acetylphenylalanine leucyl carboxamide

AcPheOEt – ethyl ester of N-acetylphenylalanine

AcPheOH – N-acetylphenylalanine

BOC – di-tert-butyl dicarbonate

DPH – 1,6-diphenyl-1,3,5-hexatriene

DTT – dithiothreitol

FMOC - fluorenylmethyloxycarbonyl

FRET – Förster (fluorescence) resonance energy transfer

HPLC – high-performance liquid chromatography

HPTS – 8-Hydroxypyrene-1,3,6-trisulfonic acid

LeuNH$_2$ – leucyl carboxamide

LUCA – last universal common ancestor

MS – mass spectrometry

NMR – nuclear magnetic resonance

OA – oleic acid

Phe-Phe – phenylalanyl-phenylalanine

PNA – peptide nucleic acid

ROS – reactive oxygen species

RPM - revolutions per minute

RT – room temperature

Ser-His – Serine-Histidine

Ser-His-Gly – Seryl-Histydyl-Glycine

TFA – trifluoroacetic acid

TMAC – tetramethylammonium chloride

UV – ultraviolet light

List of publications and conference contributions

M. Gorlero, R. Wieczorek, K. Adamala, A. Giorgi, M. E. Schinina, P. Stano and P. L. Luisi (2009). "**Ser-His catalyses the formation of peptides and PNAs.**" FEBS Lett 583(1): 153-156.

K. Adamala and P. L. Luisi (2011). "**Experimental systems to explore life origin: perspectives for understanding primitive mechanisms of cell division.**" Results Probl Cell Differ 53: 1-9.

T. F. Zhu, K. Adamala, N. Zhang and J. W. Szostak (2012). "**Photochemically driven redox chemistry induces protocell membrane pearling and division**." Proc Natl Acad Sci U S A 109(25): 9828-9832.

K. Adamala and J.W. Szostak "**Competition between model protocells driven by an encapsulated catalyst**" manuscript under consideration (submitted November 2012)

Conference contributions during the period of the PhD studies (only meetings where I presented poster or oral contribution are listed)

Bolaamphiphilic bilayer mimics; poster presentation; Mathangi Krishnamurthy, Katarzyna Adamala, Jack W. Szostak; Origin of Life Gordon Conference; January 2008, Ventura, CA, US

Enzymatic reactions in model protocells; poster presentation; Katarzyna Adamala, Jack W. Szostak; ISSOL, August 2008, Florence, Italy

The evolution of protocell membrane, poster presentation and lighting talk; Katarzyna Adamala, Jack W. Szostak; AbGradCon, July 2009, Seattle, WA, US

On the origin of life: natural selection in the protocell population; oral presentation; Katarzyna Adamala, Jack W. Szostak; June 2010, Tällberg, Sweden

Toward the beginning of Darwinian evolution; poster presentation, Katarzyna Adamala, Jack W. Szostak; AbGradCon, June 2011, Bozeman, MT, US

On the origin of life: natural selection in the protocell population; oral presentation; Katarzyna Adamala, Jack W. Szostak; ISSOL, July 2011, Montpellier, France

Vice-chairperson and program organizer, Origin of Life Gordon Research Seminar, January 2012, Galveston, TX, US

The chemical beginning of Darwinian evolution; poster presentation; Katarzyna Adamala, Jack W. Szostak; Origin of Life Gordon Research Conference, January 2012, Galveston, TX, US

Potential mechanisms of prebiotic gene regulation; oral presentation; Aaron E. Engelhart, Katarzyna Adamala, Jack W. Szostak; SERMACS November 2012, Raleigh, NC, US

1. Introduction

1.1 Origin of life

There is no commonly accepted definition of life. Most scientists working on the problem agree that life can be defined by the set of functions and features that must be possessed by the system to be called alive. Yet, the specificity of these functions remains undefined. (Luisi 1998; Cleland and Chyba 2002; Ruiz-Mirazo, Pereto et al. 2004; Ruiz-Mirazo, Pereto et al. 2010; Weber 2010) Since there is no indisputable definition of life, it is also hard to define the event of the origin of life. For the purpose of this work, it will be assumed that the origin of life was the process during which the chemical reactions spontaneously arranged into a homeostatic system undergoing Darwinian evolution. (Oro and Lazcano 1992; Lynn, Burrows et al. 2012)

Life originated on Earth at least 3.6 billion years ago. The oldest known traces of fully-developed life are dated for approximately 3.465 billion years (Schopf 1994), and some evidence show possibility of biochemical cycles existing as early as 3.8 billion years ago (Schidlowski 2001).

The time between the origin of Earth's crust and primordial ocean 4 billion years ago (Morbidelli, Chambers et al. 2000), and the first known traces of life (date back to 3.8 billion years), is the time when all processes of the origin of life must have occurred. This leaves approximately 200 – 500 million years for the chemical evolution processes. (Orgel 1998)

The first theories postulated that life originated in the water of the prebiotic ocean. The first theory of the primordial ocean, the "prebiotic soup", is commonly claimed to Aleksandr Ivanovich Oparin (1894-1980) and John B. S. Haldane (1892-1964). Oparin's book is indeed first well-known scientific publication presenting the theory of origin of life (Oparin 1957). However, the priority in developing backgrounds of modern origin of life scenario belongs to the nineteenth century chemist Ernst H. P. A. Haeckel (Haeckel 1866). In his book, in 1905, he claimed that life originated as a "simple lump of albuminous combination of carbon" (Haeckel 1905). Recently, various possible environments are considered. Prebiotic Earth provided many different sites for possible

prebiotic chemistry reactions, including open water of the prebiotic ocean, lagoons, surfaces of various minerals, thin layers of organic compounds, gaseous phase of the atmosphere or submarine hydrothermal vents. Different prebiotic processes proposed in the literature are placed in different conditions. (Miller and Schlesinger 1983; Miller 1987)

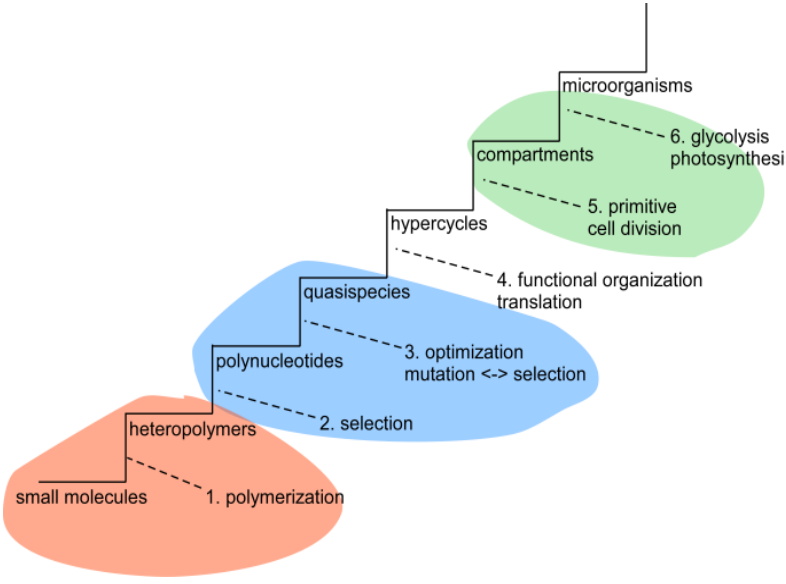


Figure 1.1.1 General schemes of possible stages of prebiotic evolution. Adapted from (Eigen and Schuster 1982)

Circled in green: focus of section 2.1 of this thesis; circled in blue: section 2.2, and circled in red problems addressed in section 2.3 of this thesis.

Nevertheless the origin of life on Earth might have not been a singular accident; only one protocell lineage succeeded and survived, proliferating into all known forms of life. There is no reason to assume that our cells' metabolism represents the only possible type of metabolic process; yet, all the evidence suggests that all known life comes from a

single ancestor. (Orgel 1998; Goldman, Bernhard et al. 2013) This organism, LUCA - the Last Universal Common Ancestor of all organisms (Penny and Poole 1999; Delaye, Becerra et al. 2005) – was most likely a population of cells resulting from previous prebiotic, and earliest biological, evolution and selection.

It is not impossible that origin-of-life processes are still occurring, although it is much more difficult on the oxidized environment, and on the planet absolutely possessed by one type of biological organisms it is practically impossible to expect any other form of metabolism to growth enough compete with "our type" of life. Therefore, no effective biogenesis processes are observed today. (Delaye and Lazcano 2005)

Generally, one can distinguish five major fields of research, corresponding to the five major stages of the origin of life. (Eigen and Schuster 1982) (**Figure 1.1.1**).

The research shown in this thesis was design to address three problems, as marked on figure 1.1.1:

1. the origin of cell division cycle (results described in section 2.1 of this thesis), using lipid vesicles as model protocells;

2. the origin of competition and mechanisms for developing adaptations (results described in section 2.2), with lipid vesicles as model protocell and small dipeptides as prebiotic catalysts;

3. the prebiotic polymerization of small organic molecules (results described in section 2.3), with small dipeptide as a catalyst.

1.2 Systems to experimentally explore the origins of life: the model of protocell membrane

This section was published as part of the chapter of the book Cell Cycle in Development (Adamala and Luisi 2011)

To study the origin of elements of the cell cycle, particularly growth and division of protocell membrane, model protocell vesicles are commonly used. (Chakrabarti, Breaker et al. 1994; Segre, Ben-Eli et al.

2001; Walde 2006) The self-assembled bilayer membranes, semipermeable to small organic molecules and able to encapsulate bigger, polar compounds, are a good model of a prebiotic protocells.

Several authors, including the group of D. Deamer, proposed that at the earliest stage of the prebiotic bilayer membrane formation, membranes consisted of simple, long chain carboxylic acids (Fig. 1.2.1). The open question about the nature of the membrane in Last Universal Common Ancestor leaves many possible routes to the origin of lipid membranes during the earliest stages of protobiological evolution. (Schopf 1994; Goldman, Bernhard et al. 2013)

In modern cells, apart from compartmentation, membranes perform several other functions, including energy transduction and transport of organic and inorganic compounds, and they are the docking site of many enzymes. Presumably, the very first role of the membranes was simple encapsulation – isolation of the reaction cycles (i.e., genetic materials or enzymatic peptides) from the environment. This could be done by the simplest amphiphiles, possibly available under the prebiotic conditions: medium-sized (up to C10) chain carboxylic acids. [Figure 1.2.2] (Luisi, Walde et al. 1999)

The main building blocks of modern cells' membranes are phospholipids and sterols. Phospholipid glycerol esters and sterols are too complex to be synthesized under abiotic conditions. However, all these compounds can be derived from simplest building block – sterols from isoprene units and lipid derivatives from simple unsaturated carboxylic acids. The simple lipids might have been synthesized under prebiotic Earth conditions (Allen and Ponnamperuma 1967; Yuen, Lawless et al. 1981) including environment of the underwater hydrothermal vents (McCollom, Ritter et al. 1999). Simple amphiphiles were also detected in carbonaceous chondrite meteorites (Yuen and Kvenvolden 1973; Deamer 1985).

Compounds based on these simplest units could have formed the first membranes encapsulating biochemical cycles of the protocell. In a water solution, with the pH close to the polar headgroup pKa, the simplest amphiphiles spontaneously self-organize into bipolar membrane sheets that close into spherical vesicles. (Luisi, Walde et al. 1999; Apel, Deamer et al. 2002; Chen and Walde 2010)

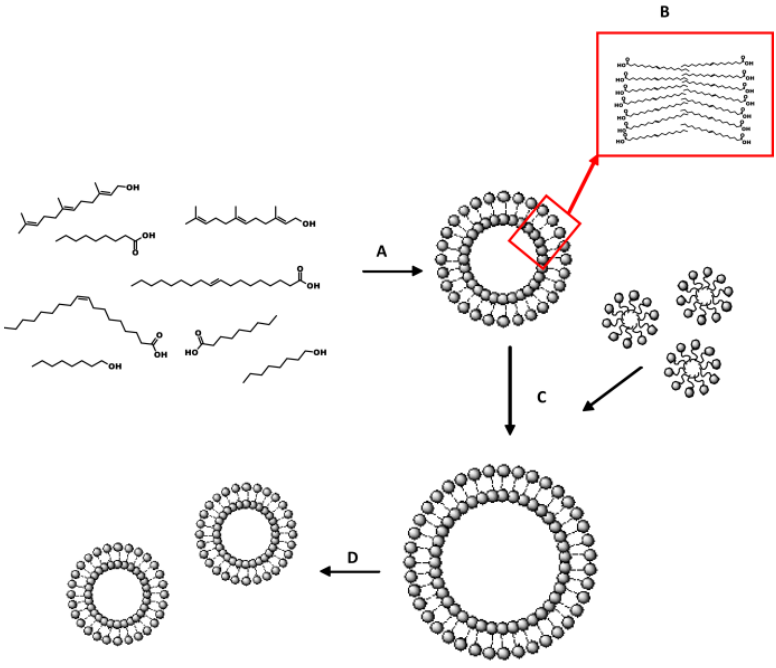


Figure 1.2.1 Protocell vesicles.

A: Vesicles are spontaneously forming from the amphiphilic monomers;

B: bilayer membrane of the vesicle, with polar, hydrophilic headgroups directed outside, and aliphatic, hydrophobic chains inside;

C: vesicles can grow upon addition of micelles;

D: vesicles can be forced to divide into daughter vesicles

Vesicles are commonly accepted as an approximation of the compartments of the earliest protocells (Walde 2006). Vesicle-like bilayer membranes were even observed in amphiphiles organic material

from Murchison carbonaceous chondrite (Deamer 1985; Deamer and Pashley 1989), making its availability on prebiotic Earth more probable.

Vesicle structures can grow (Chen and Szostak 2004; Zhu and Szostak 2009), divide (Hanczyc, Fujikawa et al. 2003; Zhu, Adamala et al. 2012), and selectively take up compounds from the environment (Chen, Roberts et al. 2004). Therefore, investigating properties of the different vesicle systems can give insight into possible routes to the origin of protobiological compartmentalization.

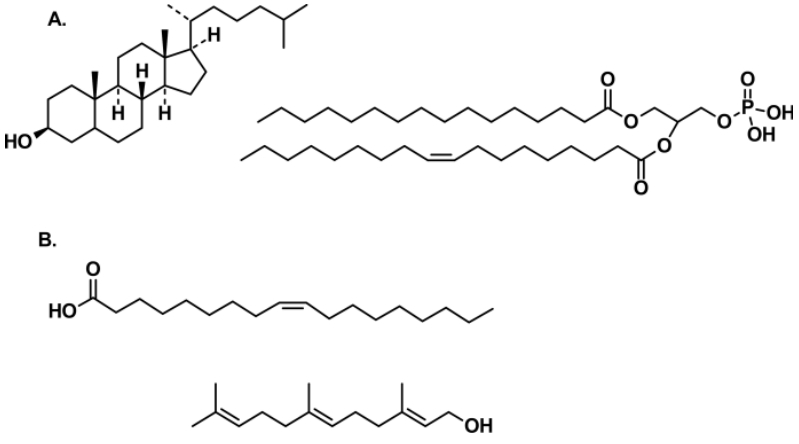


Figure 1.2.2 Amphiphilic compounds building the membranes.

A: modern cell's membrane building block;

B: possible prebiotic amphiphiles.

1.3 Exploring the chemical origins of Darwinian Evolution

Modern cells are thought to have evolved from much earlier protocells – simple replicating chemical systems, composed of a cell membrane and an encapsulated genetic polymer, that were the first cellular systems capable of Darwinian evolution. Evolvability may have emerged in such systems via competition between protocells for a limiting resource. (Szathmáry E 1987; Szostak 2001) Since protocells lacked the complex biochemical machinery of modern cells, such competition was necessarily based on simple chemical or physical processes. (Budin and Szostak 2010) In order to gain further insight into such competitive processes, a variety of model protocell systems have been developed. Fatty acid vesicles have been widely employed as models of early protocellular systems (Hargreaves 1978; Szostak 2001; Apel, Deamer et al. 2002; Noireaux V 2004; Deamer and Dworkin 2005; Mansy, Schrum et al. 2008) because fatty acids can be generated in plausibly prebiotic scenarios, and because membranes based on fatty acids have physical properties that are well suited to primitive forms of life. (Deamer and Dworkin 2005)

The nature of the primordial genetic material remains uncertain; competing schools of thought support either RNA or some alternative nucleic acid as a progenitor of RNA.

Irrespective of the nature of the original genetic material, an important question in considering the origins of cellular competition is how that genetic material could impart a selective advantage to a primitive protocell. An early model (Chen, Roberts et al. 2004) for such a scenario postulated an autocatalytic self-replicating genetic material, such as an RNA replicase, that would accumulate within vesicles at a rate corresponding to its catalytic efficiency. Mutations leading to greater replicase activity would result in a more rapid increase in internal RNA concentration and thus internal osmotic pressure, which would lead to faster vesicle swelling, which in turn would drive competitive vesicle growth. This model was supported by experimental observations that osmotically swollen vesicles could grow by absorbing fatty acid molecules from the membranes of surrounding relaxed vesicles (Chen, Roberts et al. 2004). Although the simple physical link between mutations leading to faster replication of the genetic material, and the consequent osmotic swelling and vesicle growth is attractive, this model

suffers from the lack of a plausible mechanism for the division of osmotically swollen vesicles.

More recently, we have observed that low levels of phospholipids can drive the competitive growth of fatty acid vesicles, in a manner that circumvents this problem by causing growth into filamentous structures that divide readily in response to mild shear stresses (Budin and Szostak 2011). This model implies that a catalyst for phospholipid synthesis, such as an acyltransferase ribozyme, would impart a large selective advantage to its host protocell because faster growth, coupled with division, would result in a shorter cell cycle. A model system illustrating the potential of an encapsulated catalyst to drive vesicle growth in a similar manner would therefore be a significant step towards realizing a complete model of the origin of Darwinian evolution.

Here, we show that the simple dipeptide catalyst seryl-histidine (Ser-His) can drive vesicle growth through the catalytic synthesis of a hydrophobic dipeptide, N-acetyl-L-phenylalanine leucinamide ($AcPheLeuNH_2$), which localizes to the membrane of model protocells and drives competitive vesicle growth in a manner similar to that previously demonstrated for phospholipids. Ser-His has previously been shown to catalyze the formation of peptide bonds between amino acids and between PNA monomers. (Gorlero, Wieczorek et al. 2009) Although Ser-His is a very inefficient and non-specific catalyst, we have found that Ser-His generates higher yields of peptide product in the presence of fatty acid vesicles.

As a result, vesicles containing the catalyst generate sufficient reaction product to exhibit enhanced fitness, as measured by competitive growth, relative to those lacking the catalyst [Figure 1.3.1]. We can therefore observe how a simple catalyst causes changes in the composition of the membrane of protocell vesicles and enables the origin of selection and competition between protocells.

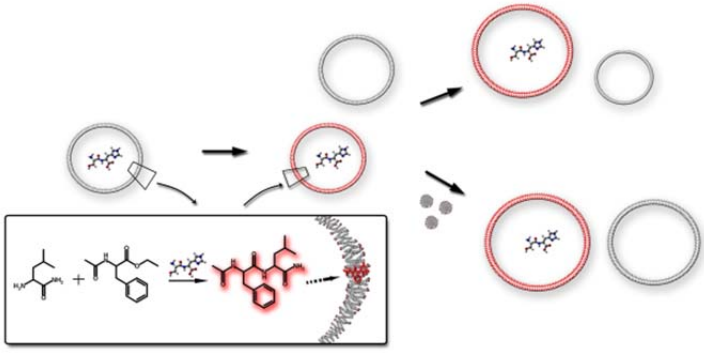


Figure 1.3.1 Schematic representation of adaptive changes and competition between protocell vesicles.

A: Synthesis of AcPheLeuNH_2 by catalyst encapsulated in fatty acid vesicles. A1: dipeptide catalyst Ser-His catalyzes the reaction between substrates LeuNH_2 and AcPheOEt, generating the product of the reaction, AcPheLeuNH_2. A2: product dipeptide AcPheLeuNH_2 localizes to the bilayer membrane.

B: Vesicles with AcPheLeuNH_2 in the membrane grow when mixed with vesicles without dipeptide, which shrink.

C: Vesicles with AcPheLeuNH_2 in the membrane grow more following micelle addition than vesicles without the dipeptide.

2. Results

2.1. Replication of protocell vesicles

The results shown in this chapter have been published in "Photochemically driven redox chemistry induces protocell membrane pearling and division" by Ting F. Zhu, Katarzyna Adamala, Na Zhang, and Jack W. Szostak, PNAS vol. 109 no. 25 p. 9828-32. (Zhu, Adamala et al. 2012)

During the course of our imaging studies of vesicle growth and division, we observed an unexpected artifact: long threadlike oleate vesicles containing an encapsulated fluorescent dye quickly (in approximately 5 s) round up into large spherical vesicles under intense illumination. The vesicles contained 2 mM HPTS (8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt, a water-soluble, membrane-impermeable fluorescent dye) (Zhu and Szostak 2009) and were illuminated by an EXFO 120 W metal halide lamp. We reasoned that since most fluorescent dyes, such as HPTS, generate reactive oxygen species (ROS) under illumination, this phenomenon might be caused by the radical mediated oxidation and fragmentation of the internal buffer solute, bicine, leading to increased internal osmolarity, as previously seen in the case of spherical vesicles that explode under similar conditions. (Zhu and Szostak 2011) The fact that the sphere-to-filament transition is reversible shows directly that growth into a filamentous form does not involve topological changes in vesicle structure. In an effort to obtain better images of filamentous vesicles, we added 10 mM dithiothreitol (DTT) to a vesicle suspension to scavenge ROS, in an attempt to block the rounding-up artifact.

To our surprise, intense illumination in the presence of 10 mM DTT caused the thread-like fatty acid vesicles to go through pearling and subsequent division (Fig. 2.1.1 and Movie S2.1.1). The long thread-like membrane first transformed into a periodic string of smaller ellipsoidal vesicles connected by narrow necks, and the smaller vesicles eventually separated into independent vesicles that moved apart by Brownian motion.

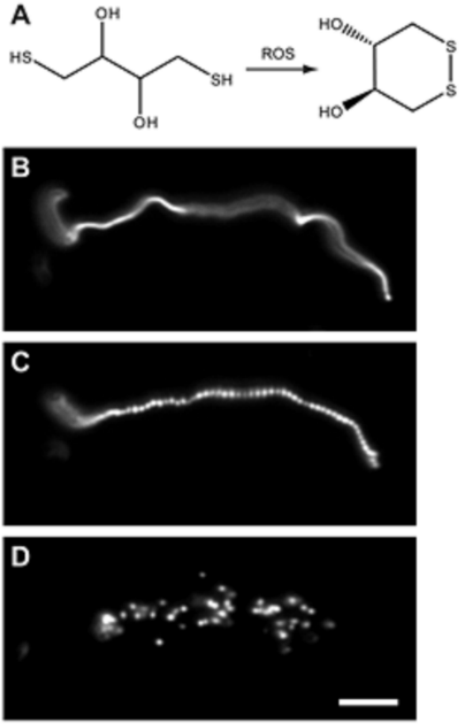


Figure 2.1.1 Oleate vesicle pearling and division.

(A) Radical-mediated oxidation of DTT.

(B) An oleate vesicle (containing 2 mM HPTS, in 0.2 M Na-glycinamide, pH 8.5, 10 mM DTT) 30 min after the addition of 5 equivalents of oleate micelles.

(C, D) Under intense illumination (for 2 sec and 12 sec, respectively), the long thread-like vesicle went through pearling and division (Movie S2.1.1). Scale bar, 10 μm

This figure was prepared by Ting Zhu.

To understand the mechanisms responsible for the pearling and division of thread-like fatty acid vesicles, we examined oleate vesicles labeled with different dyes, in either the internal aqueous space or in the membrane itself. When we prepared vesicles containing 2 mM calcein (bis[N,N-bis(carboxymethyl) aminomethyl] fluorescein) in the internal aqueous space and added 10 mM DTT 30 min after the addition of five equivalents of oleate micelles, vesicle pearling and division were again observed upon illumination.

In contrast, we found that long thread-like oleate vesicles labeled with a low concentration of membrane-localized dye [0.5 mol% Rh-DHPE (Lissamine rhodamine B 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine)] did not go through pearling and division (Fig. S3 A and B). This may be because there are fewer dye molecules associated with the vesicles in the latter case, since in a control experiment where the encapsulated water-soluble fluorescent dye was adjusted to a lower concentration (0.05 mM) comparable to that of the membrane dye used, no vesicle pearling and division were observed. We also observed that with equal concentrations (2 mM) of HPTS inside and outside of the filamentous vesicles (labeled with 1 mol% Rh-DHPE for imaging), vesicle pearling and division still occurred (Fig. 2.1.2). This result indicates that a cross-membrane concentration gradient of the fluorescent dye is not a prerequisite for the pearling and division phenomenon.

It has been shown that vesicle pearling and division can occur as a result of laser tweezers induced surface tension changes which induce a Rayleigh instability in vesicle membranes. (Bar-Ziv and Moses 1994) Recent experiments have also shown that encapsulated cationic nanoparticles can induce vesicle pearling. (Yu and Granick 2009) We hypothesized that the photochemically induced oxidation of thiols into its oxidized form, trans-4,5-dihydroxy-1,2-dithiane, resulted in an increased hydrophobicity of the compound (logP 0.12 for DTT vs. logP 0.52 for trans-4,5-dihydroxy-1,2-dithiane), and consequently increased interaction of the molecule with the membrane.

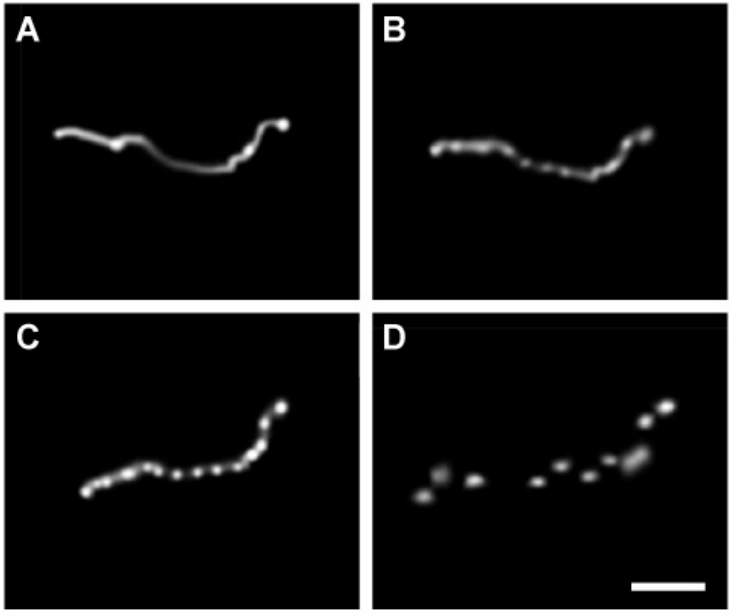


Figure 2.1.2 Vesicle pearling and division with equal concentrations of HPTS inside and outside the vesicles.

(A) An oleate vesicle with 2 mM HPTS inside and

outside the membranes (labeled by 1 mol% Rh-DHPE for imaging, in 0.2MNa-bicine, pH 8.5, 10mMDTT) 30 min after the addition of five equivalents of oleate

micelles.

(B–D) Under intense illumination (for 2 s, 7 s, and 11 s, respectively), the long thread-like vesicle went through pearling and division. Scale bar, 10 μm.

We used NMR methods to examine the interaction of DTT and its oxidation product with fatty acid vesicle membranes. Because the proton chemical shifts of both DTT and trans-4,5-dihydroxy-1,2-dithiane overlap with the strong background peaks of bicine buffer at 2–4 ppm (and other organic buffers such as tricine and TRIS), which are difficult to selectively suppress, we used oleate vesicles prepared without buffer (15mMoleate in 7.5mMNaOD solution in D_2O, pD approximately 8.5) for the following experiments.

We added 15 mM DTT and 15 mM trans-4,5-dihydroxy-1,2-dithiane to vesicles prepared as above and used two independent NMR methods to examine the interaction of these small molecules with the oleate membranes. We first used saturation transfer difference (STD) spectroscopy, a widely used method for examining the interaction of small ligands with large receptors. (Mayer and Meyer 2001; Haselhorst, Lamerz et al. 2009) Following selective saturation irradiation of the oleate vinyl protons, we observed saturation transfer to the CH protons of trans-4,5-dihydroxy-1,2-dithiane but not to the CH protons of DTT. Thus, only the oxidized form of DTT appears to interact with oleate membranes. As a further test, we used the waterLOGSY method, another sensitive NMR method commonly used for screening ligand-receptor interactions. (Dalvit, Fogliatto et al. 2001)

Again, only oxidized and not reduced DTT was observed to interact with the oleate bilayer membrane.

To further understand the effect of thiols on vesicle pearling and division, we asked whether other thiols can also cause this phenomenon.

Thiols, such as 3-mercaptopropionic acid (10 mM), 3-mercapto-1-propanol (50 mM), 1-mercapto-2-propanol (50 mM), and 3-mercapto-1,2,4-triazole (50 mM), all caused thread-like oleate vesicles containing 2 mM HPTS to undergo pearling and division. At lower thiol concentrations (2 mM 3-mercaptopropionic acid, 10 mM 3-mercapto-1-propanol, 10 mM 1-mercapto-2-propanol, and 10 mM 3-mercapto-1,2,4-triazole), vesicle pearling was observed but without subsequent division. Recent evidence suggests that the thiol-containing amino acid cysteine may have been prebiotically available, as cysteine oxidation products have been found in the material from a 1958 Miller H_2S-rich spark discharge experiment. (Parker, Cleaves et al. 2011)

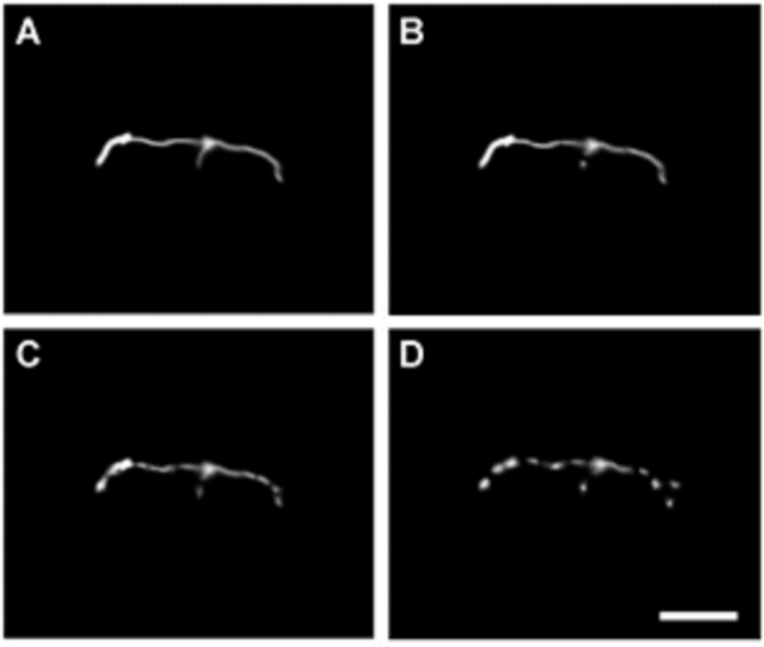


Figure 2.1.3 Oleate vesicle pearling and division mediated by the dipeptide di-Lcysteine.

(A) An oleate vesicle (containing 2 mM HPTS, in 0.2 M Na-bicine, pH 8.5, 20 mM di-L-cysteine) 30 min after the addition of five equivalents of oleate micelles. (B–D) Under intense illumination (for 3 s, 8 s, and 12 s, respectively), the long thread-like vesicle went through pearling and division.

Scale bar, 10 μm.

When we tested cysteine, we observed vesicle pearling but not division. We then considered the possibility that the cys-cys dipeptide, di-L-cysteine (Matsushita, Hinou et al. 2005), might allow pearling and division of vesicles after oxidation to generate an intramolecular disulfide

bond. When we prepared filamentous oleate vesicles containing 20 mM di-L-cysteine and 2 mM HPTS in the internal aqueous phase, we observed both vesicle pearling and division upon illumination (Fig. 2.1.3).

As an initial step toward exploring prebiotically plausible scenarios in which photochemically driven pearling and division might operate, we tested the idea that polycyclic aromatic hydrocarbons (PAHs) might replace the synthetic fluorescent dyes used in the above experiments. (Deamer 1992) PAHs are the most abundant polyatomic organic molecules in the universe, have been postulated to play important roles in the origin of life (Deamer 1992; Ehrenfreund, Rasmussen et al. 2006), and have been shown to stabilize short-chain fatty acid membranes (18).

We therefore asked whether oxygenated derivatives of PAHs, such as 1-hydroxypyrene (while HPTS itself is also a hydroxypyrene derivative, it is unlikely to be prebiotically abundant), can be incorporated into vesicle membranes and act as photosensitizers that can absorb UV radiation, generate ROS, and facilitate the division of protocells. To test this idea experimentally, we dissolved a high concentration of 1-hydroxypyrene (20 mol%), oleic acid and a low concentration of Rh-DHPE (1 mol% for imaging) in a chloroform solution, followed by rotary evaporation and resuspension in buffer (0.2 M Na-bicine, pH 8.5). After vesicle growth into filamentous form, and in the presence of 15 mM DTT and UV illumination, vesicle pearling and division were indeed observed (Fig. 2.1.4 and Movie S2.1.2).

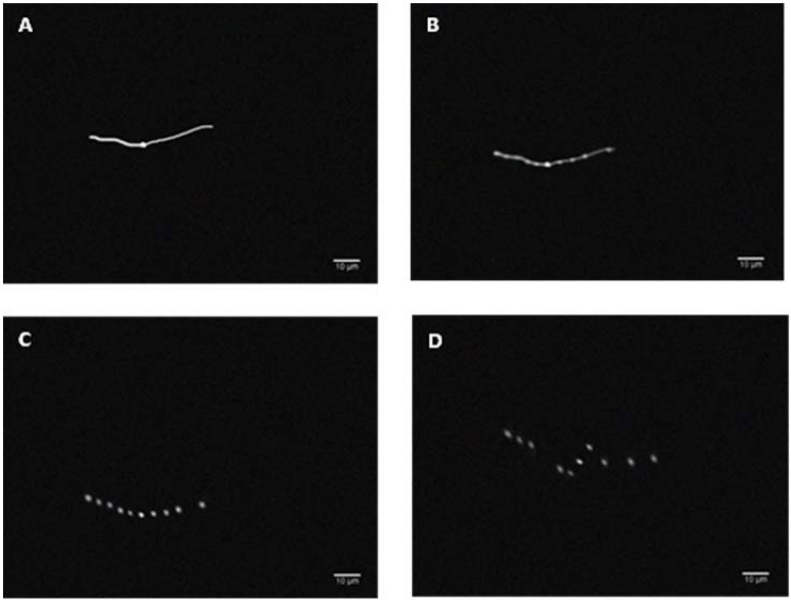


Figure 2.1.4 Pearling and division of an oleate vesicle containing 1-hydroxypyrene in the membrane.

(A) An oleate vesicle (with 20 mol %1-hydroxypyrene and 1 mol % Rh-DHPE in the membrane, in 0.2 M Na-bicine, pH 8.5, 15 mM DTT) 30 min after the addition of 5 equivalents of oleate micelles. (B-D) Under intense illumination (57 sec, 153 sec, and 213 sec after the start of illumination, respectively), the long thread-like vesicle went through pearling and division.

Scale bar, 10 μm.

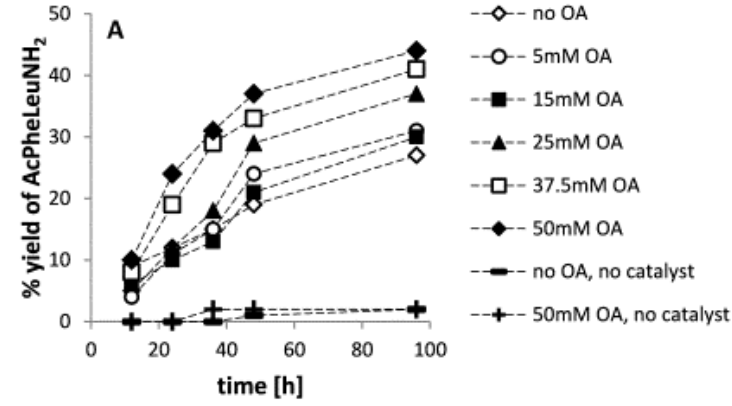
2.2. Competition between model protocells driven by an encapsulated catalyst

We have shown that activity of the prebiotic catalytic dipeptide Ser-His can be enhanced by the presence of the bilayer membrane of the protocell, and the product of the reaction catalyzed by Ser-His can cause the emergence of competitive growth of the protocell.

Vesicles Enhance Ser-His synthesis of $AcPheLeuNH_2$

The dipeptide Ser-His has been reported to synthesize the hydrophobic dipeptide $AcPheLeuNH_2$ from the ethyl ester of N-acetylphenylalanine (AcPheOEt), and leucyl carboxamide ($LeuNH_2$). (Gorlero, Wieczorek et al. 2009) However, the catalytic efficiency is extremely low, and quantitation is difficult due to precipitation of the hydrophobic product. In addition, Ser-His appears to be a more effective catalyst of the hydrolysis of the AcPheOEt ethyl ester than of peptide bond formation, which further limits product yield. We decided to characterize the Ser-His catalyzed synthesis of $AcPheLeuNH_2$ in the presence of fatty acid membranes, which we suspected would dissolve the product and prevent precipitation. We also suspected that co-localizing the hydrophobic substrates for the synthesis of the $AcPheLeuNH_2$ dipeptide within or on fatty acid membranes might improve the yield and possibly minimize substrate hydrolysis.

We therefore tested the above reaction in the presence of different concentrations of oleic acid vesicles. In these experiments, both the Ser-His catalyst and the AcPheOEt and $LeuNH_2$ substrates were present both inside and outside of the oleate vesicles. We found that the Ser-His catalyst produced progressively more $AcPheLeuNH_2$ in the presence of increasing concentrations of oleate vesicles, with the conversion of substrate to product increasing from 25% in free solution to 44% in the presence of 50 mM oleate vesicles [Figure 2.2.1A]. The presence of vesicles also diminished substrate hydrolysis from 65% in free solution to 10% in the presence of 50 mM oleate vesicles [Figure 2.2.1B].



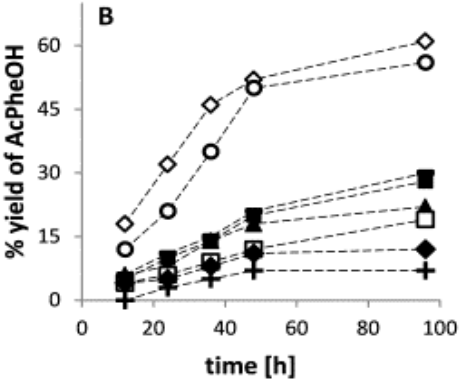


Figure 2.2.1A-B Ser-His activity in presence of fatty acid vesicles.

A: Ser-His catalyzed synthesis of $AcPheLeuNH_2$ in the presence of different concentrations of oleate vesicles.

B: Hydrolysis of substrate AcPheOEt in the same reactions.

All experiments: 10mM of each substrate, 5 mM Ser-His catalyst, 0.2 M Na+-bicine pH 8.5, 37 °C.

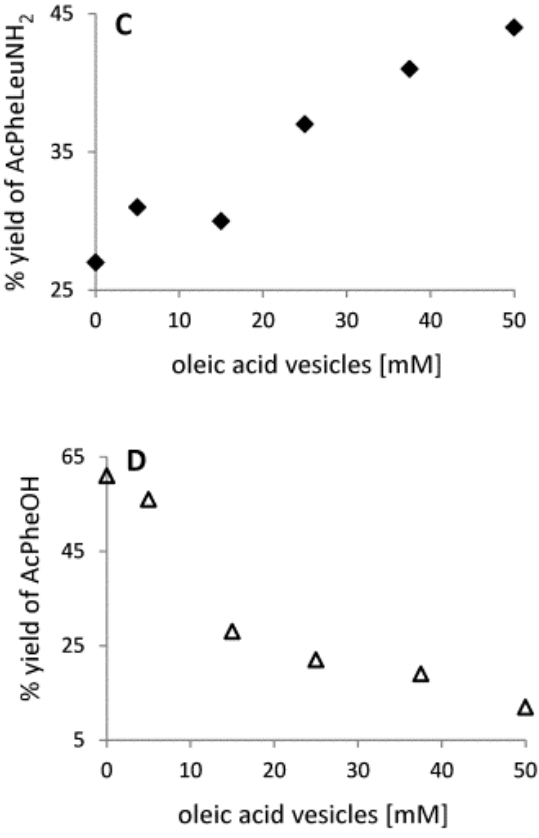


Figure 2.2.1C-D Ser-His activity in presence of fatty acid vesicles.

C: yield of dipeptide $AcPheLeuNH_2$ vs. concentration of oleate vesicles.

D: yield of hydrolyzed substrate AcPheOH vs concentration of oleate vesicles in the same reactions.

All experiments: 10mM of each substrate, 5 mM Ser-His catalyst, 0.2 M Na+-bicine pH 8.5, 37 °C.

One possible explanation for these results is that the hydrophobic substrates partition to the membrane, allowing the reaction to occur at the solvent-lipid bilayer interface, or even within the bilayer, thereby minimizing ester hydrolysis and enhancing product formation.

We also studied the catalysis of $AcPheLeuNH_2$ synthesis by the tripeptide Ser-His-Gly, which is a less effective catalyst than Ser-His, producing about half as much product dipeptide in the presence of oleate vesicles (with 50 mM vesicles, the yield of dipeptide AcPheLeuNH2 synthesis from 10 mM of each substrate AcPheOEt and LeuNH2 and with 5mM Ser-His-Gly was 27% after 96 hours in 0.2 M Na+-bicine, pH 8.5 at 37°C, compared to 44% for Ser-His).

Slow exchange of Ser-His and $AcPheLeuNH_2$ peptides between vesicles

Competition between protocells, and the origin of Darwinian evolution, leads to the selection of adaptations that are beneficial to the protocells in which those adaptations originated. Thus, the adaptive property must not be shared with other protocells in a population. It was, therefore, important to establish that the Ser-His catalyst and the dipeptide product $AcPheLeuNH_2$ remain localized within the vesicles that originally contained the catalyst.

We first asked whether the hydrophobic peptide $AcPheLeuNH_2$ remained localized within an initial set of vesicles, or exchanged between vesicles. We prepared two sets of vesicles, both at a concentration of 50 mM oleate, one with 5 mol% of $AcPheLeuNH_2$ and one without the dipeptide. After placing samples of each vesicle suspension on opposing sides of a dialysis membrane, we removed aliquots of each solution at a series of time points, and measured the concentration of $AcPheLeuNH_2$ in each solution using HPLC analysis.

Our results indicate that dipeptide $AcPheLeuNH_2$ exchanges only slowly between vesicles when the vesicles are prepared from oleic acid plus 0.5 equivalents of NaOH, with no additional buffer, remaining

mainly in the original vesicles over a period of several hours [Figure 2.2.2A]. In contrast the presence of additional buffer (0.2 M Na+-bicine, pH 8.5) leads to accelerated exchange of peptide between vesicles. All subsequent experiments were therefore carried out over time scales that were short relative to peptide exchange. We performed similar experiments to show that encapsulated Ser-His and Ser-His-Gly were also retained within vesicles and did not exchange between vesicles [Figure 2.2.1B and 2.2.2C].

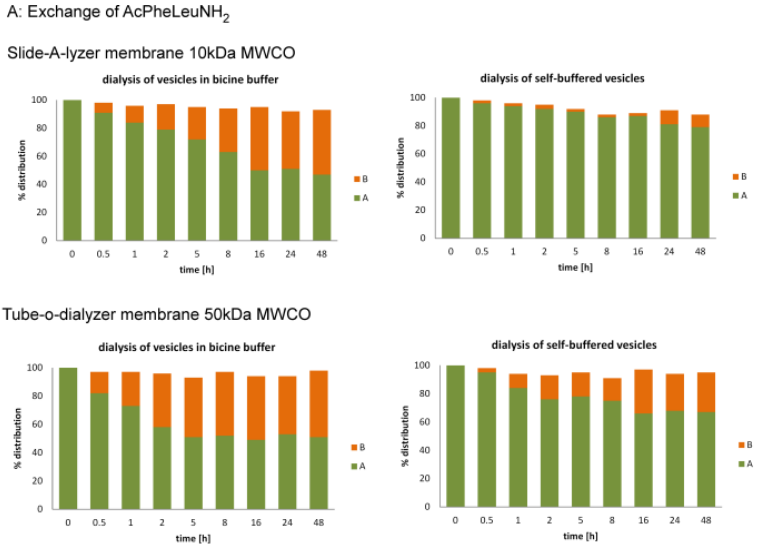


Figure 2.2.2A Exchange of compounds between vesicles

A: Exchange of $AcPheLeuNH_2$ between two populations of vesicles.

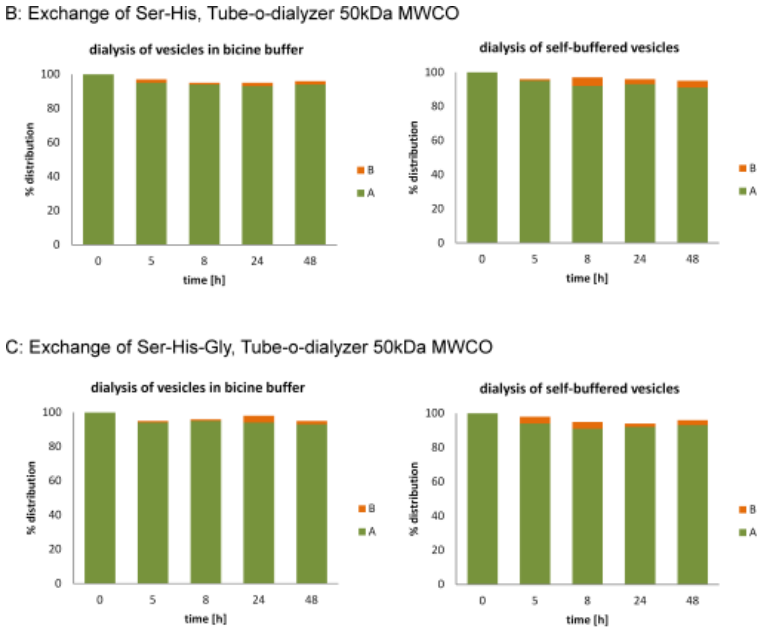


Figure 2.2.2BC Exchange of compounds between vesicles

B: Exchange of Ser-His between two populations of vesicles.;

C: exchange of Ser-His-Gly between two populations of vesicles.

Vesicles prepared separately, with and without $AcPheLeuNH_2$, and with and without bicine buffer. After 24 h of tumbling, extrusion, and another 5 h of tumbling, vesicles were transferred to a dialysis chamber. Two different dialysis membranes were tested in two different conditions: self-buffered (50 mol% NaOH) or 0.2 M Na+-bicine pH 8.5. Vesicles with peptide contained 5 mol% $AcPheLeuNH_2$.

Equal volumes were present on both sides of the dialysis membrane. $AcPheLeuNH_2$ distribution between vesicles on either side of the dialysis membrane were measured by HPLC after dialysis for indicated times.

Interaction of AcPheLeuNH2 with Membranes

As a means of examining the interaction of AcPheLeuNH2 with the vesicle membrane, we measured the fluorescence anisotropy of the molecular probe 1,6-diphenyl-1,3,5-hexatriene (DPH) as a probe of membrane order.

This assay has previously been used to demonstrate that the addition of phospholipids to fatty acid membranes leads to an increase in membrane viscosity (Budin and Szostak 2011) and has frequently been used to determine viscosity changes in biological membranes resulting from the presence of different peptides. (Juhan-Vague I 1986 ; Miyamoto A 1990) We have observed that the fluidity of oleate membranes also decreases in the presence of the dipeptide $AcPheLeuNH_2$ [Figure 2.2.3], demonstrating the interaction of this compound with the membrane.

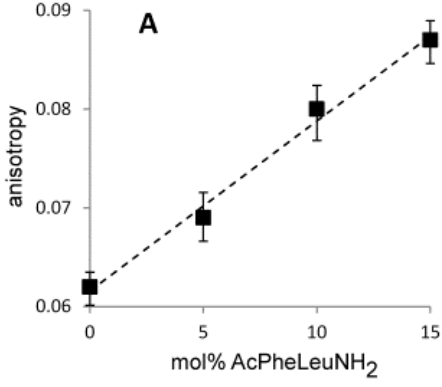


Figure 2.2.3A DPH anisotropy in oleate membranes with and without $AcPheLeuNH_2$

A: self-buffered vesicles (0.5 eq NaOH)

Dashed lines are linear regression fits, R2 > 0.98. Error bars indicate SEM (N=5).

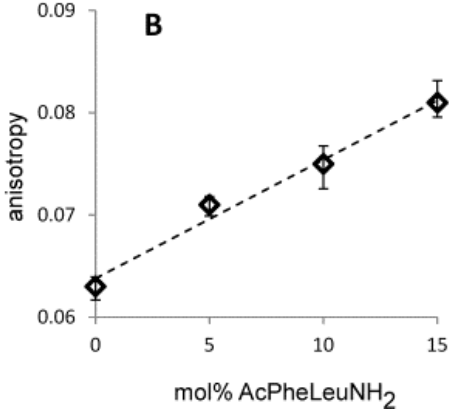


Figure 2.2.3B DPH anisotropy in oleate membranes with and without AcPheLeuNH$_2$

B: vesicles in high salt buffer (0.2M Na+-bicine, pH 8.5).

Dashed lines are linear regression fits, R2 > 0.98. Error bars indicate SEM (N=5).

Consistent with previous phospholipid effects (Budin and Szostak 2011), the presence of the hydrophobic dipeptide also decreases the off-rate of fatty acids from fatty acid membranes [Figure 2.2.4]. The rate of desorption of lipids from fatty acid vesicles containing dipeptide AcPheLeuNH2 was measured using a population of phospholipid reporter vesicles containing the pH sensitive dye HPTS encapsulated in their internal aqueous space.

After oleic acid vesicles were mixed with reporter vesicles, oleic acid monomers desorbed from the fatty acid vesicles and were then rapidly adsorbed into the reporter phospholipid vesicles, followed by flip-flop and ionization of the oleic acid, causing acidification of the interior of the phospholipid vesicle.

The pH decrease inside the phospholipid reporter vesicles was followed by changes in the fluorescence of the HPTS dye (see Materials

and Methods). We found that the desorption rate of oleate monomers from fatty acid vesicles decreases with increasing concentration of dipeptide $AcPheLeuNH_2$ in the membrane. This is consistent with previously reported observations of the effect of phospholipid on fatty acid vesicles. The similar effect of $AcPheLeuNH_2$ and phospholipid on fatty acid desorption suggested that the dipeptide might, like phospholipid, also drive vesicle growth at the expense of surrounding pure fatty acid vesicles.

We also examined the effects of the peptide on membrane permeability; neither $AcPheLeuNH_2$, nor reaction substrates or catalyst affected vesicle permeability to the small molecule calcein or to oligonucleotides [Figure 2.2.5].

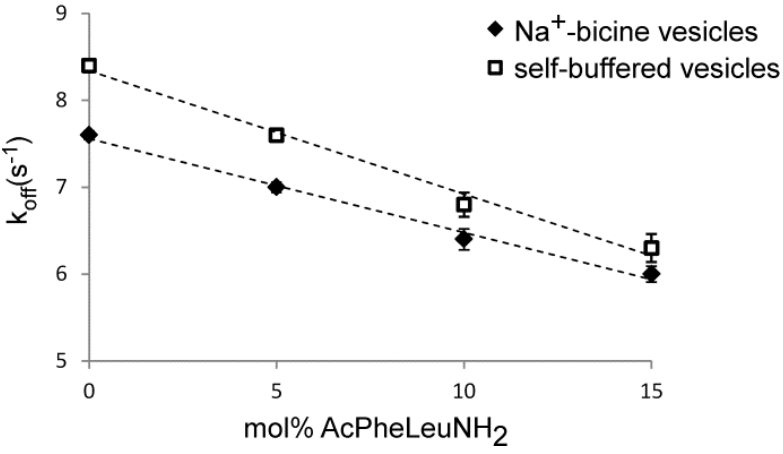


Figure 2.2.4 The desorption rate of oleate from oleate vesicles as a function of $AcPheLeuNH_2$ content.

Fatty acid desorption rates are derived from the rate of pH change inside reporter phospholipid vesicles. The pH change inside reporter vesicles was measured with the pH-sensitive dye HPTS.

The dotted lines indicate linear regression fits, $R2 \geq 0.98$. Error bars indicate SEM (n=4).

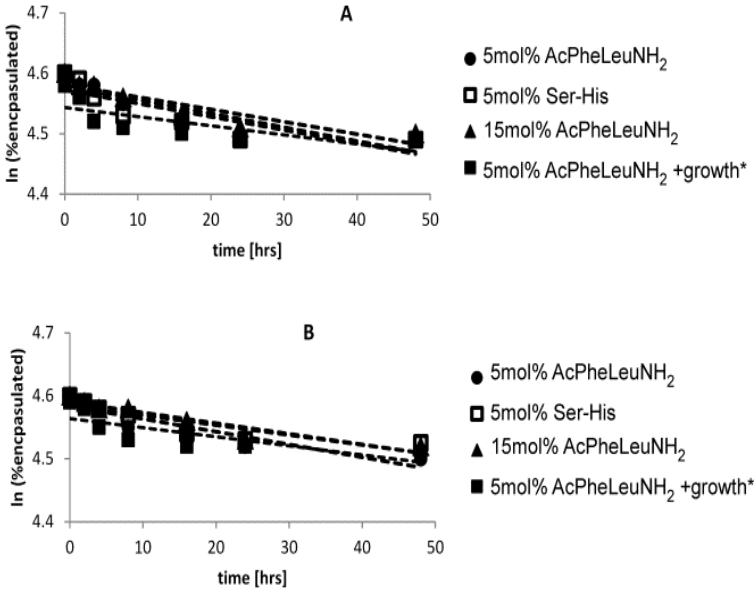


Figure 2.2.5 Stability of vesicles with $AcPheLeuNH_2$ in the membrane.

A: Leakage of small molecules; vesicles in high salt buffer (0.2M bicine Na+ pH=8.5) were prepared with 5mM calcein dye. Unencapsulated dye was removed on size exclusion column. Vesicles were then tumbled for the specified amount of time and each sample was purified again on size exclusion column. Leakage was calculated by quantifying amount of encapsulated vs. free dye.

B: Leakage of oligonucleotide; vesicles were prepared as described above, but with d(Cy3-CAGCAG) oligonucleotide encapsulated instead of calcein dye.

Competitive Growth of Vesicles Containing $AcPheLeuNH_2$

Given that $AcPheLeuNH_2$ decreases membrane fluidity and fatty acid dissociation in a manner similar to that previously observed for phospholipids, we investigated whether $AcPheLeuNH_2$ also affected membrane growth dynamics. The dipeptide $AcPheLeuNH_2$ is practically insoluble in water, so the dilution of the insoluble peptide fraction present in fatty acid membrane is entropically favored. That, in addition to the decreased fatty acid off rate (monomer efflux from the membrane), could lead to the accumulation of fatty acids in the dipeptide-containing membrane, when fatty acid vesicles without the dipeptide are present to provide the oleate monomer.

We monitored vesicle growth using a FRET-based assay for the real-time measurement of membrane surface area (Chen and Szostak 2004), in which the dilution of membrane localized fluorescent donor and acceptor dyes causes decreased FRET (see the Materials and Methods for details). We found that vesicles containing $AcPheLeuNH_2$ grew at the expense of those lacking this dipeptide, when the two were incubated together [Figure 2.2.6A, Table 2.2.1A].

Similarly, when "fed" with added fatty acid micelles, $AcPheLeuNH_2$-containing vesicles grew preferentially, taking up more micelles than vesicles without dipeptide [Figure 2.2.6B, Table 2.2.1B]. The time course of competitive vesicle growth after mixing 1 equivalent of vesicles with and without peptide, and the corresponding time course of shrinking of vesicles without the dipeptide, match previously reported phospholipid-driven growth, suggesting a similar fatty acid exchange mechanism [Figure 2.2.7].

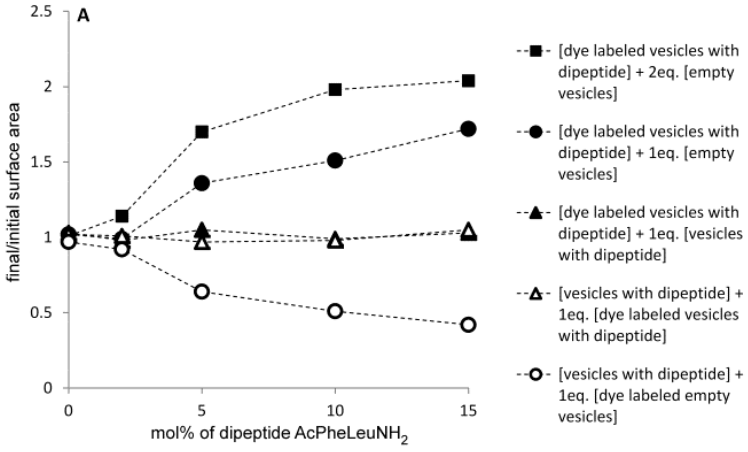


Figure 2.2.6A Competition between vesicles in different environments.

A: In the absence of excess salt (self-buffered oleate), vesicles with $AcPheLeuNH_2$ mixed with 1 or 2 equivalents of empty vesicles; surface area measured after 15min. Filled markers: with FRET dyes on vesicles with $AcPheLeuNH_2$ growth is observed; open markers: with FRET dyes on vesicles without $AcPheLeuNH_2$ shrinkage is observed.

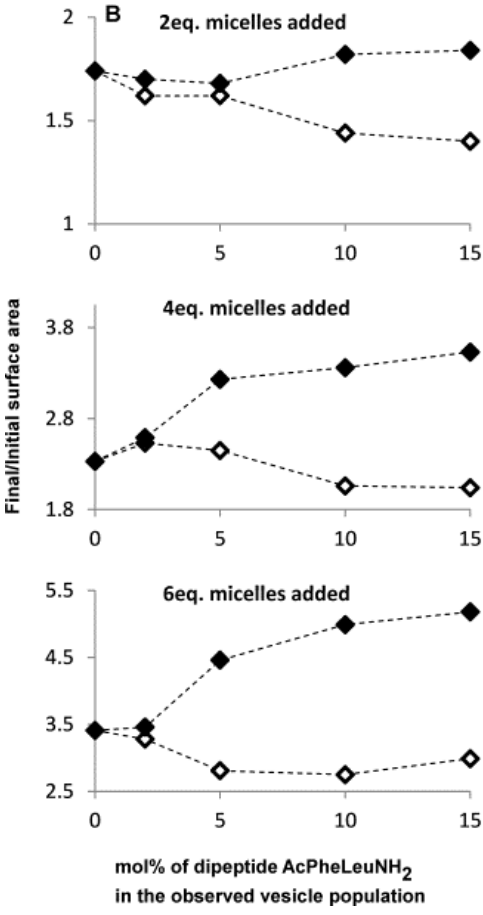


Figure 2.2.6B Competition between vesicles in different environments.

B: In a high-salt environment (0.2M Na+-bicine, pH=8.5), equal amounts of vesicles with and without $AcPheLeuNH_2$ were mixed, and to that sample oleate micelles were added surface area measured after 15min. Filled markers: FRET dyes on vesicles with $AcPheLeuNH_2$, growth is observed. Open markers: with FRET dyes on vesicles without $AcPheLeuNH_2$, less growth is observed than for the corresponding peptide-containing sample.

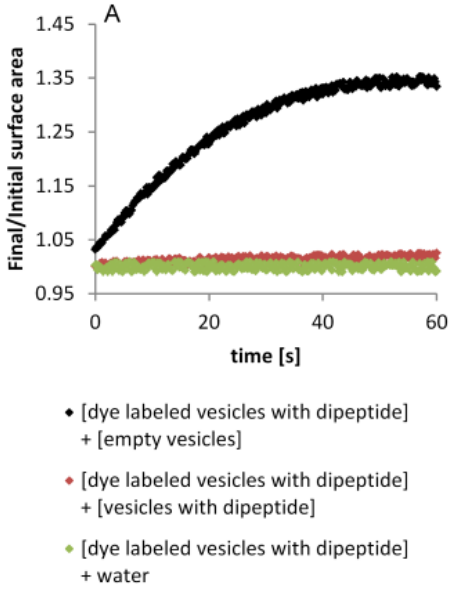


Figure 2.2.7A Time course of competitive vesicle growth and shrinking.

A: dye labeled oleic acid vesicles with 5 mol% of dipeptide $AcPheLeuNH_2$ in the membrane were mixed with: 1 equivalent of oleic acid vesicles without the dipeptide (black trace), 1 equivalent of oleic acid vesicles with 5 mol% of dipeptide $AcPheLeuNH_2$ (red trace), or with water (green trace).

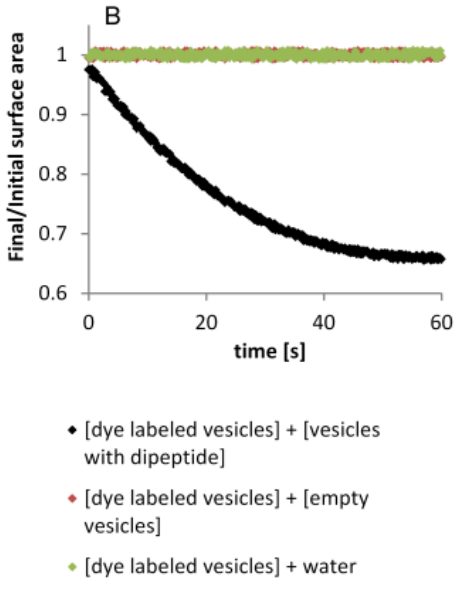


Figure 2.2.7B Time course of competitive vesicle growth and shrinking.

B: dye labeled oleic acid vesicles were mixed with: 1 equivalent of oleic acid vesicles with 5 mol% of dipeptide $AcPheLeuNH_2$ (black trace), 1 equivalent of oleic acid vesicles without the dipeptide (red trace), or with water (green trace).

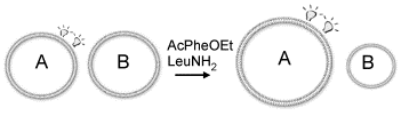
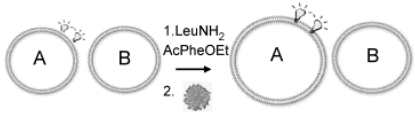
reaction scheme	encapsulated peptide in vesicles A	vesicles B	final/initial surface area
A. Competitive growth and corresponding shrinking.			
	S-H	none	1.24
	S-H	S-H	1.00
	S-H-G	none	1.14
	S-H-G	S-H-G	1.00
	S-H	none	0.74
	S-H	S-H	1.01
	S-H-G	none	0.85
	S-H-G	S-H-G	1.00
B. Competitive micelle uptake.			
	S-H	none	4.18
	S-H	S-H	3.41
	S-H-G	none	4.01
	S-H-G	S-H-G	3.43
	S-H	none	2.78
	S-H	S-H	3.44
	S-H-G	none	2.96
	S-H-G	S-H-G	3.41

Table 2.2.1 Changes of vesicle size

A: during competitive growth and shrinkage, following 1:1 mixing of indicated vesicle populations, and

B: during competitive oleate uptake after addition of 6 equivalents of oleate micelles.

Indicated populations of vesicles contained either Ser-His (S-H), Ser-His-Gly (S-H-G), or no catalyst. All vesicle populations were incubated separately with amino acid substrates for 48 h to allow for synthesis of hydrophobic dipeptide product prior to mixing.

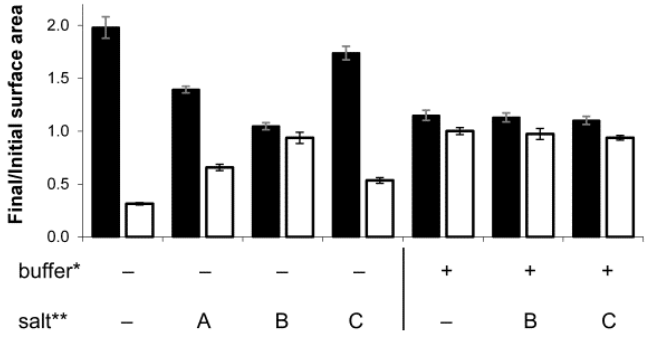
Surprisingly, we only observed competitive growth between vesicles with and without peptide in self-buffered vesicles. Following the addition of ½ molar equivalent of NaCl to self-buffered vesicles (relative to oleate), less growth was observed, and when 1 equivalent of NaCl was added to self-buffered vesicles, no significant competitive growth was observed [Figure 2.2.8].

The addition of 1 equivalent of tetramethylammonium chloride (TMAC) affected the yield of lipid transfer (observed surface area change) to a lesser extent [Figure 2.2.8], suggesting that surface ionic interactions strongly affect fatty acid exchange processes. In contrast to competitive vesicle-vesicle growth, we were only able to measure competitive micelle-induced growth in high-salt Na+-bicine buffered vesicle samples [Figure 2.2.6B].

Without additional buffer, the alkaline oleate micelles (with 1 equivalent of NaOH per fatty acid, vs. the ½ equivalent per fatty acid in a vesicle) quickly and excessively changed the pH of the mixture, destabilizing the pre-formed vesicles.

In a preliminary effort to correlate structure and activity, we examined the effect of several different small peptides on vesicle growth. While other small hydrophobic dipeptides (e.g., Phe-Phe) led to some competitive growth, none was as efficient as $AcPheLeuNH_2$ [Figure 2.2.9]. N-terminal peptide acetylation was quite important, as $PheLeuNH_2$ was less effective than $AcPheLeuNH_2$.

LogPcalc (calculated logarithm of octanol-water partition coefficient) values suggest that the more lipophilic peptides induce a greater competitive growth effect [Figure 2.2.9].



* +: vesicles in high salt buffer (0.2M Na^+-bicine, pH 8.5), –: self-buffered vesicles (50 mol% NaOH)

** –: no salt added; A: 50 mol% NaCl (relative to oleate), B: 100 mol% NaCl, C: 100 mol% TMAC

Figure 2.2.8 Effect of salt/buffer on competitive growth of vesicles.

In black columns, vesicles containing $AcPheLeuNH_2$ were labeled with FRET dyes (growth was monitored); in white columns, vesicles without $AcPheLeuNH_2$ were labeled with FRET dyes (shrinking was monitored).

Error bars indicate SEM (N=5).

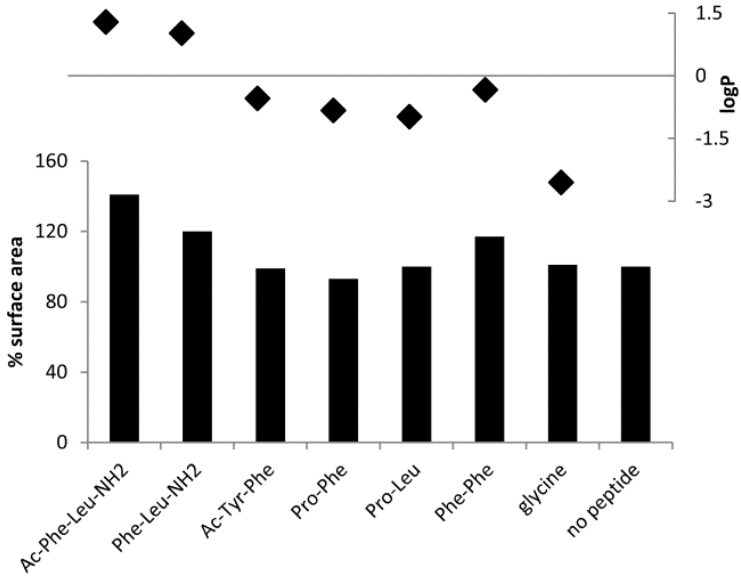


Figure 2.2.9 Competitive growth with other small hydrophobic peptides, and corresponding partition coefficients (logPcalc).

Two populations of self-buffered oleate vesicles prepared as described in Materials and methods, one with 5 mol% of tested peptide and with FRET dye pair, and one population of empty vesicles without any additives. Equal amounts of each vesicle population were mixed and the size of the vesicles with peptide was measured after 1h using the FRET assay.

Partition coefficients logPcalc were calculated using Molinspiration Property Calculation Service (www.molinspiration.com).

Competitive growth can facilitate protocell vesicle division

It has previously been shown that the growth of oleate vesicles following micelle addition results in the development of fragile, thread-like structures that can easily fragment, producing daughter vesicles. (Zhu and Szostak 2009) Similar filamentous growth of phospholipid containing vesicles is observed following mixing with excess pure fatty acid vesicles. (Budin and Szostak 2011) We therefore asked whether competitive growth caused by the presence of the hydrophobic $AcPheLeuNH_2$ dipeptide can also result in the formation of filamentous vesicles and subsequent division.

The development of thread-like filamentous vesicles from initially spherical vesicles is caused by the more rapid increase of surface area relative to volume increase, which is osmotically controlled by solute permeability. To recreate this effect in the absence of additional buffer, we used sucrose, a non-ionic osmolyte providing an osmotic constraint on vesicle volume. We found that initially spherical vesicles with 10 mol% of $AcPheLeuNH_2$ in their membranes develop into thread-like filamentous vesicles after mixing with 100 equivalents of empty oleate vesicles [Figure 2.2.10A-B].

Gentle agitation of the filamentous vesicles resulted in vesicle division into multiple smaller daughter vesicles [Figure 2.2.10C]. We have previously shown that division of filamentous vesicles into daughter vesicles occurs without significant loss of encapsulated content, and therefore is a plausible mechanism for spontaneous protocell division. (Budin and Szostak 2011; Zhu, Adamala et al. 2012)

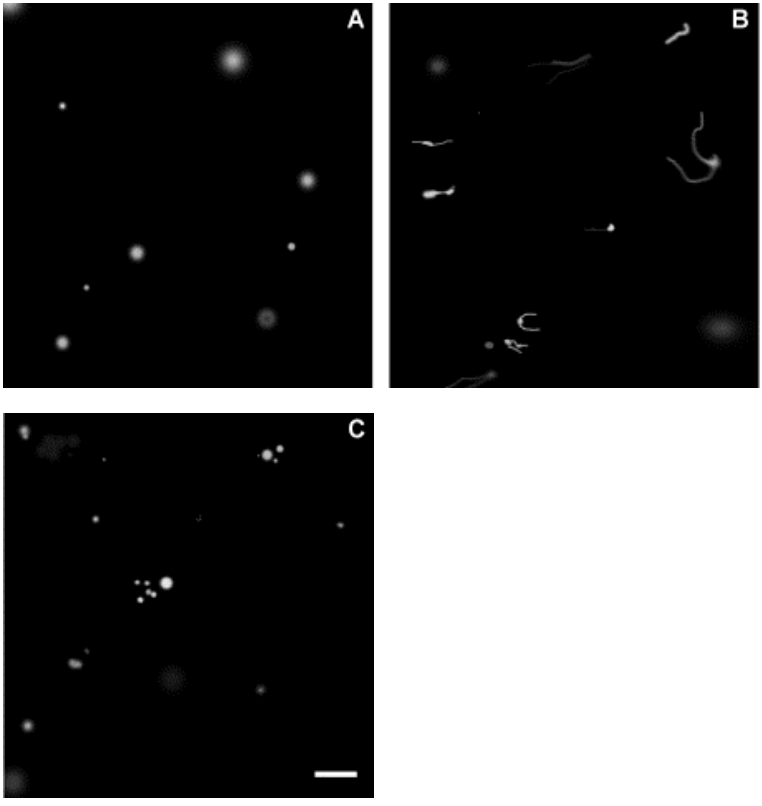


Figure 2.2.10 Vesicle growth and division.

A: Large multilamellar vesicles with 0.2 mol% Rh-DHPE dye and 10 mol% of dipeptide AcPheLeuNH2 in the membrane are initially spherical.

B: 10 minutes after mixing with a 100 equivalents of unlabeled, empty oleic acid vesicles without the dipeptide; thread-like filamentous structures develop.

C: after gentle agitation of the sample, threads break producing small daughter vesicles.

Scale bar, 10 um.

Competitive vesicle growth induced by Ser-His catalyzed synthesis of AcPheLeuNH2

The results described above show that Ser-His can catalyze the formation of $AcPheLeuNH_2$ in vesicles and that $AcPheLeuNH_2$ can cause or enhance vesicle growth. We sought to combine these phenomena to effect Ser-His-driven vesicle growth. We prepared oleate vesicles containing encapsulated Ser-His dipeptide and FRET dyes in their membranes. Unencapsulated catalyst was removed either by dialysis against a vesicle solution of the same amphiphile concentration lacking the catalyst, or, alternatively, by purification on a Sepharose 4B size exclusion column.

Purified vesicles containing 5 mM Ser-His were then mixed with 10 mM of the amino acid substrates AcPheOEt and $LeuNH_2$, and incubated at 37 °C for 24 to 48 hours to allow for synthesis of the dipeptide $AcPheLeuNH_2$. HPLC analysis of vesicles after 48h of incubation showed that inside vesicles with Ser-His the dipeptide product $AcPheLeuNH_2$ was synthesized with 28% yield; parallel experiments with vesicles containing Ser-His-Gly showed that the product was synthesized in 16% yield [Figure 2.2.11].

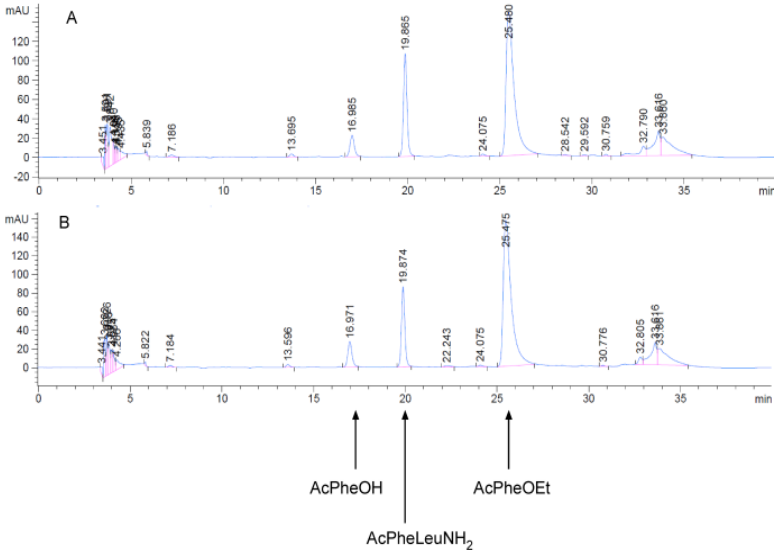


Figure 2.2.11 Synthesis of dipeptide AcPheLeuNH$_2$ in vesicles.

A: HPLC analysis of sample with catalyst Ser-His incubated for 48h at 37°C. Product dipeptide AcPheLeuNH$_2$ elutes at 19.8min.

B: HPLC analysis of sample with catalyst Ser-His-Gly incubated for 48h at 37°C. Product dipeptide AcPheLeuNH$_2$ elutes at 19.8min, marked with an arrow.

HPLC analysis conditions as described in Materials and methods.

After incubation, samples with catalyst were mixed with one equivalent of oleate vesicles that had been incubated with amino acid substrates, but without Ser-His catalyst. Vesicle size changes were measured following mixing using the FRET assay for surface area, as described above. We found that vesicles containing the peptide catalyst Ser-His that had been incubated with substrates to allow for the accumulation of AcPheLeuNH$_2$ increased in surface area by 24% when mixed with one equivalent of empty oleate vesicles. When the added

empty oleate vesicles were labeled with FRET dyes, we saw a decrease in their surface area, as expected. In parallel experiments, vesicles lacking Ser-His, or incubated without substrates, did not grow following the addition of oleate vesicles [Table 2.2.1A]. We also examined vesicles containing the less active catalyst Ser-His-Gly, which grew, but to a lesser extent than the Ser-His containing vesicles [Table 2.2.1A].

We then examined the ability of Ser-His (and Ser-His-Gly) to enhance vesicle growth following $AcPheLeuNH_2$ synthesis and then oleate micelle addition. As expected from previous experiments in which vesicles were prepared with $AcPheLeuNH_2$ directly, vesicles in which $AcPheLeuNH_2$ was synthesized internally also showed enhanced oleate uptake from added micelles, and they therefore grew more than empty vesicles [Table 2.2.1B]. Vesicles prepared with Ser-His-Gly showed a similar but smaller growth enhancement.

$AcPheLeuNH_2$-containing Vesicles Exhibit Enhanced Chemical Potential Generation During Competitive Micelle Uptake

As fatty acid vesicles grow, protonated fatty acid molecules flip across the membrane to maintain equilibrium between the inner and outer leaflets. Subsequent ionization of a fraction of the protonated fatty acids that flipped to the inside of the vesicle acidifies the vesicle interior, generating a pH gradient across the membrane.

This pH gradient normally decays rapidly due to H^+/Na^+ exchange, but we have previously shown that by employing a membrane-impermeable counterion, such as arginine, the pH gradient can be maintained for many hours ($t_{1/2} \approx 16h$). (Chen and Szostak 2004) We have therefore used arginine as a counterion (arg+) to study the effect of $AcPheLeuNH_2$ on the generation of a trans-membrane electrochemical potential during micelle-mediated vesicle growth.

In order to monitor the effect of the peptide on vesicle growth-induced pH gradient formation, we prepared two populations of vesicles in arg+-bicine buffer, one with and one without the hydrophobic dipeptide $AcPheLeuNH_2$ in the membrane. In one experiment, the peptide-containing vesicles also carried the pH-sensitive water-soluble dye HPTS encapsulated in the vesicle interior; in a second, separate experiment, the HPTS was in the peptide-free vesicles. In both

experiments, the two vesicle populations were mixed together and incubated for 30 min. No competitive growth between those two populations occurred, since vesicles were in a high-salt arg+-bicine buffer. We then added 1 equivalent of oleate-arg+ micelles to the mixed vesicle sample, triggering growth of both sets of vesicles.

We measured the pH change inside the vesicles by monitoring the change in the fluorescence emission of the HPTS dye (see Materials and Methods). In the two parallel experiments, pH change was monitored either inside the vesicles containing $AcPheLeuNH_2$ or inside vesicles without dipeptide (by having HPTS dye encapsulated in either of those populations). Consistent with the enhanced surface area growth of $AcPheLeuNH_2$-containing vesicles [Figure 2.2.6B], the peptide-containing vesicles developed a larger transmembrane pH gradient than the vesicles lacking peptide, following growth induced by addition of oleate-arg+ micelles [Figure 2.2.12].

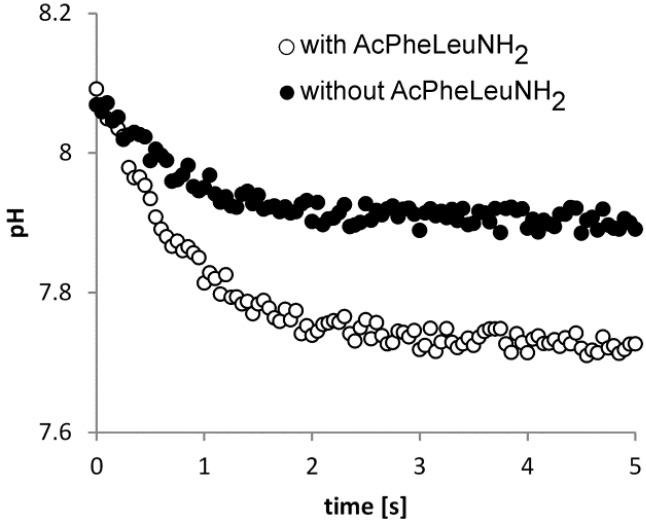


Figure 2.2.12 Transmembrane pH gradient generated by growth of vesicles during competitive micelle uptake.

Equal amounts of vesicles with and without 10 mol% $AcPheLeuNH_2$ were mixed in high salt buffer (arg+ -bicine, pH=8.1), and 1 equivalent of arginine-oleate micelles was added to the mixture. The internal pH of the vesicles was measured using the pH-sensitive fluorescent dye HPTS.

Competition between vesicles containing different catalysts

In order to examine competition between vesicles harboring peptides of varying catalytic efficiencies, we prepared self-buffered Ser-His-containing vesicles and Ser-His-Gly-containing vesicles and incubated them separately in the presence of $LeuNH_2$ and AcPheOEt to allow for the synthesis of the hydrophobic dipeptide $AcPheLeuNH_2$. These vesicles were then mixed, and after 60 min incubation the change in the surface area of vesicles labeled with dye was determined from the change in the FRET signal. The vesicles containing Ser-His increased in

surface area, while those containing Ser-His-Gly shrunk. This directly demonstrates competition between protocells containing two catalysts of varying efficiency [Table 2.2.2].

In a separate experiment, we mixed vesicles with both catalysts and added one equivalent of empty oleate vesicles, to serve as a "feedstock" for the vesicles with the dipeptide $AcPheLeuNH_2$. In this case, both vesicles with Ser-His and with Ser-His-Gly increased in size, but vesicles with Ser-His grew more than those with Ser-His-Gly. This demonstrates that in case where "feedstock" is readily available (from empty vesicles), both populations of protocell grow, but the more efficient catalyst allows for more growth [Table 2.2.2].

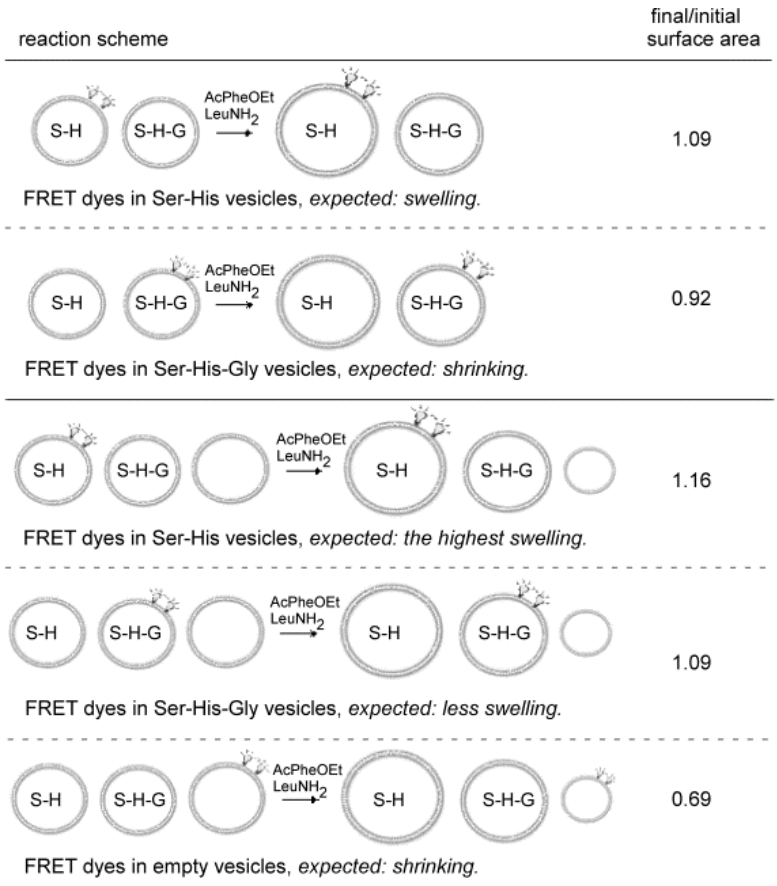


Table 2.2.2 Competition between vesicles with two different catalysts.

Vesicle size changes during competitive growth and shrinking in populations of vesicles with and without different catalysts. Vesicle populations were incubated separately with substrates for 48 h, as described in Materials and Methods, prior to mixing.

Each sample contained two or three populations of vesicles, as indicated, in a 1:1 or 1:1:1 ratio. In each case, the FRET dye pair was placed in one of the populations, to measure the size change after the reaction.

2.3. Synthesis of genetic polymer PNA catalyzed by the prebiotic catalyst Ser-His

We have shown that the dipeptide catalyst Ser-His can catalyze synthesis of a prebiotically plausible genetic polymer PNA.

The results shown in this chapter have been published in Gorlero, M., R. Wieczorek, K. Adamala, A. Giorgi, M.E. Schinina, P. Stano, and P.L. Luisi, "Ser-His catalyses the formation of peptides and PNAs" FEBS Lett, 2009. 583(1): p. 153-6. (Gorlero, Wieczorek et al. 2009)

This thesis contains only results from the part of the article that have been prepared by the author of this thesis.

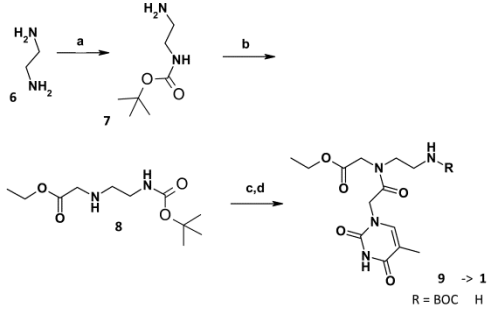


Figure 2.3.1 Synthesis the PNA monomer

a) $(BOC)_2O$, THF, 0°C -> RT, 4h; 91%;

b) $BrCH_2COOEt$, K_2CO_3, CH_2Cl_2; 79%;

c) Thymine acetic acid, EDC*HCl, Et_3N, DMF, 61%;

d) TFA : H2O 1:1, RT, 45 min; 96%

As part of the project of investigating catalytic activity of Ser-His and other simple prebiotically plausible peptides, we have asked would Ser-His catalyze synthesis of peptide nucleic acids.

Since PNA monomers were not commercially available at the time, we have synthesized PNA thymine monomer, with ethyl ester activating group on the carbonyl end.

The PNA monomers were synthesized according to the previously described procedure. (Fader, Boyd et al. 2001)

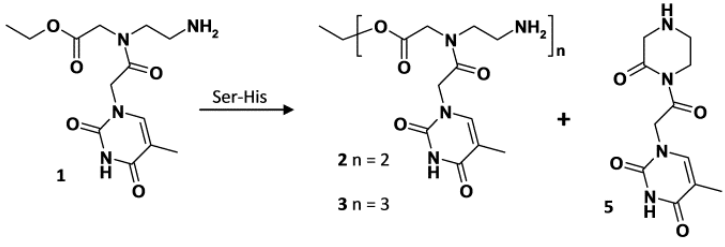


Figure 2.3.2 Oligomerization of the PNA monomer

(1) the monomer oligomerizes to give PNA dimer (2), trimer (3) and tetramer (4).

Ketopiperazine (5) derivatives are also formed.

Ser-His catalyzed synthesis of the PNA oligomers

The thymine PNA monomer was readily soluble in water. Upon addition of Ser-His to the PNA monomer, at pH 8.3, after 35h we have observed formation of PNA oligomers.

The PNA monomer polymerized to the corresponding PNA dimer 2, trimer 3, and tetramer 4 in moderate yields. [Table 2.3.1]

It is interesting to note that the tetramer product is formed in a very high yield compared to the lower products.

The control reaction, carried out under the same conditions but with alpha-chymotrypsin enzyme instead of Ser-His catalyst, gave about 30% substrate conversion, compared to 19% with Ser-His. In the chymotrypsin control reaction we observed the unusually high tetramer product accumulation again.

In all reactions, we have observed the accumulation of the internally cyclized PNA T monomer, the ketopiperazine product.

T monomer with Ser-His

no.[a]	c of Ser-His [mM]	c of substrate **1** [mM]	Rt [min]; (isolated product mg, yield[c]) substrate **1**	dimer **2**	trimer **3**	tetramer **4**	% conversion[b]
1.	-	10	4.36	-	-	-	0
2.	1.5	10	4.11	4.92 (0.38, 6.5%)	6.45 (0.20, 3.6%)	10.82 (0.51, 9.1%)	19%
controls							
3.	substrate **1**		4.27				
4.	1 mg enzyme[d]	10	4.36	4.81 (0.29, 10.3%)	6.24 (0.15, 5.2%)	10.22 (0.42, 15.3%)	30.8%

[a]HPLC chromatogram no.; [b]% of monomer **1** conversion calculated from the total yield of isolated products (**2**, **3** and **4**); [c]calculated from the theoretical 100% yield of that product as the only product in the mixture; [d]alpha-chymotrypsin, reaction time 25 min, reaction volume 1 ml.

Table 2.3.1 Results of the Ser-His catalyzed oligomerization of PNA monomers.

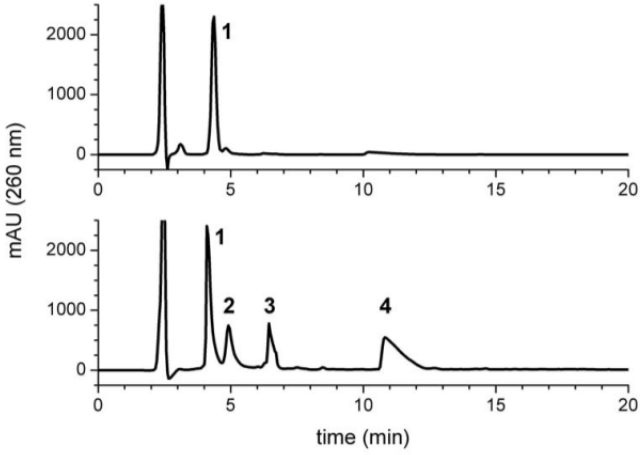


Figure 2.3.3 HPLC analysis of PNA oligomers

1: monomer; 2: dimer; 3: trimer; 4: tetramer

3. Discussion

3.1 On the replication of the protocells

Our observations strongly suggest that oxidized, but not reduced, thiols can associate with membranes, triggering division in our protocell model. While the mechanism by which disulfide-containing compounds cause membrane pearling and division is not entirely clear, it is plausible that disulfide-membrane interactions lead to changes in surface tension. Changes in membrane surface tension due to externally applied force from optical tweezers have been shown to induce pearling in tubular vesicles (9, 19), as well as vesicle expulsion (20).

Vesicle pearling has also been demonstrated by insertion of amphiphilic polymers in the outer leaflet of the membrane, inducing changes in spontaneous curvature (21, 22). In addition, lipid phase separation has been shown to drive vesicle budding and division as a consequence of minimizing interdomain edge energy (23). While our results suggest that the oxidation of dithiothreitol to trans-4,5-dihydroxy-1,2- dithiane results in increased interaction of the molecule with the membrane, how such interactions lead to membrane pearling and division remains unclear. Future studies will be required to investigate the location and homogeneity or heterogeneity of disulfide association with the bilayer membrane in order to quantitatively understand the resulting changes in morphology of the vesicle membrane. The observation that thiols and UV-absorbing PAHs facilitate vesicle pearling and division may have important implications for understanding the origin of cellular life.

Thiols are likely to have been abundant in prebiotic hydrothermal systems, such as nearsurface hydrothermal vents (24). Oxygenated PAH fragments could be generated by hydrothermal processing of meteoritic kerogen or by pyrolysis of organic materials. Such hydrophobic molecules are likely to have been selectively solubilized and concentrated in primitive membranes. Indeed, hydroxypyrene, a simple oxygenated PAH, has been shown to integrate into and stabilize membranes composed of short chain fatty acids (18).

In a high UV surface environment, membrane-localized PAHs would be expected to absorb UV radiation and generate ROS, even under anaerobic conditions (16). Our observations show that in the presence of thiols, generation of ROS could lead to the pearling and subsequent division of filamentous vesicles composed of fatty acids and PAHs, thus providing an independent pathway for protocell division that is distinct from the previously described shear force-dependent pathway (6).The control of the timing of cell division by thiol redox state provides a mechanism by which an initial dependence on environmental fluctuations could transition to an internally controlled process, through the evolution of cellular metabolism. For example, we have shown that the dipeptide cys–cys can result in vesicle pearling and division, whereas the amino acid cysteine by itself cannot. Therefore, metabolic control of dipeptide synthesis and degradation could potentially mediate the control of the timing of cell division in primitive cells. Alternatively, as cellular metabolism evolved to the point that cells derived energy from environmental redox disequilibrium, they could potentially use the control of their internal thiol:disulfide redox state as a means of controlling cell division. Finally, it would be interesting to investigate the possibility that intracellularly produced small molecules, peptides, or structured RNAs that interact with protocell membranes might also lead to pearling and division. Such processes would result in competition between protocells on the basis of faster or more appropriately timed cell division, and thus control of cell size, foreshadowing the rise of more complex and more tightly regulated modes of cell division.

3.2. On the catalytic activity of protoenzymes, leading to the origin of protocell competition and synthesis of genetic polymer

The ubiquitous role of proteins as the catalysts of metabolic reactions raises the question of the origins of protein enzymes. The fact that all modern proteins are synthesized through the catalytic activity of the RNA component of the large ribosomal subunit (Moore and Steitz 2011) suggests that primitive enzymes might have been peptides synthesized by ribozymes. By extension, the earliest enzyme progenitors might have been simple peptides synthesized in a non-coding fashion by

one or more ribozymes acting sequentially. If short, prebiotically available peptides played useful roles in the growth or division of early protocells, then there would have been a strong selective advantage conferred by ribozymes able to synthesize more of such useful peptides. We have found that a short hydrophobic peptide, whether supplied exogenously or synthesized internally, can confer a growth advantage to fatty acid vesicles. Thus, a ribozyme capable of synthesizing short hydrophobic peptides could accelerate protocell growth, thereby conferring a strong selective advantage. The short peptide Ser-His confers similar effects through its catalytic synthesis of the hydrophobic peptide product, supporting the idea that a ribozyme with similar catalytic activity would confer a selective advantage, but also raising the possibility that a ribozyme that made a catalytic peptide product could amplify its own efficacy through the indirect synthesis of the functional end-product. Similarly, a ribozyme that synthesized a peptide with nascent phospholipid synthase activity would confer a strong selective advantage through phospholipid-driven growth. (Budin and Szostak 2011)

Because of the polarity of nucleic acids, ribozymes might have difficulty catalyzing reactions between membrane-localized substrates; thus the synthesis of intermediate catalytic peptides could be an effective strategy for the synthesis of membrane-modifying products. The hydrophobic environment found in the interior of vesicle bilayers could provide a favorable reaction milieu for many chemical reactions, similar to that afforded (in much more sophisticated fashion) by the interior of folded proteins in contemporary biochemistry. This speculation is supported by our observation that the presence of vesicles increases the yield of $AcPheLeuNH_2$ from amino acid substrates in the presence of Ser-His as a catalyst. This increased yield most likely results from the greatly decreased extent of AcPheOEt substrate hydrolysis when it is localized to the hydrophobic membrane interior and is thereby protected from attack by water [Figure 2.2.1], much as labile intermediates are protected from water within the active sites of enzymes. Membrane localization of the leucyl-carboxamide substrate may also decrease the pKa of the N-terminal amino group, enhancing its reactivity by increasing the fraction of nucleophilic deprotonated amine. The phenomena of enhancing yields of chemical reactions by co-localizing substrates, altering pKas, and limiting side-reactions are likely to be observed for many other membrane-associated substrates, allowing

protocells to start membrane-localized metabolism with assistance from very simple peptide catalysts.

Direct vesicle-vesicle competitive growth is analogous to a predator-prey interaction, with one population acquiring nutrients from another population so that the "predatory" population grows while the "prey" population shrinks. The competitive micelle uptake is analogous to a "competition for feedstock" among two populations. It is interesting to note that these two mechanisms operate under different environmental conditions, with direct competitive growth occurring only under low-salt conditions, while competitive micelle uptake occurs only under high-salt/buffer conditions. In each case, adaptive changes occur as a result of an encapsulated reaction, the rate of which is enhanced by an encapsulated catalyst. Thus, this system links intra-protocell chemistry (and catalysis) to the ability of a model protocell to adapt to selective pressure. Because $AcPheLeuNH_2$ exchanges only slowly between vesicles, the presence of this hydrophobic dipeptide is truly adaptive, in that the product produced by the encapsulated catalyst remains with its original vesicle at least for a few hours, affording it an advantage - here, an enhanced affinity for membrane components, allowing for growth. Competitive protocell vesicle growth can also result, under certain circumstances, in the development of a higher trans-membrane pH gradient [Figure 2.2.12], which could potentially be linked to the development of useful energy sources for protocell metabolism. (Chen and Szostak 2004) Furthermore, rapid competitive vesicle growth leads to the development of thread-like filamentous vesicles, which can subsequently divide into small daughter vesicles as a result of gentle agitation [Figure 2.2.10]. This process has previously been observed for oleate vesicles grown by either micelle addition (Zhu and Szostak 2009) or competitive lipid uptake driven by membrane composition (Budin and Szostak 2010), and thus appears to be a general route to a cycle of growth and division.

Here we have shown that growth can be induced by activity of a catalyst encapsulated within the protocell, thus bringing the system one step closer to an internally controlled cell cycle. We have also demonstrated how Ser-His is capable of synthesizing simple genetic polymer. It has also been recently demonstrated that Ser-His can also catalyze synthesis of short RNA oligomers. (Wieczorek, Dorr et al. 2012)

It is possible that some small changes of the Ser-His sequence, or the chemistry of the activating group of the monomers used in the reaction, could lead to much higher yields.

We have demonstrated adaptive changes arising from encapsulated primitive catalysts acting at the protocell level, as well as competition between populations of protocells containing two different encapsulated catalysts differing in primary structure (Ser-His and Ser-His-Gly), and this catalyst is capable of synthesizing potential genetic polymer.

If such a system exhibited heredity, for example, via the activity of a self-replicating, peptide bond-forming ribozyme, it would amount to a fully functioning protocell, capable of Darwinian evolution.

Conclusions

Origin of life on Earth is one of the most fundamental questions in biology, and it leads to the myriad of other, equally interesting questions, including the issue of ubiquity of life in the Universe. (Rasmussen, Chen et al. 2004) Building an artificial cell would allow to draw a most probable scenario of events leading to the origin of life on the early Earth, and it would make possible more reliable speculations about the chances of finding life elsewhere. (Bedau, McCaskill et al. 2000; Szostak 2001)

The experimental route to protocell, or so-called minimal cell, would also enable artificial, programmable evolution of synthetic cells, with many possible applications. (Luisi, Ferri et al. 2006; Luisi 2007)

Artificially build synthetic cells have been proposed as possible small reactor vessels for efficient, highly controlled biochemical reactions (Noireaux V 2004; Jesorka and Orwar 2008) and liposomes have been extensively used in drug delivery (Langner and Kral 1999; Lian and Ho 2001).

The results presented in this thesis contribute to developing a plausible, complete scenario of the prebiotic evolution, from the polymerization of organic monomers, through the origin of adaptation and competition mechanisms, to the origin of the cell division. This research provides proof of principle that the smallest, prebiotically plausible, catalysts could have been versatile and efficient enough to catalyze multiple steps on the route from prebiotic small molecules to the living cell.

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Materials, methods and supplementary information

For the “Investigation on the origin of biological enzymatic catalysis”

Katarzyna Adamala

1. Replication of protocell vesicles

Vesicle Preparation.

Oleate vesicles were prepared by resuspending a dried film of oleic acid (Nu-Chek Prep) in 0.2 M Na-bicine (Sigma-Aldrich) containing 2 mM HPTS at pH 8.5, to a final concentration of 10 mM oleic acid.

Large (approximately 4 μm in diameter) monodisperse multilamellar vesicles were prepared by extrusion and large-pore dialysis as described. [1] Oleate vesicles containing 1-hydroxypyrene (Sigma-Aldrich) in the membrane were prepared by co-dissolving 1-hydroxypyrene (20 mol%), oleic acid, and Rh-DHPE (1 mol%) in a chloroform solution, followed by rotary evaporation and resuspension in buffer (0.2 M Na-bicine, pH 8.5, 15 mM DTT). Oleate vesicles containing di-L-cysteine were prepared by resuspension of 10 mM oleic acid in 0.2 M Na-bicine (pH 8.5) containing 2 mM HPTS and 20 mM di-L-cysteine. Di-L-cysteine (cysteinylcysteine) was prepared using F-moc solid phase synthesis on a microwave peptide synthesizer as described. [2]

Imaging Vesicle Pearling and Division.

Vesicle growth experiments were performed as described [3], in a buffer solution containing DTT, 3-mercaptopropionic acid, 3-

mercapto-1-propanol, 1-mercapto-2-propanol, 3-mercapto-1,2,4-triazole, or di-L-cysteine. Vesicle pearling and division were imaged using a Nikon TE2000S inverted epifluorescence microscope with extralong working distance (ELWD) objective lenses (Nikon). The illumination source was a metal halide lamp (EXFO) with a 480 20 nm (for HPTS), 546 5 nm (for Rh-DHPE), or 360 20 nm (for UV) optical filter (Chroma).

NMR Measurements.

All NMR spectra were recorded at 293 K with a Varian 400 MHz NMR spectrometer (Oxford AS-400) equipped with a 5 mm broadband PFG (z-gradient) probe. DTT and trans-4,5-dihydroxy-1,2-dithiane (Sigma-Aldrich) at concentrations of 15–20 mM were added to sonicated vesicle suspensions prior to NMR measurements.

Oleate vesicles were prepared without buffer by mixing 15 mM oleate with 7.5 mM NaOD (pH approximately 8.5, in 100% D_2O for STD experiments and in 10% D2O for waterLOGSY experiments).

Supplementary movies

Movie S1.1.1.

This real-time movie shows that a long thread-like oleate vesicle (containing 2 mM HPTS, in 0.2 M Na-glycinamide, pH 8.5, 10 mM DTT, 30 min after the addition of 5 equivalents of oleate micelles) went through pearling and division under intense illumination (QuickTime; 9 FPS; 2 MB). Scale bar, 10 μm.

Movie S1.1.2.

This real-time movie shows that the filamentous portions of an oleate vesicle (containing 20 mol% hydroxy-pyrene in the membrane, 0.5mol% Lissamine rhodamine B for visualisation, in 0.2 M Na-bicine, pH 8.5, 10 mM DTT, 30 min after the addition of 1 equivalent of oleate micelles), under intense illumination, went through pearling and division (QuickTime; 25 FPS; 5 MB). Scale bar, 10 μm.

2. Competition between model protocells driven by an encapsulated catalyst

FRET dyes N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)- 1,2-dihexadecanoyl-*sn*-glycero-3- phosphoethanolamine (NBD-PE) and Lissamine rhodamine B 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (Rh-DHPE) were purchased from Invitrogen Life Technologies. Amino acid substrate leucinamide ($LeuNH_2$) and dipeptides Ser-His and Ser-His-Gly were purchased from Bachem. All other compounds were purchased from Sigma Aldrich. All compounds were used without further purification. $LogP_{calc}$ values of peptides were determined using the Molinspiration Property Calculation Service (www.molinspiration.com).

In all experiments pH was monitored using a Corning 430 pH-meter.

Preparation of vesicles

Oleic acid/oleate vesicles were prepared as previously described [16] by direct re-suspension of oleic acid, either in buffer (0.2M Na^+-bicine, pH 8.5, for high-salt buffer samples) or in water with 0.5 equivalents of NaOH (for self-buffered samples). Samples were briefly vortexed, tumbled at room temperature for a minimum of 12 hrs, then extruded with 7 passages through a 100

nm pore membrane (Avanti Polar Lipids) using a Mini-Extruder (Avanti Polar Lipids). Vesicles were tumbled for at least 1 h after extrusion, before performing measurements.

Ser-His and Ser-His-Gly activity in presence of vesicles (HPLC assay)

Vesicles were prepared by re-suspending varying amounts of oleic acid in 0.2M Na^+-bicine, pH=8.5, containing 5mM Ser-His and 10mM of each substrate, vortexed, briefly sonicated, and incubated at 37°C without separating vesicles from the unencapsulated solution, to avoid diluting either vesicles or catalyst. Aliquots were taken at a series of time points and analyzed on an Agilent 1100 Analytical HPLC with a Varian Microsorb-mv 100-8 C_{18} 250 x 4.6 mm column with a gradient of solvent A: 0.1% TFA in H_2O, and solvent: B 0.1% TFA in acetonitrile, 3%-80%B over 40 minutes, with UV detection at 265 nm. Under these conditions, the retention times were: hydrolyzed substrate (N-Acetyl-L-phenylalanine, AcPheOH) 16.7-16.8 min, product ($AcPheLeuNH_2$) 19.3-19.5 min, unreacted substrate (N-Acetyl-L-phenylalanine ethyl ester, AcPheOEt) 25.3-25.4 min. Reaction samples containing fatty acid vesicles were sufficiently homogeneous to inject directly. Reaction samples lacking vesicles were not homogeneous, owing to the insolubility of the reaction substrate; they were mixed with

50% DMSO, sonicated, and vortexed to obtain a homogeneous sample prior to HPLC analysis.

Vesicle size change measurements (FRET assay)

Changes in membrane surface area were measured by FRET as previously described [1], using membrane-anchored donor (NBD-PE) and acceptor (Rh-DHPE) fluorescent dyes. Fluorescence was recorded using a Cary Eclipse fluorimeter (Varian, Mulgrave, Australia). Fluorescence was measured at excitation wavelength of 430nm and emission wavelengths of 530nm and 586nm. Vesicles with membrane-localized FRET dyes were prepared by mixing oleic acid with a chloroform solution of the FRET dyes (0.1 mol% of each FRET dye, relative to oleate) evaporating the solvent and rehydrating the mixture with 0.5 eq of aqueous NaOH (for self-buffered vesicles) or 0.2 M Na^+-bicine buffer (for high salt buffer). The samples were then briefly vortexed, tumbled at RT for at least 12 h, extruded as described above, and allowed to tumble for at least 1 hr before performing any measurements. All samples were diluted to an A_{500} of 0.1 OD or less, to avoid artifacts due to light scattering. FRET signal was calculated as the ratio of the fluorescence of the donor (NBD-PE) to the fluorescence of the acceptor (Rh-DHPE), with both excited at the donor excitation-maximum wavelength of 430 nm. Membrane surface area change was

calculated using calibration curves of FRET signal vs. lipid to dye ratio. Calibration curves of FRET signal were obtained for both self-buffered vesicles and vesicles in Na^+-bicine buffer, and a separate calibration curve was made for vesicles with the dipeptide $AcPheLeuNH_2$ in the membrane, to check for any possible peptide influence on the FRET signal [Figure S2.1].

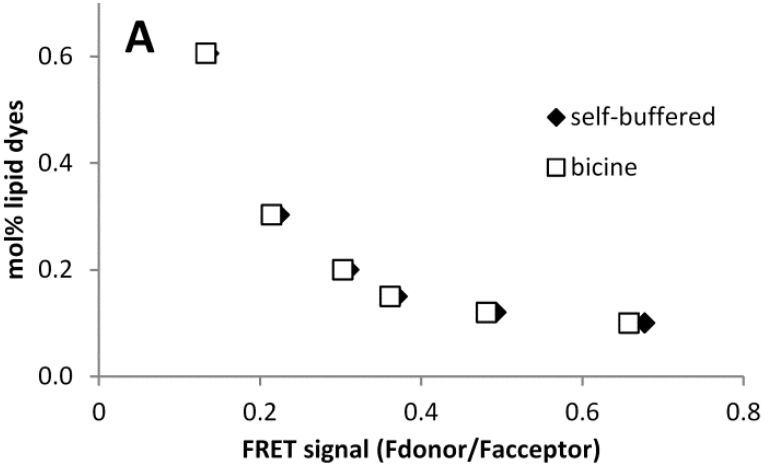

Figure S2.1A. Calibration curves for FRET size change measurements.

A: in 0.2 M Na^+-bicine buffer and self-buffered oleate. Samples were prepared at the specified oleate: lipid dye ratio and processed as described in materials and methods ("Vesicle size change measurements" section). Briefly, vesicles were tumbled overnight, extruded, tumbled at RT for 1h, and the signal was measured.

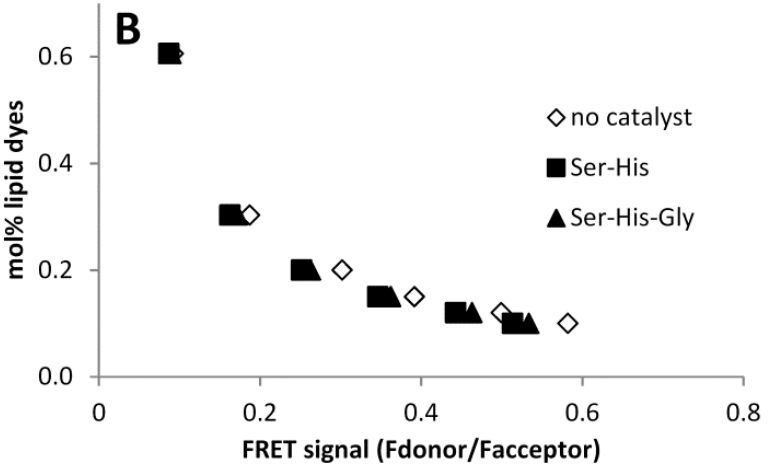

Figure S2.1B. Calibration curves for FRET size change measurements.

B: Samples were prepared as described in the materials and methods ("Competitive vesicle growth" section). Each sample was incubated with FRET dyes and the specified catalyst (without substrates). FRET efficiency was measured after 48h of incubation at 37°C.

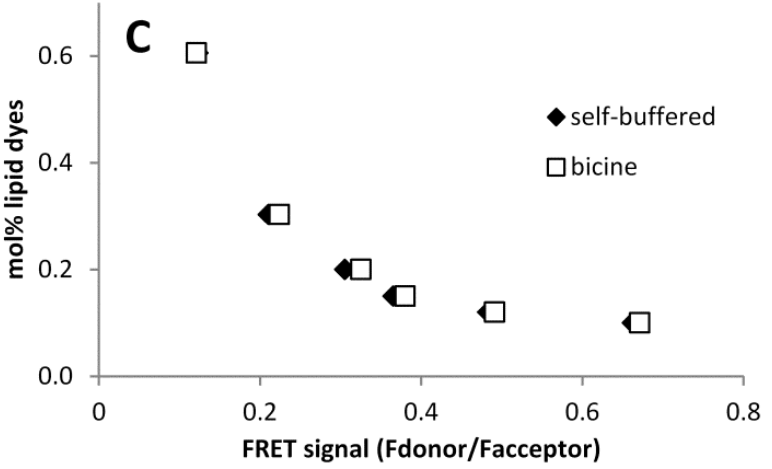

Figure S2.1C. Calibration curves for FRET size change measurements.

C: Vesicles in 0.2 M Na^+-bicine buffer and self-buffered oleate, all with 10mol% of dipeptide $AcPheLeuNH_2$ in the membrane. Samples prepared at varying ratio of oleate to FRET dyes and processed as described in Materials and methods (section Vesicle size change measurements), briefly: vesicles tumbled overnight, extruded, tumbled at RT for 1h and FRET signal measured. The presence of dipeptide in the membrane does not affect the measured FRET signal.

FRET signal is given as the ratio of the fluorescence of the donor (NBD-PE) measured at 530nm to the fluorescence of the acceptor (Rh-DHPE) measured at 586nm, with both excited at the donor excitation maximum of 430nm.

Vesicle Growth Imaging

Approximately monodisperse large multilamellar vesicles were prepared as previously described [19]. Briefly, a dried film of oleic acid was prepared by evaporating solvent from a chloroform solution of oleic acid with 0.2 mol% of Rh-DHPE and 10 mol% of dipeptide AcPheLeuNH_2; the lipid film was suspended in water with 0.5 equivalents of NaOH and 50mM sucrose. Vesicles were tumbled for 19 hr to equilibrate, extruded through a 5 um pore filter, and then dialyzed (using a 3 um pore dialysis membrane) against vesicles with dipeptide but without rhodamine dye (10mM lipid in both solutions), resulting in a population of multilamellar vesicles approximately 4μm diameter. Vesicle growth was initiated by mixing 1μL of 5mM oleate vesicles with dipeptide AcPheLeuNH_2 with 100 equivalents of 5mM oleate vesicles without dipeptide and dye. Vesicles were imaged using a Nikon TE2000S inverted epifluorescence microscope with extralong working distance (ELWD) objective lenses (Nikon).

Synthesis of AcPheLeuNH_2

10 mL of a solution of 25 mM AcPheOEt, 25 mM LeuNH_2 and 1 mg/mL α-chymotrypsin in 9.5:0.5 EPPS (0.2 M, pH 8.5) : DMF was incubated at 37^O C for 1.5 hr. The sample was then centrifuged (4000 rpm, 10 min), the supernatant decanted, and

the product, a white precipitate, was washed twice with 10 ml of 9.5:0.5 EPPS (0.2 M, pH 8.5) : DMF and centrifuged after each wash. The precipitate was dried overnight *in vacuo*. The product was obtained as a white powder in 87% yield and used without further purification. HPLC analysis of the product showed only a trace (<0.5%) of the hydrolyzed substrate AcPheOH. The NMR spectrum was recorded using a Varian VXR-400 spectrometer. ^{1}H NMR ($CDCl_3$, 400MHz): 0.83-0.89 (m, 6H), 1.75 (s, 1H), 2.42 (brs, 2H), 2.50 (s, 3H), 3.36-3.50 (m, 2H), 4.2 (1H), 4.46-4.49 (m, 1H), 7.15-7.18 (m, 5H), 7.97 (brs, 1H), 8.12 (brs, 1H). The ESI-MS spectrum was recorded on a Bruker Daltonics Esquire 6000; mass calculated for $C_{17}H_{25}N_3O_3$+H: 320.20 Da; observed [M+H] 320.6 Da.

Exchange of catalyst and product between vesicles

Vesicles were prepared as described above, with and without $AcPheLeuNH_2$. Equal volumes of samples of each vesicle type at the same concentration were placed on either side of a dialysis membrane mounted between two 1.5 ml Eppendorf tubes with insulating o-rings. Samples were tumbled at RT and at the given time points, the concentration of $AcPheLeuNH_2$ was measured on both sides of the dialysis membrane on an Agilent Analytical HPLC with a Varian Microsorb-mv 100-8 C_{18} 250 x 4.6 mm column with a gradient of solvent A: 0.1% TFA in H2O, and

solvent: B 0.1% TFA in acetonitrile, 3%-80% B over 40 minutes, with UV detection at 265 nm.

Membrane anisotropy

Oleate vesicles were prepared as described above, with varying amounts of $AcPheLeuNH_2$ and with 1,6-diphenyl-l,3,5-hexatriene (DPH) at a ratio of 1:400 DPH:oleate.

Samples were diluted to 4 mM oleate for measurements. The steady state fluorescence anisotropy was measured at excitation wavelength 360 nm and emission wavelength 430 nm, with g factor = 1, using a Cary Eclipse fluorimeter (Varian, Mulgrave, Australia) with Manual Polarizer Accessory by Varian. Fluorescence anisotropy was calculated as a unitless ratio defined as $R = (I_{=} - I_{\perp})/(I_{=} + 2I_{\perp})$, where I is the emission intensity parallel ($I_{=}$) or perpendicular ($I_{\perp}$) to the direction of polarization of the excitation source.

CMC measurements

The CMC (Critical Micelle Concentration) was measured at high pH (pH ~11) using pyrene as a reporter. Oleate micelles (20 mM) were prepared with 20 mM NaOH, 50 uM pyrene and 0, 5, 10 or 15 mol% $AcPheLeuNH_2$ (relative to oleate). Oleic acid was mixed with a dichloromethane solution of pyrene and dipeptide, organic solvent was evaporated, and the aqueous NaOH solution

was added. Samples were left tumbling to equilibrate for 18 h, and then samples from 10 mM to 4.8 uM oleate were prepared by serial dilution. Pyrene fluorescence was recorded using a Cary Eclipse fluorimeter (Varian, Mulgrave, Australia) at excitation wavelength 345 nm and emission wavelengths 374 nm (F1) and 385 nm (F2). The intensity of pyrene emission at either of the maxima (F1 and F2) depends on the polarity of the local environment, with F1 being higher than F2 in an aqueous environment, while in a nonpolar environment F1 decreases significantly. The ratio of F1/F2 was calculated and plotted against the oleate concentration [Figure S2.2].

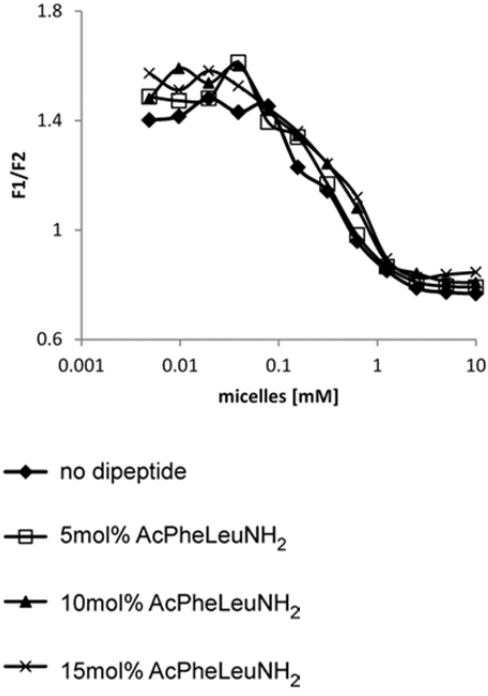

Figure S2.2. CMC, critical micelle concentration, measured using pyrene fluorescence.

Oleate micelles (20 mM) were prepared with 20 mM NaOH, 50 μM pyrene and 0, 5, 10 or 15 mol % $AcPheLeuNH_2$ (relative to oleate). Samples from 10 mM to 4.8 μM oleate were prepared by serial dilution. Pyrene fluorescence was recorded with an excitation wavelength of 345nm and emission wavelengths of 374nm (F1) and 385nm (F2).

Vesicle trans-membrane pH gradient measurement

Dried films were prepared by evaporating solvent from a chloroform solution of oleic acid with or without 10 mol% $AcPheLeuNH_2$. Vesicles were formed by adding arg^+-bicine buffer, pH 8.1, with and without 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS), to the dried lipid film, and tumbling for 18 hours. Arg^+-oleate micelles were prepared by mixing a 15% methanol in water solution of arginine with one equivalent of oleic acid, as previously described. pH gradient measurements were performed as previously described.[4]

Briefly, vesicles with and without dipeptide $AcPheLeuNH_2$ were mixed together in high salt buffer with arginine as a membrane-impermeable counter-ion (0.2M arg^+-bicine, pH=8.1). After mixing the two populations of vesicles, one equivalent of arg^+-oleate micelles was added, and the pH change was monitored through the change in HPTS fluorescence with excitation at 460 nm and emission measured at 510 nm. Because the absorption spectrum of HPTS is pH-sensitive around neutrality, the emission intensity depends on the pH. [5] A calibration curve was prepared using vesicles containing HPTS at known pH, with and without $AcPheLeuNH_2$, which was found not to affect the measured pH inside the vesicles.

Fatty acid desorption rate measurement

The fatty acid desorption rate from oleic acid vesicles with different concentrations of dipeptide $AcPheLeuNH_2$ in the membrane was measured as previously described. [6]

Briefly, vesicles were prepared as described above, with varying concentrations of dipeptide $AcPheLeuNH_2$, in either 0.2M Na^+-bicine pH 8.5 or as self-buffered samples with 50 mol% of NaOH. Reporter vesicles were prepared from 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 0.5 mM HPTS [10, 18] in 0.2 M Na^+-bicine pH 8.5 or as self-buffered vesicles with pH adjusted to pH 8.5. Fatty acid vesicles (0.25 mM oleate) with dipeptide were mixed with phospholipid reporter vesicles (1 mM POPC), and the resulting pH change was monitored through the change in HPTS fluorescence with excitation at 460 nm, and emission at 510 nm [Figure S2.3]. The decrease of HPTS fluorescence over time was fitted to a first-order exponential decay. The reported k_{off} (s^{-1}) is an average of four independent measurements for each peptide concentration.

Changes in HPTS fluorescence were converted to changes in pH using a standard curve generated by using POPC vesicles prepared at different pH values in either 0.2M Na^+-bicine or as self-buffered vesicles (the pH of self-buffered vesicles was adjusted by adding 1M NaOH and monitoring the pH with a pH-meter).

Figure S2.3

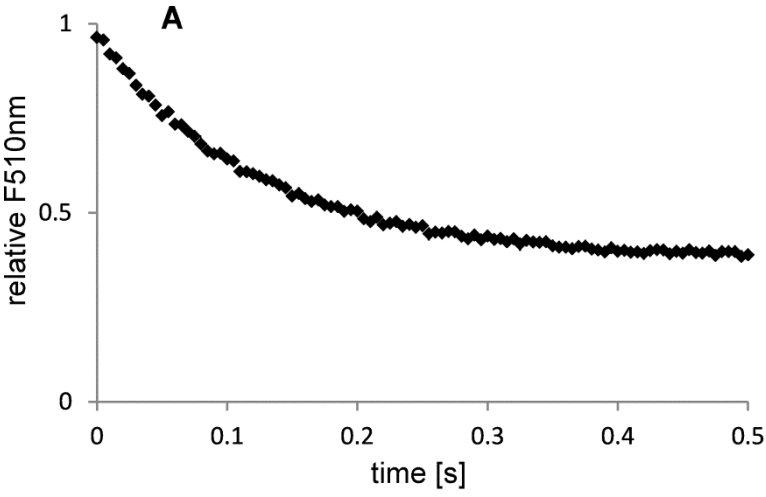

Figure S2.3. An example of fatty acid desorption rate measurement.

Change in HPTS emission vs. time following mixing of reporter vesicles with oleic acid vesicles without the dipeptide.

Fatty acid desorption rate is derived from the pH change inside reporter phospholipid vesicles. The pH change inside reporter vesicles is measured by pH-sensitive HPTS dye, and the observed fluorescence decay is fitted to the first order exponential decay equation.

Competitive Vesicle Growth

Vesicles with catalyst were prepared as follows: a thin film of oleic acid was resuspended in a solution containing 0.50 eq of NaOH and the catalyst; typically the resulting solution was 100 mM oleate in 50 mM NaOH. If vesicles were FRET dye labeled, the thin film of lipids was prepared by mixing chloroform solutions of oleic acid and the FRET dyes (NBD-PE and Rh-DHPE, 0.1 mol% of each) followed by rotary evaporation and resuspension as above. All samples were tumbled at RT for 18 to 20hr, then vesicles were extruded through 100 nm pore membrane.

Extruded vesicles were left tumbling for at least 1h and purified on a Sepharose 4B size exclusion column to remove unencapsulated catalyst. After purification vesicles were tumbled at RT for at least 1 h, and then mixed with substrates AcPheOEt and $LeuNH_2$. Each vesicle sample was tumbled at 37 °C for 48 hours. Vesicles without the catalyst were prepared the same way, except the size exclusion column purification was not necessary since there was no encapsulated catalyst to remove.

For competitive growth between vesicles with Ser-His and vesicles without the catalyst, after incubation at 37 °C for 48 hours with reaction substrates, vesicles with Ser-His were mixed with an equal volume of vesicles without Ser-His (but also incubated with substrates). Samples were tumbled for 1h and

then diluted to an absorbance of 0.1 or less at 500 nm (to avoid artifacts due to light scattering). Fluorescence of the dyes was recorded and averaged over a period of 10 minutes, and FRET signal was calculated as described above. Surface area change was calculated using calibration curves for surface area change vs. FRET signal prepared by incubating vesicles with the FRET dye pairs and with and without the tested catalyst. Separate calibration curves for each catalyst were used to correct for hydrolysis of FRET dyes during the incubation.

For competitive growth between vesicles with Ser-His, vesicles with Ser-His-Gly and vesicles without the catalyst: all three populations of vesicles (with Ser-His, with Ser-His-Gly, and empty) were incubated at 37 °C for 48 hours with reaction substrates and then mixed together, tumbled for 1h and then FRET signal was recorded as described above.

Micelle-mediated vesicle growth

In a typical experiment, vesicles were prepared with $AcPheLeuNH_2$ in the membrane by mixing oleic acid with dipeptide $AcPheLeuNH_2$ in chloroform, evaporating organic solvent and adding high salt bicine buffer. Vesicles with peptide were mixed with an equal volume of oleate vesicles lacking $AcPheLeuNH_2$.

Mixed vesicle samples were equilibrated for ~10 minutes; during that time no growth of peptide vesicles was observed (since competitive growth does not happen in high salt buffer). A small volume (approximately 10 to 15% of initial sample volume) of Na^+-oleate micelles was then added and membrane surface area changes were monitored using FRET.

3. Synthesis of genetic polymer PNA catalyzed by the prebiotic catalyst Ser-His

The PNA monomers were synthesized according to the previously described procedure. [7]

Ser-His catalyzed synthesis of the PNA oligomers

Reaction conditions: 1 ml of 20.0 mM 1 in 0.2 M HEPES Na+ buffer pH=8.3 was mixed with 1 ml of 3.0 mM Ser-His in 0.2 M HEPES Na+ buffer pH=8.3 at RT. Reaction time was 35h.

After the completion of the reaction solvent (water) was removed on rotary evaporator, sample was dissolved in 0.2 ml H2O and purified on HPLC.

HPLC: Varian VariTide RPC 6M column was used. Elution was performed at a room temperature with a flow rate of 1.5 ml/min. The used gradient of buffer B in buffer A was: 2% B to 50% B in 35 minutes, then 50% B to 98% B in 5 minutes. Buffer A: 0.1% TFA in H2O, buffer B: 0.1% TFA in acetonitrile.

Detection wavelength was set to 260nm.

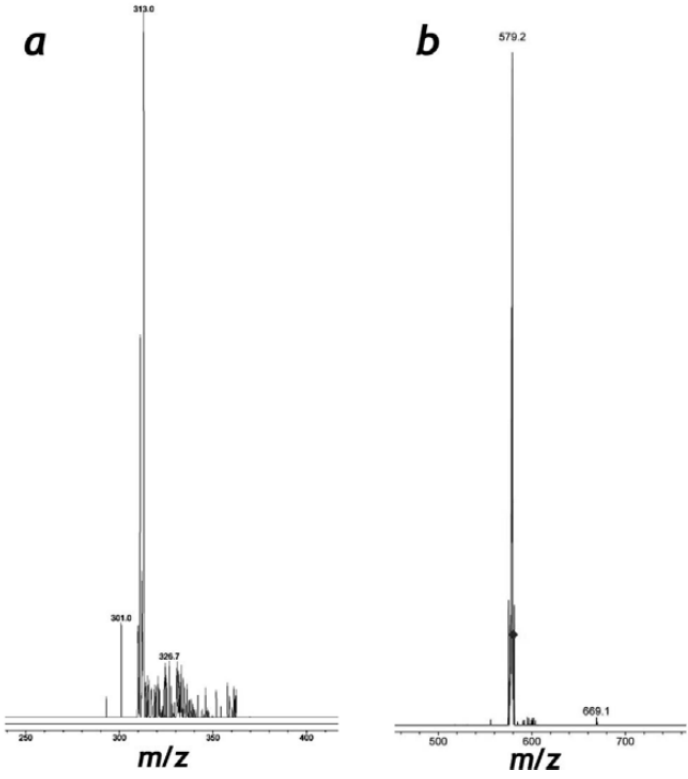

Figure S3.1 Mass spectra of PNA monomer (a) and dimer (b).

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