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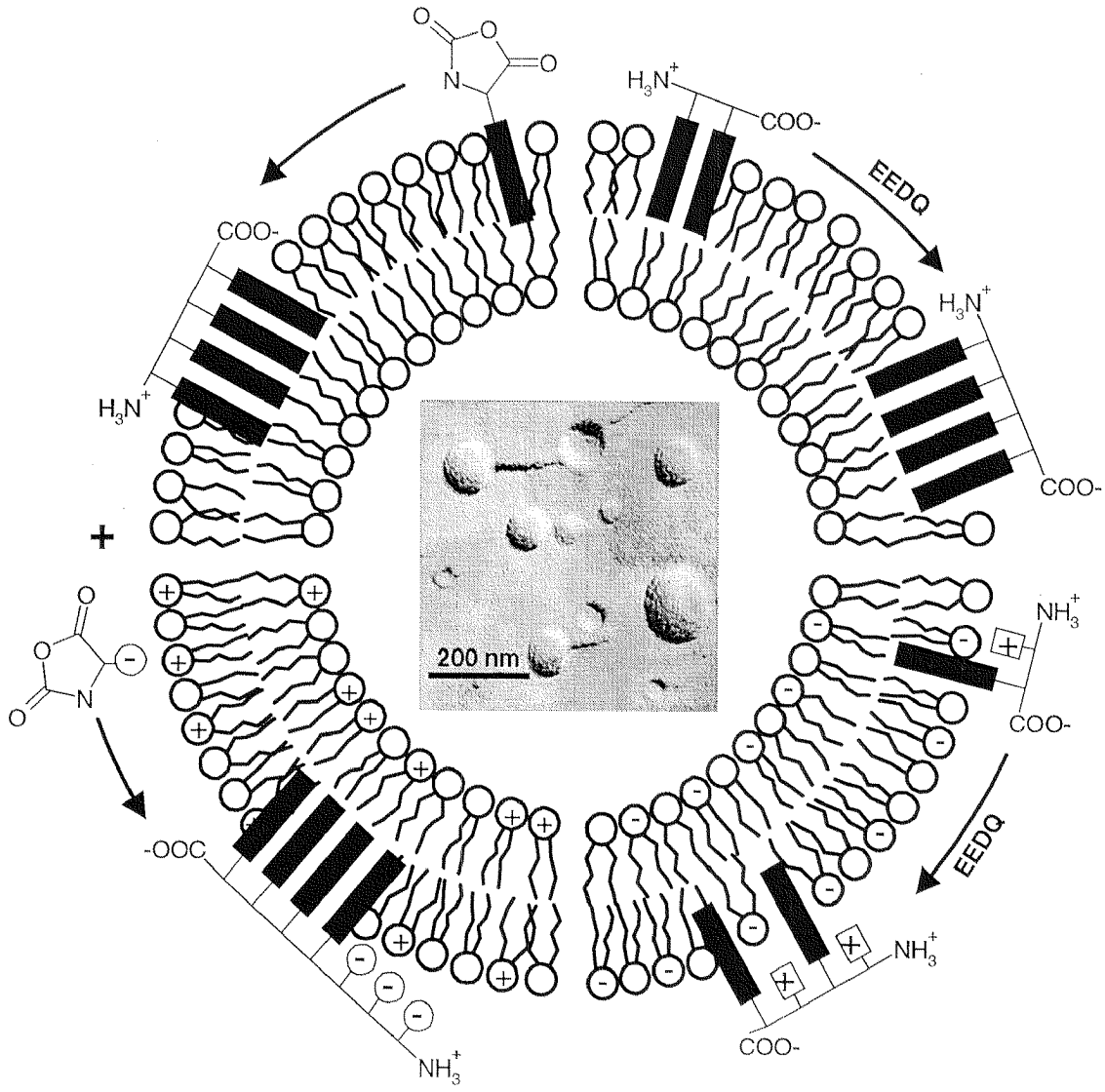
Liposome-Assisted Polycondensation of Amino Acids and Peptides

Markus Christoph Blocher



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Liposome-Assisted Polycondensation of Amino Acids and Peptides

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presented by
MARKUS CHRISTOPH BLOCHER
Dipl. Chem. ETH
born April 16, 1971
Citizen of Meilen (ZH), Zürich, and Schattenhalb (BE)

accepted on the recommendation of
Prof. Dr. Pier Luigi Luisi, examiner
Prof. Dr. Donald Hilvert, co-examiner
Prof. Dr. Peter Walde, co-examiner

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To Daniela

"Things perfected by nature are better than those finished by art."

Marcus Tullius Cicero (106 - 43 BC)

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Abbreviations

ACN:	Acetonitrile
Ala:	Alanine
APCI:	Atmospheric pressure chemical ionization
API:	Atmospheric pressure ionization
Arg:	Arginine
Asn:	Asparagine
Asp:	Aspartic acid
CAC:	Critical aggregation concentration
CAD:	Collision activated dissociation
CDI:	N,N'-carbonyldiimidazole
CI:	Chemical ionization
CID:	Collision-induced dissociation
CLN:	Nitrogen pyro-chemiluminescence
CRM:	Charged-residue model
CTAB:	Cetyltrimethyl ammonium bromide
Cys:	Cysteine
DAD:	Diode array detector
DC:	Direct current
DCC:	N,N'-Dicyclohexylcarbodiimide
DCP:	Dicetyl phosphate
DDAB:	Dimethyl didodecylammonium bromide
DLS:	Dynamic light scattering
DOPA:	1,2-Dioleoyl-sn-glycero-3-phosphate
DOPC:	1,2-Dioleoyl-sn-glycero-3-phosphocholine
DPPC:	1,2-Dipalmitoyl-sn-glycero-3-phosphocholine
EDAC:	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide
EEDQ:	2-Ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline
EI:	Electron impact
EM:	Electron microscopy
ESI:	Electrospray ionization
FAB:	Fast atom bombardment
FD:	Field desorption
FeS2:	Pyrite
ff-EM:	Freeze-fracture electron microscopy
FI:	Field ionization
FIB:	Fast ion bombardment
Gln:	Glutamine
Glu:	Glutamic acid
Gly:	Glycine
GV:	Giant vesicle
His:	Histidine
HPLC:	High-performance liquid chromatography
IEM:	Ion evaporation model
Ile:	Isoleucine
ISF:	In-source fragmentation
Ka:	Association constant

LC:	Liquid chromatography
LD:	Laser desorption
Leu:	Leucine
LOD:	Limit of detection
LS:	Light scattering
LSIMS:	Liquid secondary ion mass spectrometry
LV:	Large vesicle
Lys:	Lysine
M:	Magnet
MALDI:	Matrix assisted laser desorption ionization
Met:	Methionine
MLV:	Multilamellar vesicles
MS:	Mass spectrometer
MS:	Mass spectrometry
MSD:	Mass spectrometer detector
MVV:	Multivesicular vesicles
MW:	Molecular weight
NCA:	N-Carboxyanhydride
NIR:	Near infrared
NMR:	Nuclear magnetic resonance
NTA:	2-Thio-N-carboxyanhydride
P:	Partition coefficient
PC:	Phosphatidylcholine
PD:	Plasma desorption
Phe:	Phenylalanine
POPC:	1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
Pro:	Proline
Q:	Quadrupole
rf:	Radio-frequency
RI:	Refractive index
RPC:	Reversed-phase chromatography
Ser:	Serine
SIM:	Selected ion monitoring
SIR:	Selected ion recording
SV:	Small vesicle
TFA:	Trifluoroacetic acid
Thr:	Threonine
TIC:	Total ion current
Tm:	Phase transition temperature
TOF:	Time-of-flight
Trp:	Tryptophan
TSP:	Thermospray
Tyr:	Tyrosine
UV:	Ultra violet
UV:	Unilamellar vesicles
UV-vis:	Ultra-violet-visible
Val:	Valine

Aims

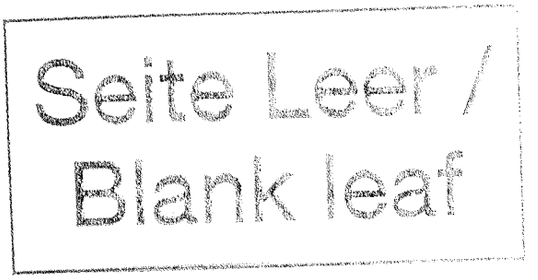
The observation that spontaneous formation of order and compartmentalization - which are central to all living structures - can be achieved by simple surfactant molecules. This made surfactant aggregates the focal point in research on the origin of life at Prof. Luisi's laboratory. This dissertation is built on the interest in prebiotic macromolecule formation in the presence of surfactant aggregates and focuses on condensation experiments of amino acids and peptides in the presence of liposomes.

No updated, extensive review of the literature in the field of prebiotic peptide condensation existed prior to this study. The aim of the theoretical section of this thesis was:

- to order and summarize the experiments presented to date in the literature;
- to provide a critical, but brief overview of the origin of life research and its main hypotheses;
- to introduce the LC-MS technique into Prof. Luisi's group and to use this analytical method to analyze the products formed in the condensation experiments which were carried out as part of this study.

The aim of the experimental work was to examine the condensation of amino acids and peptides in aqueous solution in the presence of liposomes, using *N*-carboxyanhydride-activated amino acids on the one hand, and on the other the lipophilic condensing agent 2-ethoxy-1-ethoxycarbonyl-1,2-dihydrochinoline (EEDQ). This research should clarify:

- whether and to what extent liposomes can aid the formation of hydrophobic oligopeptides in condensation experiments;
- whether the presence of liposomes might lead to preferential, selective formation of certain peptide sequences, starting from a pool of different monomers;
- whether there is a stereochemical influence of the chiral lipid 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) on the condensation of different amino acid enantiomers (L or D) and their racemates.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Abstract

One of the main questions in the field of the origin of life is how macromolecules with specific sequences might have formed prior to the existence of the replication or translation machinery. It is of particular interest to find simple mechanisms (in a molecular evolution scenario) that permit selective formation of a restricted number of polypeptide (or polynucleotide) primary sequences out of the incommensurably large number of theoretically possible sequences. The present dissertation aims to contribute to this question using liposomes as matrices for the polycondensation of amino acids and peptides. The study is influenced by the compartmentalistic view of the origin of life, where compartments such as liposomes (resembling the structure of the extant biological cell membranes) are believed to have appeared early in the transition to life. The present study, although originating from the origin of life perspective, is also of interest to the general field of macromolecular chemistry. It addresses the general questions of whether and to what extent liposomes can assist in the formation of specific oligopeptides in condensation experiments and whether there is any stereochemical influence of the chiral 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) on the condensation of different amino acid enantiomers and their racemates.

At the beginning of the theoretical section of this thesis, an introduction into liquid chromatography mass spectrometry (LC-MS) is given. This analytical method was the main tool used to identify and quantify the products formed in the condensation reactions. Other methods such as dynamic light scattering (DLS), freeze-fracture electron microscopy (ff-EM), and equilibrium dialysis were also used. A critical but brief overview of origin of life research and its main hypotheses is given, followed by a review of prebiotic amino acid and peptide condensation experiments published to date in the literature.

In the experimental section, the results of two complementary polycondensations in the presence of lipid bilayers as matrices are discussed and compared to the aqueous reference system. In the first approach, the CDI (*N,N'*-carbonyldiimidazole)-induced, classic polycondensation method with *N*-carboxyanhydride (NCA) amino acids for the polymerization of hydrophobic amino acids was used. In the second approach, the hydrophobic condensing agent EEDQ (2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline), which exhibits an affinity for the hydrophobic membrane of lipid

bilayers, was used for the condensation of Trp-containing dipeptides. These polycondensation reactions were found to be promoted by a selection process based on the hydrophobic interaction between the bilayer and peptides and can lead to the formation of much longer and sequence specific oligomers when compared to the aqueous reference system. In the case of NCA-Trp, multiple-feeding to POPC liposomes led to much longer oligomers (up to 29mer) when compared to the aqueous reference system, where the maximal length of oligo-Trp formed was never higher than the 8mer. In the case of EEDQ-induced condensation, H-TrpTrp-OH in the presence of POPC liposomes also led to higher oligomers (up to H-Trp_8-OH), whereas in the aqueous control experiment only traces of H-Trp_4-OH were found. If the liposomes were exposed to a small library of four different dipeptides (H-TrpTrp-OH, H-TrpGly-OH, H-TrpAsp-OH, H-TrpGlu-OH), only the most hydrophobic H-TrpTrp-OH was selected by the membrane and underwent oligomerization. Out of the 16 theoretically possible tetrapeptides, H-Trp_4-OH made up about 70% of all the tetrapeptides formed.

POPC membranes can also display electrostatic interactions once positively or negatively charged cosurfactants are added to them. Mixed POPC liposomes with different charge densities were prepared using positively charged dimethyl didodecylammonium bromide (DDAB) or negatively charged 1,2-dioleoyl-*sn*-glycero-3-phosphate (DOPA). Such charged membranes were used to bind oppositely charged amino acids (NCA-Glu) or peptides (H-ArgTrp-OH or H-HisTrp-OH) in order to oligomerize them. The polycondensation of mixtures of amino acids and peptides based on both hydrophobic and electrostatic interactions was possible. The subsequent feeding of NCA-Trp and NCA-Glu to either POPC or mixed DDAB/POPC liposomes led to the formation of longer block oligomers in the latter case. For example, for block oligomers containing five Trps, the decapeptide H-Glu_5Trp_5-OH is formed in a 0.2% yield based on initial Trp, whereas in the POPC system H-Glu_2Trp_5-OH (0.2%) is the longest co-oligomer detected. The EEDQ-induced condensation of H-ArgTrp-OH in the presence of DOPA/POPC liposomes led to yields (of initial dipeptides reacted) of about 70% in oligopeptides, whereas in the presence of POPC liposomes the yield in oligomers was less than 10%. The syntheses of oligopeptides consisting of polar (i.e. Arg, Glu, or His) and hydrophobic (i.e. Trp) amino acid residues in the same oligomer chain were performed in this way.

Finally, it was shown that in the case of NCA-amino acid condensation, the different stereochemical interaction between the chiral POPC liposome matrix in the fluid state and either one of the enantiomeric amino acid monomers had no particular influence on the product distribution. However, the nature of the racemic NCA condensation of amino acids showed some interesting features. The presence of liposomes enhances the formation of longer oligomers, but a preferential formation of homochiral (all D or all L) oligomers at higher degrees of oligomerization was observed for NCA-Trp in the absence, as well as in the presence of POPC liposomes. Similar results were obtained for Leu and Ile in the absence of liposomes. For the first time, relative stereoisomer distributions of racemic NCA-amino acid oligomers were determined by LC-MS quantification, using isotopic labelling of one of the enantiomers. The distributions were symmetrical, indicating no significant stereochemical influence of the chiral POPC bilayer surface in the fluid state on the racemate condensation. The overrepresentation of homochiral oligomers in such stereoisomer distributions at higher n-mers was found to be several times greater (i.e. for racemic NCA-Trp in the presence of liposomes, 40 times overrepresented for the Trp-decamer) than expected based on a random distribution. This effect was attributed to second-order Markov oligomerization behavior of the growing oligomer chain.

In conclusion, liposomes can assist the condensation of amino acids and peptides. Depending on the chemical nature of the amino acids or peptides and the lipids used, liposomes show a selectivity towards formation of certain oligopeptide sequences. The POPC liposomes assist the formation of long hydrophobic oligopeptides (i.e. H-Trp_{29}-OH) which can not be obtained in aqueous buffer (where the longest oligomer was H-Trp_8-OH). The selectivity principle of hydrophobic interactions, where the main factor was the assistance due to membrane solubility, was extended to electrostatic interactions. This allowed the selective formation of peptides containing different types of amino acids in the presence of liposomes. Such a process might be a key to the formation of specific oligopeptide sequences in an origin of life scenario. The chirality of the fluid POPC liposomal membrane has no significantly different stereochemical influence on the condensation of either the NCA-L- or NCA-D-amino acid or its racemate. Racemic NCA-amino acid condensation in the absence and presence of POPC liposomes leads to preferential formation of homochiral stereoisomers for oligomers above a certain chain

length. LC-MS in combination with isotopic labelling is a good and novel method for the determination of relative stereoisomer distributions of NCA-amino acid oligomers.

Zusammenfassung

Wie konnten sich Makromoleküle mit spezifischen primären Sequenzen gebildet haben, bevor weder die Replikation- noch die Translations-Maschinerie erfunden war? Dies ist eine der Hauptfragen im Forschungsgebiet über den Ursprung des Lebens. Es ist von besonderem Interesse einfache Mechanismen (im Rahmen der molekularen Evolution) zu finden, die eine selektive Bildung einer begrenzten Anzahl von Polypeptidsequenzen (oder Polynukleotidsequenzen), aus der unvorstellbar grossen Zahl theoretisch möglicher Sequenzen, erlauben. Die vorliegende Dissertation möchte sich dieser Frage annehmen, unter der Verwendung von Liposomen als Matrizen für die Polykondensation von Aminosäuren und Peptiden. Die Arbeit ist durch eine Ansicht des Ursprungs des Lebens geprägt, die eine Kompartimentierung durch supramolekulare Aggregate wie Liposomen (welche Ähnlichkeiten mit den heutigen biologischen Membranen aufweisen) in einem frühen Stadium der Entwicklung zum Leben vorsieht. Obwohl aus der Perspektive des Ursprungs des Lebens entstanden, ist die vorliegende Doktorarbeit auch für das allgemeine Gebiet der Makromolekularen Chemie von Interesse. Sie befasst sich hauptsächlich mit den Fragen ob und in welchem Ausmass die Anwesenheit von Liposomen die Bildung von spezifischen Oligopeptiden in Kondensationsexperimenten unterstützt und ob die chirale Oberfläche der 1-Palmitoyl-2-oleoyl-*sn*-3-phosphocholin (POPC) Membran einen stereochemischen Einfluss auf die Kondensation des einen oder anderen Enantiomers einer Aminosäure oder das Racemate hat.

Am Anfang des theoretischen Teils dieser Arbeit wird in die Flüssigchromatographie-Massenspektrometrie (LC-MS) eingeführt. LC-MS war die Hauptmethode der Analytik, die zur Identifizierung und Quantifizierung der Kondensationsprodukte verwendet wurde. Zusätzlich zu ihr wurden andere Methoden wie die Dynamische Lichtstreuung, die Gefrierbruch-Elektronenmikroskopie oder die Gleichgewichtsdialyse verwendet. Im theoretischen Teil ist auch ein kurzer, kritischer Überblick über die Forschung des Ursprungs des Lebens und ihre wichtigsten Hypothesen dargestellt. Anschliessend folgt eine Zusammenstellung, der bis heute publizierten Arbeiten, über präbiotische Aminosäure- und Peptidkondensationsexperimente.

Im experimentellen Teil werden die Resultate, von zwei sich ergänzenden Polykondensationsmethoden in der Anwesenheit von Liposomen als Matrizen diskutiert und mit dem wässerigen Referenzsystem verglichen. Einerseits wurde die CDI (*N,N'*-

Carbonyldiimidazol) induzierte, klassische Polykondensation von *N*-Carboxyanhydriden (NCA) verwendet um hydrophobe Aminosäuren zu polymerisieren. Andererseits wurde das hydrophobe Kondensreagens EEDQ (2-Ethoxy-1-ethoxycarbonyl-1,2-dihydrochinolin), welches selbst Affinität zur Lipidmembran besitzt, für die Oligomerisierung von Trp-enthaltenden Dipeptiden eingesetzt. Beide Typen von Polykondensationen zeigten eine bevorzugte Bildung von längeren und sequenzspezifischen Oligomeren. Dies wird hauptsächlich durch einen, auf hydrophoben Wechselwirkungen beruhenden, Selektionsprozess zwischen der Membran und den Peptiden bewirkt. Im Falle einer mehrfachen, hintereinander erfolgten Zugabe von NCA-Trp zu POPC Liposomen wurden viel längere Oligomere erhalten (bis zum 29mer) als im wässerigen Referenzsystem, in welchem die maximale Länge der Oligomere das 8mer nie überschritt. Die EEDQ-induzierte Kondensation von H-TrpTrp-OH in der Anwesenheit von POPC Liposomen führte ebenfalls zu längeren Oligomeren (bis zum H-Trp_8-OH). In der wässerigen Kontrolle wurden hingegen höchstens Spuren von H-Trp_4-OH gefunden. Führte man die Kondensation von vier verschiedenen Dipeptiden (H-TrpTrp-OH, H-TrpGly-OH, H-TrpAsp-OH, H-TrpGlu-OH) in Anwesenheit von Liposomen durch, so wurde nur das hydrophobe H-TrpTrp-OH durch die Membran gebunden. Seine Aktivierung, durch das ebenfalls an das Liposom gebundene EEDQ, endete schliesslich in dessen Oligomerisierung und Oligo-Trp entstand dadurch selektiv in guter Ausbeute. Von den 16 verschiedenen, theoretisch möglichen Tetrapeptiden, machte H-Trp_4-OH etwa 70% aus.

Durch die Einführung von geladenen Cotensiden, kann die Membran der POPC Liposomen mit positiven oder negativen Ladungen versehen werden. Dies ermöglicht die Ausbildung von elektrostatischen Wechselwirkungen mit Aminosäuren (NCA-Glu) oder Peptiden (H-ArgTrp-OH oder H-HisTrp-OH) entgegengesetzter Ladung und schliesslich deren Oligomerisierung. Zu diesem Zweck wurden gemischte POPC Liposomen hergestellt unter Verwendung von Dimethyldidodecylammoniumbromid (DDAB) oder 1,2-Dioleoyl-*sn*-glycero-3-phosphat (DOPA) als Cotensid, um die Membranen mit positiver, respektive negativer Ladungsdichte zu versetzen. Es zeigte sich, dass sich mittels hydrophober und/oder elektrostatischer Wechselwirkungen die Polykondensation von Aminosäuren und Peptiden erfolgreich an der Oberfläche von Liposomen durchführen lässt. Bei sequentieller Zugabe von NCA-Trp und NCA-Glu zu POPC oder gemischten DDAB/POPC Liposomen wurden nur im letzteren Fall höhere

Blockoligomere gebildet. Betrachtete man zum Beispiel alle Blockoligomere, die fünf Trp enthalten, so wurde das Decapeptid H-Glu_5Trp_5-OH mit einer Ausbeute von 0.2% des ursprünglich vorhanden Trp gebildet. Hingegen im POPC Liposomen-System war H-Glu_2Trp_5-OH (0.2%) das höchste Blockoligomer das gefunden wurde. Die EEDQ-induzierte Kondensation in der Anwesenheit von DOPA/POPC Liposomen von H-ArgTrp-OH führte zu Oligopeptiden mit rund 70% Ausbeute (von reagiertem Dipeptid). Wurde die Reaktion jedoch mit POPC Liposomen durchgeführt war die Ausbeute weniger als 10%. Auf diese Weise konnten Oligopeptide hergestellt werden, die sowohl aus polaren (z. Bsp. Arg, Glu oder His) als auch aus hydrophoben (z. Bsp. Trp) Aminosäureresten bestanden.

Schliesslich konnte gezeigt werden, dass im Falle der NCA-Aminosäure-Kondensation die unterschiedlichen stereochemischen Wechselwirkungen der Aminosäure-Enantiomere mit den chiralen POPC Liposomen im fluiden Zustand keinen signifikanten Einfluss auf die Produkteverteilung haben. Es wurden hingegen interessante Eigenschaften für die racemische NCA-Kondensation beobachtet. Wie zuvor führt die Anwesenheit der Liposomen erneut zu längeren Oligomeren, aber für höhere Oligomerisationgrade wurde eine deutlich bevorzugte Bildung von homochiralen (alles L oder alles D) Oligomersequenzen für die NCA-Trp Kondensation in der Ab- und Anwesenheit von POPC Liposomen beobachtet. Ähnliche Resultate wurden für Leu und Ile in der Abwesenheit von Liposomen gefunden. Für das erste Mal wurde LC-MS zur Quantifizierung der relativen Stereoisomerverteilung für NCA-Aminosäure-Oligomere eingesetzt und zwar durch Isotopenmarkierung eines der beiden Enantiomeren. Die Verteilung erwies sich als symmetrisch, was wiederum keinen signifikanten, stereochemischen Einfluss der chiralen POPC Membran im fluiden Zustand auf die racemische Kondensation bestätigt. Die homochiralen Stereoisomere waren für höhere Oligomere mehrfach übervertreten als man dies für eine statistisch Verteilung erwarten würde. Für das 10mer der racemischen NCA-Trp Kondensation mit POPC Liposomen zeigte sich die Konzentration der homochiralen Stereoisomere als 40-fach höher als statistisch erwartet. Dieser Effekt wurde dem Markov zweiter Ordnung Wachstumsverhalten der wachsenden Oligomerkette zugeschrieben.

Zusammenfassend, Liposomen können die Kondensation von Aminosäuren und Peptiden unterstützen. Abhängig von der chemischen Art der Aminosäuren oder der Peptide und den Lipiden, zeigen die Liposomen eine Selektivität zur Bildung gewisser

Oligopeptidsequenzen. Die POPC Liposomen unterstützen die Bildung von langen hydrophoben Oligopeptiden (z. Bsp. H-Trp_{29}-OH), die im wässerigen Puffer nicht erhalten werden können (H-Trp_8-OH war das längste Oligomer das erhalten wurde). Das Selektivitätsprinzip der hydrophoben Wechselwirkungen, mit der Solubilisationshilfe der Membran als Hauptfaktor, kann durch die Einführung von elektrostatischen Wechselwirkungen erweitert werden. Dies erlaubte die selektive Bildung von Peptiden, bestehend aus verschiedenen Aminosäuren, in der Anwesenheit von Liposomen.

Die Chiralität der fluiden POPC Liposomen-Membranoberfläche hat keinen signifikanten unterschiedlichen stereochemischen Einfluss auf die Kondensation von entweder NCA-L-, NCA-D-Aminosäuren oder ihr Racemat. Die racemische NCA-Aminosäurekondensation mit und ohne Liposomen führt ab einer gewissen Kettenlänge zu einer bevorzugten Bildung von homochiralen Oligomersequenzen. LC-MS in Kombination mit Isotopenmarkierung ist eine neuartige Methode und eignet sich ausgezeichnet zur Bestimmung relativer Stereoisomerenverteilungen von NCA-Aminosäure-Oligomeren.

1. Liquid Chromatography Mass Spectrometry

Low concentrations, interference from the matrix, difficulties associated with low selectivity, and limited structural information are problems for conventional detectors (i.e. UV detectors) used in liquid chromatography (LC).

The development of the diode array detector (DAD) [1], [2] was a significant step in improving sensitivity and selectivity, and fluorescence [3], [4] and electrochemical detectors [5] have also been successfully applied to increase the selectivity of detection. However, the signals of such detectors can not be unambiguously assigned to a particular substance such as a peptide with a specific primary structure.

Recent, the vigorous development of mass spectrometry (MS) has opened a new era in structure elucidation. The on-line coupling of mass spectrometry detectors (MSD) to liquid chromatography systems (see Figure 1) since the late 1980's has led to the powerful liquid chromatography mass spectrometry (LC-MS) technique, which is now widely used in peptide laboratories. Whitehouse et al. [6] first interfaced LC to an electrospray ionization (ESI) mass spectrometer.

In the following paragraphs, the principles of high-performance liquid chromatography (HPLC) (focusing on reverse-phase separation), diode array detection, liquid chromatography mass spectrometry coupling (focusing on the electrospray ionization technique), and of the mass analyzer (focusing on the single quadrupole instrument) will be discussed.

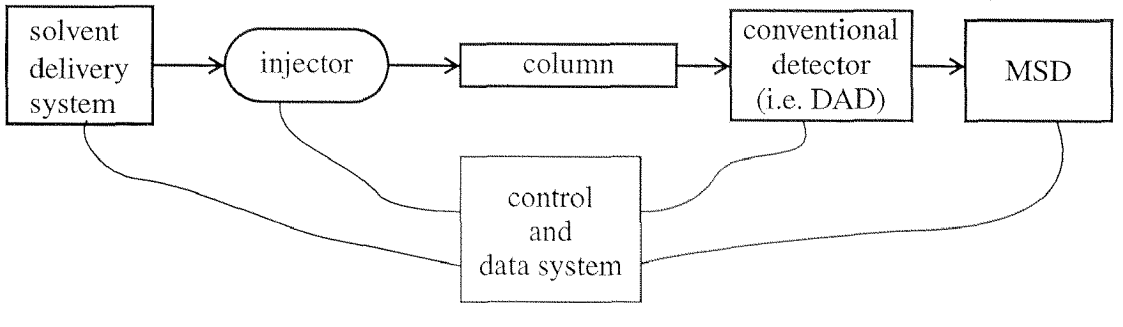


Figure 1: HPLC-MS scheme: Solvent delivery system consisting of a degasser, pumps with the facility to drive gradients, an injector to inject sample onto the column, a column (e.g. with reversed phase material) to separate the different components of the sample, and a diode array as well as a mass spectrometry detector (MSD) to detect and characterize the eluting components. All of these components are controlled by a control and data system unit.

1.1. Reversed-Phase High-Performance Liquid Chromatography

The versatility of the stationary and mobile phases that are available, the resolving power, robustness, and ease of operation make HPLC an extremely powerful technique for the analysis of peptides and many other organic compounds. Several different physico-chemical properties may be used for separation by HPLC, i.e. molecular size (gel permeation or size exclusion chromatography), charge (ion-exchange chromatography), hydrophobicity (reversed-phase chromatography (RPC)), or a combination of these. The present work made extensive use of RPC and therefore the following paragraph will focus on this type of separation.

RPC, the most widely used chromatographic technique, is mainly utilized to separate neutral molecules in solution on the basis of their hydrophobicity [7]. It has proved to be the most powerful available chromatographic method for the separation of peptides and proteins. It provides the flexibility of changing the stationary phase, the pH of the mobile phase, the ionic strength of the aqueous buffer, the nature and concentration of the ion-pairing agent, the type of organic modifier, the gradient shape, and the temperature at which the separation is performed.

Reversed-phase chromatography, as the name suggests, is the reverse of normal-phase chromatography in the sense that it involves the use of a non-polar stationary phase and a polar mobile phase.

1.1.1. Reversed-Phase Stationary Phases

The most common type of stationary phase employed in RPC consists of non-polar, hydrophobic organic species (e.g. octyl, octadecyl, or phenyl groups) attached by siloxane (or silyl ether) bonds (-Si-O-Si-) to the surface of a silica support. These stationary phases are prepared by reacting organochlorosilanes (or organoalkoxysilanes) with reactive silanol (SiOH) groups on the surface of the silica (see Figure 2). Inorganic network silica (SiO_2) is the most common support material because of its many beneficial properties. Small, spherical particles of silica are available in a variety of particle diameters (typically 3, 5, or 10 μm) and pore sizes (typically 60-300 Å) of narrow distribution. The mechanical strength of silica allows porous particles with very large surface areas (typically 60-500 m^2g^{-1}) to be used at high mobile-phase pressures, which allow fast mass-transfer during chromatographic separation as described below. In addition, the silica surface is easily modified owing to reactive SiOH sites. [8]

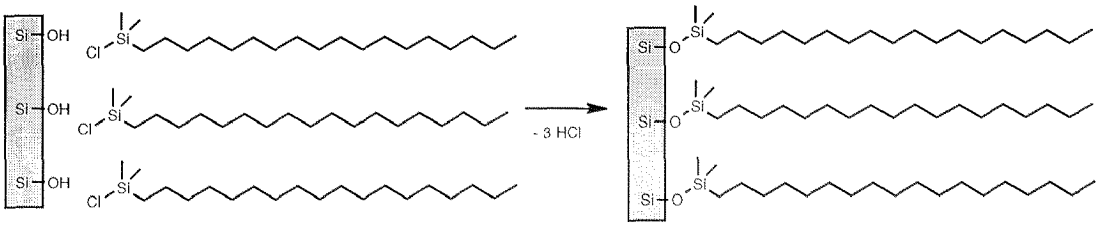


Figure 2: Reaction of silica gel with a functional group to produce a C_{18} reversed-phase stationary phase.

1.1.2. Mobile Phases

The mobile phase in HPLC refers to the solvent being continuously applied to the column (containing the stationary phase). In RPC, mobile phases are based on a polar solvent, typically water, to which a less polar solvent such as acetonitrile or methanol is added. A sample solution is injected into the mobile phase through an injector port (see Figure 1). As the sample solution flows through the column with the mobile phase, the components of that solution migrate according to the non-covalent interactions of each compound with the stationary phase. The chemical interactions of the mobile phase and sample with the column determine the degree of migration and separation of the components contained in the sample. Those components having stronger interactions with the mobile phase than with the stationary phase, will elute faster from the column than those components having stronger interactions with the latter. Therefore the first components (hydrophilic) will have a shorter retention time than the second ones (more hydrophobic). The mobile phase can be altered in order to manipulate the interactions of the sample and the stationary phase. The two most common variations are isocratic (constant mobile phase composition) and gradient elution (increasing strength of the organic solvent).

Two main theories have been developed to explain the mechanism of retention: the solvophobic and the partitioning model (see Figure 3) [7].

In the solvophobic theory, the stationary phase is thought to behave more like a solid than a liquid, and retention is considered to be related primarily to solvophobic interactions between the solutes and the mobile phase (solvophobic effects). The solute binds to the surface of the stationary phase, thereby reducing the surface area of the analyte exposed to the mobile phase. Adsorption to the solid-phase increases as the surface tension of the mobile phase increases. Hence, solutes are retained more as a result of solvophobic

interactions with the mobile phase than through specific interactions with the stationary phase.

In the partitioning model, the stationary phase plays a more important role in the retention process. The solute is thought to be fully embedded in the stationary phase chains, rather than adsorbed on the surface. It is therefore considered to be partitioned between the mobile phase and a "liquid-like" stationary phase.

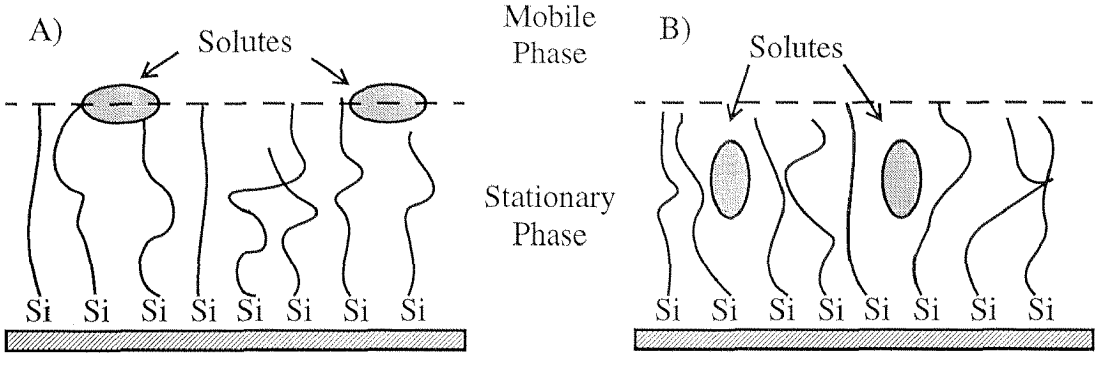


Figure 3: A) Solvophobic and B) partitioning models of solute retention (freely adapted from [7]).

Although reversed-phase chromatography is used routinely for separating non-polar, non-ionic compounds, it is also possible and practical to separate ionic compounds on standard reversed-phase stationary-phase materials by using secondary equilibria, such as ion-suppression, ion-pair formation, metal complexation, and micelle formation. To take advantage of these secondary equilibria, additives are introduced into the mobile phase. In the present work, much use was made of ion-suppression and ion-pair formation, therefore only these two will be briefly discussed.

1.1.3. Ion-Suppression

Weak acids and bases, for which ionization can be suppressed, may be separated on RP-columns by a technique known as ion-suppression. In this technique, a buffer of appropriate pH is added to the mobile phase to render the analyte neutral or only partially charged. Suppression of ionization decreases the molecular solvation by the polar solvent of the mobile phase and exposes the now more hydrophobic part of the molecule to the stationary reversed-phase. As a result of the added buffer (with pH separated by at least two units from the pK of the component), the constituents of a sample are eluted as

sharper peaks, due to the presence of only the protonated (or deprotonated) form of the component. [9]

1.1.4. Ion-Pairing

The analysis of strong acids and bases, on the other hand, is typically accomplished by the technique known as ion-pairing. In this case, the pH of the eluent is adjusted in order to encourage ionization of the sample. Retention is then altered by including in the mobile phase a bulky organic molecule having a charge opposite to that of the ion to be analyzed. This counterion is called the ion-pairing agent. Three basic models have been proposed to describe the ion-pair mechanism: [7] the ion-pair model, where the postulated ion-pair is hydrophobic in character and will therefore adsorb onto the stationary phase causing greater retention; the dynamic ion-exchange model, where the dynamic equilibrium between an unpaired ion-pairing agent in the mobile phase and ion-pairing agent adsorbed to the stationary phase causes the column to behave like as an ion exchanger, and sample ions are separated on the basis of conventional ion-exchange mechanisms; and the ion-interaction model, which can be viewed as an intermediate between the two previously mentioned models.

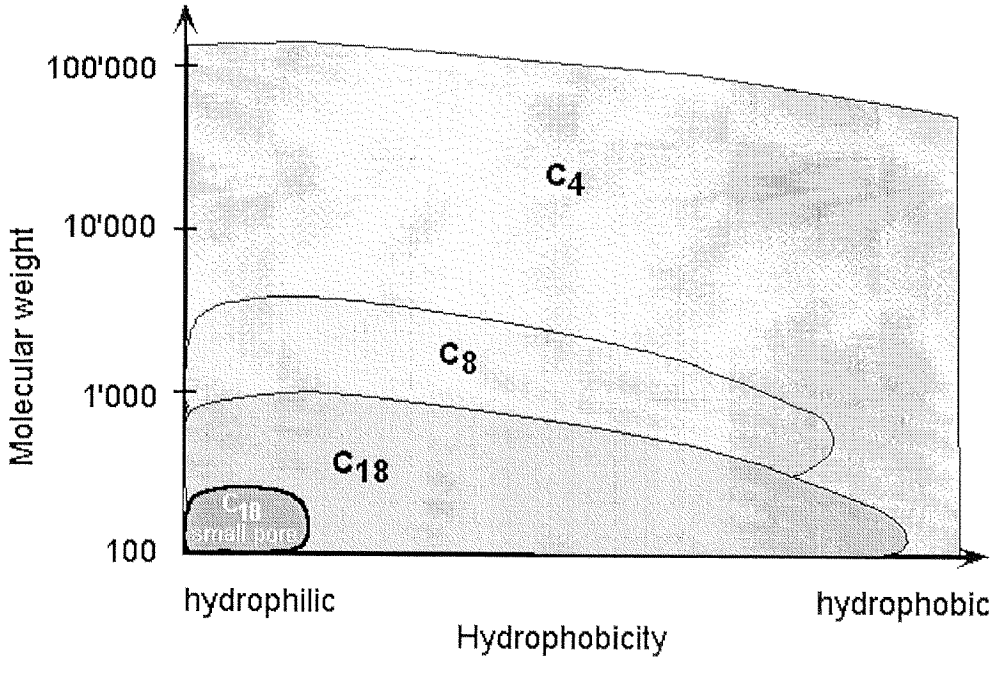


Figure 4: Guide to selection of reversed-phase columns for peptide and protein separation (freely adapted from [10]).

1.2. Peptides in RP-HPLC

Optimal RPC conditions depend on the type of application. Nevertheless, some general guidelines for peptide mapping have been described: [11] 3-5 μm stationary phase particles with C_8 or C_{18}-type bonded phases (see Figure 4) and 125-300 Å pores, a column of 100-250 mm in length with an internal diameter of 1-2 mm, ca. 0.06% trifluoroacetic acid (TFA) in a water-acetonitrile gradient and an ambient temperature up to 50°C. UV detection is best performed at 215 nm. At this wavelength, absorption by the peptide bonds can be detected, and the baseline shift caused by gradually changing the composition of the mobile phase is minimal [12].

TFA (pK ~ 0.3) in the mobile phase acts as an ion-suppressor (protonation of acidic groups in the peptides) as well as an ion-pairing agent (charge-screening of protonated basic groups), and consequently has an impact on peptide retention by the column (see above). The main factor determining the retention time in RPC is the hydrophobicity of a peptide. The more hydrophobic the peptides are (determined by their amino acid composition and the eluting conditions), the stronger the hydrophobic interaction between each peptide and the solid-phase material, leading to longer retention times. A disadvantage of using TFA, however, is its negative effect on the silica support of the solid-phase (hydrolysis of the bonded functional groups). Formic acid (pK ~ 3.8) has been found to be less damaging to the column and can be used instead of TFA [13]. However, the resolution is lower than when TFA is employed.

1.3. HPLC Detectors

In general, an HPLC detector converts a physico-chemical change in the column effluent into an electrical signal that is recorded by the data system (see Figure 1). Detectors are classified as selective (only compounds in the eluent with the physical or chemical property being measured will be detected) or universal (the physical bulk property is measured and changes in the bulk will be detected), depending on the property measured. There are many types of detectors that can be combined with HPLC (see Table 1).

Detectors may also be classified according to whether they are destructive or non-destructive. In a non-destructive detector the sample is unaltered by the detection process. The commonly used optical detectors, such as those that measure ultra-violet absorbance and refractive index, fall into that category. Non-destructive detectors are

often used in series to obtain additional qualitative information. Destructive detectors include electrochemical, mass spectrometric, and chemiluminescence nitrogen detectors, in which at least part of the sample is altered in the detector itself.

Table 1: HPLC detectors and their classification in terms of sensitivity, selectivity, and typical limit of detection (LOD). ++: very good, +: good, -/+: sufficient, -: poor, --: very poor, n.d.: not determinable.

Detectors:	**Sensitivity:**	**Selectivity:**	**LOD:** (ng/injection)
Electrochemical	++	+	10^{-3}-10^{-4}
Fluorescent	++	+	0.01-1
Nitrogen Pyro-Chemiluminescence (CLN)	++	+	> 0.3 (nitrogen)
Radiochemical	++	++	0.1-1
Mass Spectrometer (MS)	+	++	0.1-10
Ultra-Violet-Visible (UV-Vis)	+	-/+	1-10
Refractive Index (RI)	-	-	100
Nuclear Magnetic Resonance (NMR)	--	++	10^{6}-10^{3}
Near Infrared (NIR)	--	+	10^{6}
Light Scattering (LS)	-	--	n.d.

The non-destructive diode array detector and the destructive mass spectrometry detector, which were used in the present work, are discussed in greater detail below.

1.4. Diode Array Detector (DAD)

Absorbance detectors are non-destructive and respond only to substances that absorb radiation at the wavelengths of the light passed through the sample. UV (190-350 nm) or UV-Vis (190-700 nm) detectors are most widely used, not only because of the relative insensitivity of the detector to temperature and gradient changes, but also because of the great number of compounds that absorb radiation in the UV range. The diode array detector measures an entire spectrum of wavelengths simultaneously.

To detect an entire spectrum, a detector must function in one of two ways: (i) it must scan across the entire spectral region (scanning monochromator spectrometer) or, (ii) it must

monitor the entire UV-Vis region simultaneously (linear UV-Vis diode array spectrophotometer), see Figure 5.

An achromatic lens system focuses polychromatic light from the lamp into the flow cell. The light transmitted from the beam disperses on the surface of a holographic grating and is projected onto a linear diode array. The resolution of the detector will depend on the number of diodes in the array, and on the range of wavelengths covered. In the theoretical case there is an infinite number of diodes, each of which covers the absorbance of one wavelength. Thus the diode array detector records a UV-Vis spectrum. It follows that a chromatogram can be reconstructed from the three-dimensional data matrix by choosing a specific wavelength and depicting those substances that absorb light at that wavelength.

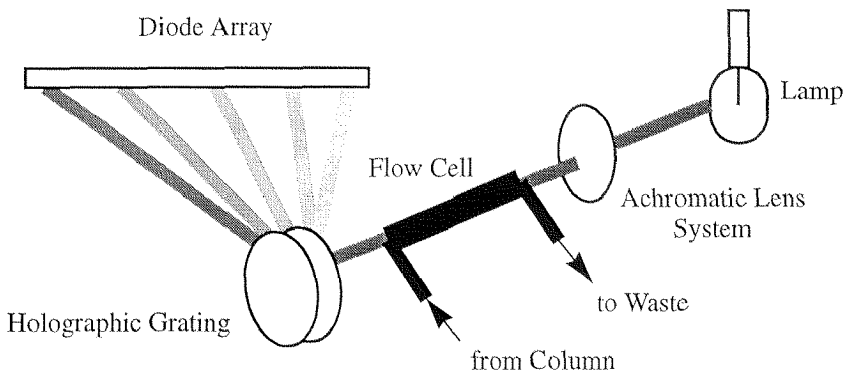


Figure 5: The principle of a typical HPLC UV-Vis photo diode array detector.

The sensitivity of the diode array detector, expressed as the minimum concentration that can be detected, will depend on the extinction coefficient of the solute. A mean value for sensitivity can be taken as about 1.5×10^{-7} g ml^{-1}. [14]

1.5. Peptides and Proteins and Their UV-Vis Spectra

Typical chromophores found in peptides and proteins (if no prosthetic groups are present) absorb light only at wavelengths below 300 nm (see Table 2). Aqueous solvents usually restrict absorption spectral measurements to wavelengths longer than 170 nm. Peptide chromophores can be conveniently divided into two classes: (i) the peptide bond itself and (ii) the amino acid side chains.

Peptide bond UV absorption is typically observed at 210-220 nm (weak absorption) and at 190 nm (strong absorption). A number of amino acid side chains, such as Asp, Glu,

Asn, Gln, Arg and His, have electronic transitions in the spectral region in which strong peptide absorption also occurs. Because their absorption is not as strong as the peptide $\pi \rightarrow \pi^*$ band, it is nearly impossible to detect these in a protein or polypeptide, if they are not present in the range of the number of peptide bonds of the analyte. Thus, the most useful side chain optical properties are those that occur at wavelengths longer than 230 nm, where the peptide bond absorption is reduced to negligible values. Between 230 and 300 nm, in the near UV, one must consider the effects of the aromatic amino acids: Phe, Tyr, and Trp (see Table 2). Variations in pH have little effect on the absorption spectrum of an isolated peptide chromophore. In contrast, major effects are seen with Tyr and Trp, because the sites of protonation directly affect the conjugated electronic system of the chromophore. Most dramatic, is the spectral drift of Tyr when the OH proton is removed (pK_a ~10.9). The effects on Trp absorbance are smaller and have not been exploited to any great extend. [15]

Table 2: Absorbance of isolated chromophores and amino acids. [15]

Chromophoric group or Amino acid:	λ_{max} / **[nm]:**	ε_{max} / **[$M^{-1}cm^{-1}$]:**	**Transition:**
Peptide bond (RCONHR')	210-220	100	$n \rightarrow \pi^*$
	190	7000	$\pi \rightarrow \pi^*$
Phenylalanine	257	200	$\pi \rightarrow \pi^*$
Tyrosine	275	1400	$\pi \rightarrow \pi^*$
Tryptophan	280	5600	$n \rightarrow \pi^*$, $\pi \rightarrow \pi^*$
Histidine	211	5900	$\pi \rightarrow \pi^*$
Cysteine	230	-	-
Disulphides (Cysteine)	250, 270	300	-

A typical protein with no prosthetic groups has a λ_{max} in the near UV range, at about 280 nm. At this wavelength, Trp and Tyr have extinction coefficients of about 5'600 and 1'300 $M^{-1}cm^{-1}$, respectively. As a rough approximation, one can assume that the extinction coefficients do not change drastically when these amino acids are incorporated into a protein. Therefore, since no other amino acid makes an appreciable contribution to the absorbance at 280 nm, the extinction coefficient can be estimated as the sum of the

extinction coefficients of its absorbing amino acids at that wavelength. [15] The same holds true for a peptide containing Trp or Tyr. The UV-Vis spectra of peptides are therefore characteristic of their component amino acids and especially of the aromatic amino acids. The spectra of the aromatic amino acids are rather broad and partially overlapping. Therefore, if more than one type of them is present in the analyzed peptide, it is almost impossible to identify one of them from the zero order spectrum of the peptide. These problems can be overcome when the derivatives are taken; this increases the resolution between spectral differences. The second order derivative, in particular, which transforms peaks and shoulders into minima, is widely used in derivative spectroscopy under non-chromatographic conditions. [16]

1.6. Mass Spectrometer Detector (MSD)

A mass spectrometer can be divided into three fundamental parts, namely the ionization source, the analyzer, and the detector. In coupled LC-MS, the interface between LC and the MSD is fused with the ionization source to the ionization chamber.

Mass spectrometers are used primarily to provide information about the molecular weight of a compound, and in order to achieve this the sample under investigation has to be introduced into the ionization chamber of the MSD. In this chamber, the sample molecules are ionized and these ions are extracted into the analyzer region of the mass spectrometer where they are separated according to their mass (m) to charge (z) ratios (m/z). The separate ions are detected and the signal fed to a data system.

Many ionization methods are available and each has its own advantages and disadvantages (see Table 3). But only a few are compatible with LC and will be discussed in the following paragraph.

1.6.1. Atmospheric Pressure Ionization (API) LC-MS Interfaces

In general, samples were originally introduced as solids or gases into the mass spectrometer. The direct coupling of samples in solution could, however, not be achieved for a long time due of vacuum problems. The great breakthrough was made by the concept of vaporizing and ionizing the liquid samples under atmospheric pressure.

Table 3: Ionization methods and their compatibility with different types of mass spectrometers, according to [19]. [a] M: molecular weight; [b] M: magnet, Q: quadrupole, TOF: time-of-flight.

Ionization method:	**Principle ions detected (+/-):**[a]	**Mass spectrometer:**[b]	**Sample classes (approx. MW limit):**
EI (electron impact)	$M^{+\cdot}$ and some fragment ions	M, Q	non-polar and some polar organic compounds (< ca. 1'000 Da)
CI (chemical ionization)	MH^+ M^-, $(M - H)^-$, $M^{-\cdot}$	M, Q	non-polar and some polar organic compounds (< ca. 1'000 Da)
ESI (electrospray ionization)	MH^+, $(M + nH)^{n+}$ $(M - H)^-$, $(M - nH)^{n-}$	M, Q, TOF	polar organics, proteins, biopolymers, organometallics (< ca. 200'000 Da)
APCI (atmospheric pressure chemical ionization)	MH^+ $(M - H)^-$	M, Q	polar and some non-polar organic compounds (< ca. 1'000 Da)
FAB/FIB/LSIMS (fast atom/ion bombardment/liquid secondary ion mass spectrometry)	MH^+ $(M - H)^-$	M, Q	polar organics, proteins, organometallics (< ca. 10'000 Da, but depends on *m/z* range of MS)
FD/FI (field desorption/ ionization)	MH^+ $(M - H)^-$	M, (Q)	non-polar and some polar organics, incl. synthetic polymers (< ca. 10'000 Da, but depends on *m/z* range of MS)
TSP (thermospray)	MH^+, $M{\cdot}NH_4^+$ $(M - H)^-$	M, Q	polar and some non-polar organic compounds (< ca. 1'000 Da)
MALDI (matrix assisted laser desorption ionization)	MH^+ $(M - H)^-$	TOF	polar and some non-polar biopolymers, synthetic polymers (ca. 200'000 Da and higher)

The development and commercialization of atmospheric pressure ionization (API) coupled with mass spectrometry brought the combination of liquid chromatography with mass spectrometry into the realm of routine analytical practice. The combination of LC with the API techniques of electrospray ionization (ESI), [6] pneumatically assisted ESI, [17] or atmospheric pressure chemical ionization (APCI) [18] has been extremely successful. ESI is a desorption process, which uses electrical fields to generate charged droplets and subsequent analyte ions by ion-evaporation ionization (see also below); pneumatically assisted ESI is the same as ESI except for the initial droplet formation, which is the result of pneumatic nebulization; and APCI is a gas-phase ionization process initiated by a discharge sustained by the LC mobile phase vapor. The advantages of these systems include: (i) API approaches can handle volumes of liquid typically used in LC, (ii) API is suitable for the analysis of nonvolatile, polar, and thermally unstable compounds typically analyzed by LC, (iii) API-MS systems are sensitive, offering comparable or better detection limits than achieved by gas chromatography/MS, and (iv) API systems are rugged and relatively easy to use.

LC-MS is still limited to conditions that are suitable for MS measurements. There are restrictions on pH, solvent choice, solvent additives, and flow rates for LC in order to achieve optimal API-MS sensitivity (see Table 4). Furthermore, these restrictions are dependent on the API operation mode (ESI, pneumatically assisted ESI, or APCI).

In general, most LC solvents are compatible with API. However, solvents suitable for ES permit the formation of ions in solution. Also, ease in nebulization and desolvation play a role in solvent suitability. For example, water easily supports the formation of ions in solution, but its surface tension and solvation energy make ion desorption more difficult than a solvent like isopropanol or acetonitrile. API techniques also require the use of volatile solvent additives, such as those listed in Table 4, [20] to prevent API chamber contamination or plugging of the sampling orifice. Phosphate, sulfate, or borate additives typically used in LC are not suitable for API-MS. Electrospray operation further requires that the solvent additives do not form strong ion pairs which could result in neutralization of ions after desorption. Solvent additives are also important in controlling pH. This is particularly important in ES operations because the protonation or deprotonation of the analyte in solution greatly enhances ion formation. In general, for compounds with basic sites (e.g. amines) the analysis should be performed at low pH using positive-ion detection. Components containing acidic sites (e.g. carboxylic acids) are analyzed at

high pH using negative-ion detection. Neutral species can often be more effectively ionized with ES through cationization by the addition of micromolar levels of sodium or potassium acetate.

The following paragraph will discuss electrospray ionization in more detail, since this was the method of choice in the present work.

Table 4: Suitable API-MS Solvents and Additives [21]

Buffers for pH setting: (typically in the range 0.1-1% (v/v))	for ESI positive-ion detection	Acetic acid, formic acid, trifluoro-acetic acid (TFA)
	for ESI negative-ion detection	Ammonium hydroxide
Ion-pair reagents: (10-100 mM)		Ammonium acetate, ammonium formate, triethylamine heptafluo-robutyric acid (HFBA), tetraethyl or tetrabutylammonium hydroxide (TEAH or TBAH)
Cationization reagents: (20-50 µM)		Potassium or sodium acetate
Solvents:	for API-MS	Methanol, ethanol, propanol, iso-propanol, butanol, acetonitrile, water, acetic acid, formic acid, acetone, dimethylformamide, dimethyl sulfoxide, 2-methoxy ethanol, tetrahydrofuran, chloro-form
	for APCI only	Hydrocarbon solvents (e.g. hex-ane, cyclohexane, toluene), car-bon disulphide, tetrachloromethane

1.6.1.1. Electrospray Ionization (ESI)

Electrospray was proposed as a source of gas-phase ions and their analysis by mass spectrometry in the early 70's by Dole et al. [22], [23] However Dole's experiments were too narrowly focused. They aimed at the detection of polymeric species such as polystyrenes, which are not themselves ionized in solution, and the experimental results obtained were not convincing. The systematic study of the influence of the different parameters on the ionization of small molecules by electrospray and analysis with a

small commercially available quadrupole mass spectrometer by Fenn et al. [24], [25] led to the introduction of electrospray ionization. A very similar, independent development was reported at approximately the same time by Aleksandrov et al. [26]. In the early work by Fenn et al., positively [24] as well as negatively [25] charged ions were detected. At the same time, sensitivity was drastically increased by direct coupling to LC. [6]

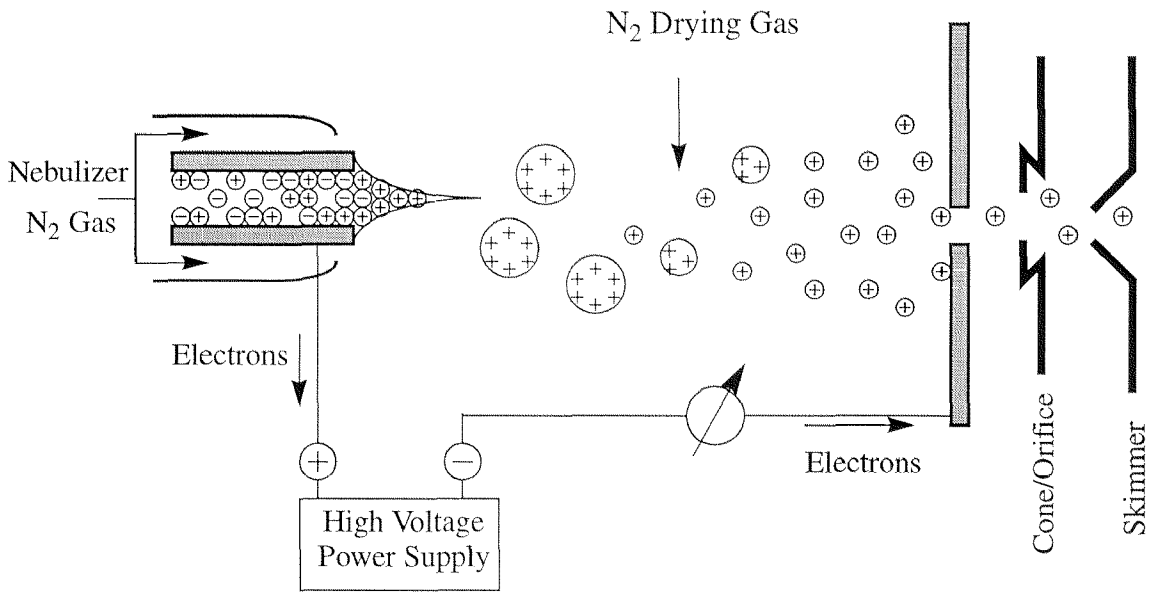


Figure 6: Schematic picture of major processes occurring in electrospray for the case of positive-ion detection (freely adapted from [27]). Penetration of the imposed electric field into the liquid at the capillary leads to the formation of an electric double layer liquid. Enrichment at the surface of the liquid by positive ions leads to the destabilization of the meniscus and to the formation of a cone and jet emitting droplets with an excess of positive ions. Charged droplets shrink by evaporation, split into smaller droplets and finally emit gas-phase ions. In the case of reverse voltages, negative ions will pass to the analyzer.

In ESI (see Figure 6), the sample in solution is introduced into the ionization source through a stainless steel capillary. A high voltage is applied to the tip of the capillary. As a consequence of this strong electric field, the sample solution emerging from the capillary is dispersed into an aerosol of highly charged droplets. This process is called electrospraying, and is supported by a co-axial nebulizing gas also flowing down the probe, around the capillary. This gas, usually nitrogen, helps to direct the spray emerging from the capillary tip. The charged droplets diminish in size by evaporation, assisted by a flow of warm nitrogen gas known as the drying gas, which passes across the front of the source. Eventually, charged sample ions, free from solvent, are released from the droplets.

Some of these ions pass through a sampling cone or orifice into an intermediate region under vacuum and then through a small hole, via the skimmer into the analyzer of the mass spectrometer, where their *m/z* ratios are measured. The analyzer is under vacuum. The skimmer acts as a momentum separator, the heavier sample ions pass through, while the lighter solvent and gas molecules are pumped away in the intermediate vacuum stage. [19]

One characteristic of ESI is the formation of multiply charged ions for analytes with molecular masses higher than 2'000 Da. Analytes with molecular masses below 1'000 Da are normally singly charged. To account for this observation, two mechanisms for the formation of gas-phase ions from very small and highly charged droplets have been proposed. The charged-residue model (CRM) was introduced by Dole et al. [22] and a newer model known as the ion evaporation model (IEM) was proposed by Iribarne and Thomson. [28]-[30] CRM assumes that in a series of coulomb-explosions - as a consequence of the high surface charge of about $5*10^4$ elementary charges per droplet [31] - the small highly charged droplets are transformed into very small charged droplets containing one single analyte molecule. The gas-phase ions are then formed out of these droplets, due to desolvation processes. On the other hand, in IEM, ion emission occurs directly from the small and highly charged droplets containing several analyte molecules.

1.6.2. Mass Analyzer

The ions produced in the ionization chamber pass into the analyzer region, and a mass spectrometer is generally classified by the type of analyzer it accommodates. There are a variety of analyzers. The ones most frequently encountered in organic and bioorganic mass spectrometry are the magnetic sector (where ion separation is achieved by magnetic deflection), the quadrupole (see below), and the time-of-flight analyzer (where separation of the ions depends on the travel-time after acceleration in an electrostatic field). The detector can be one of several possibilities, including photomultipliers, electron multipliers, microchannel plates, and diode array detectors.

The main function of the mass analyzer is to separate, or resolve, the ions formed in the ionization source by their mass to charge ratios (*m/z*). The resolution (*R*) of a mass analyzer, or its ability to separate two peaks, is defined as the ratio of the mass of a peak

(M_1) to the difference in mass between this peak and the adjacent peak of higher mass (M_2). [32]

$$R = \frac{M_1}{M_2 - M_1}$$

In the simplest terms, a single charged ion at *m/z* 1'000 could be separated from another single charged ion at *m/z* 1'001 if a resolution of 1'000 is available.

There are different methods of acquiring data when using a mass spectrometer. The most usual method is by scanning the mass analyzer over an appropriate *m/z* range, thus producing a mass spectrum of the molecular or quasimolecular (molecular related) ions, which will provide an indication of the molecular weight of the sample. If the sample has fragmented in the ionization source, then these fragment ions will also end up in the analyzer. The type of fragment ions produced can provide useful information for structure elucidation. Almost all samples are analyzed with a full scanning experiment initially to obtain as much information as possible. Subsequently, sequential fragmentation experiments, selected ion monitoring (SIM) or selected ion recording (SIR) analysis, can provide additional information about sample composition. The ions scanned for, can either be molecular ions or fragments. With the help of SIM and SIR, it is possible to resolve compounds of different masses but similar LC elution times. On the other hand the MSD might be used in the total ion current (TIC) mode, where eluting compounds with no detectable absorption in the UV-Vis region can be detected.

1.6.2.1. Quadrupole Analyzer

Mass spectrometers with quadrupole analyzers have the reputation of being easier to use than classic magnetic sector mass spectrometers, and are popular instruments for a diverse range of applications (see Table 3). Quadrupole mass spectrometers are ideal for coupling with both liquid and gas chromatography. Electrospray ionization (see above) which generates multiply charged ions, which are detected at much lower *m/z* values than the molecular weight of large biomolecules, has been largely responsible for removing the mass range limitations which traditionally restricted analysis to only singly charged compounds. Quadrupole instruments are limited in their resolution, but their strong points are high sensitivity, ease of use, reliability, and ability to cope with large volumes of solvent (LC coupling) flowing into the ionization source for extended periods of time.

The quadrupole analyzer, as the name suggests, consists of four poles. These poles are parallel, cylindrical rods which are arranged symmetrically, as shown in Figure 7. The voltage connections to the rods are such that opposite rods have the same polarity while adjacent rods have opposite polarity. The voltage applied has two components: a direct current (DC) component (U) and a radio-frequency (rf) component ($V_0[\cos(\omega t)]$, where ω is the frequency of the rf voltage). The ions produced in the ionization source exit with only a small accelerating voltage (hence a relative low energy) pass through the quadrupole assembly. On entering the electric field the ions oscillate and, at a certain radio-frequency, ions of a certain *m/z* will be in a state of stable oscillation which enables them to proceed straight through the quadrupole assembly, reach the detector and trigger a signal. Under these conditions, all other ions, with *m/z* values different from the resonant one, will not undergo stable oscillation. They will be lost on the rods of the quadrupole. In this way mass separation is achieved.

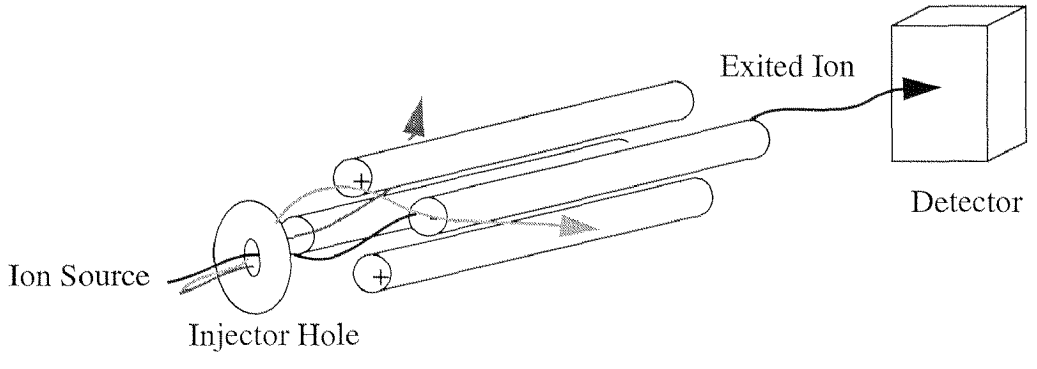


Figure 7: Arrangement of a quadrupole analyzer: the applied voltages ($U+V_0\cos(\omega t)$) affect the trajectory of the ions traveling down the flight path centered between the four rods. [33]

In order to produce a mass spectrum, U and V_0 must be varied, while the ratio U/V_0 is kept essentially constant. The mass of single charged ions (the *m/z*-ratio, respectively) being analyzed at any time is proportional to V_0, and so a linear increase in V_0 produces a linear increase in mass (*m/z*). This linear mass scale is relatively simple to calibrate by analyzing known standards, and calibration tends to remain reliable for long periods of time if the same set of operating parameters is maintained. The DC voltage is varied to effect a change in the resolution, and the higher the resolution, the fewer the number of ions detected and hence the lower the sensitivity. Resolution *vs.* sensitivity is a common compromise in mass spectrometry.

If the DC voltage is switched off, the quadrupole is described as operating in the rf mode only, and all ions will perform stable oscillations if the rf voltage is sufficiently low. In cases such as this, the quadrupole is not being used as a mass analyzer, but as a high transmission lens. As such, it has often been employed as a collision cell situated between two different mass analyzers (i.e. two quadrupole analyzers), filled with a collision gas such as argon, in tandem mass spectrometers (MS/MS) (i.e. triple quadrupole mass spectrometer).

1.7. Biomolecules in Mass Spectrometry

Determination of molecular weight is one of the first measurements used to characterize biopolymers. Up to the end of the 1970's, the common techniques that provided this information were electrophoretic, chromatographic, viscosimetric, osmotic pressure or ultracentrifugation methods. However, these were not very accurate (10-100% relative error on average) since they also depended on other characteristics than mass. Thus, the only possibility of knowing the exact molecular weight of a macromolecule remained a calculation based on its chemical structure. [34]

At that time, mass spectrometric ionization techniques EI [35] and CI [36] required the analyte molecules to be present in the gas phase and thus were suitable only for volatile compounds or for samples subjected to derivatization to make them volatile. Moreover, the FD ionization method, [37] which allows the ionization of non-volatile molecules with masses up to 5'000 da was a delicate technique that required an experienced operator. [38] This limited the field of application of mass spectrometry to large non-volatile, and often thermolabile biological molecules.

The development of desorption ionization methods based on the ejection of pre-existing ions from a liquid or solid surface (plasma desorption (PD) [39], [40], FAB [41], laser desorption (LD) [42]) was a breakthrough for mass spectrometry in the field of biomolecules. Since their implementations, the problem was no longer the production of ions, but rather that of analyzing high-mass singly charged species, which are technically difficult to detect with good sensitivity and difficult to analyze with good resolution.

At the beginning of the 1990s, two new ionization methods, ESI [43] and MALDI [44], that avoided such inconveniences were developed and continue to revolutionize the role of mass spectrometry in biological and biochemical research. These methods allow the high-precision analysis of biomolecules of very high molecular weight.

1.7.1. Peptides and Proteins

The ionization methods that are most often used to study proteins and peptides through mass spectrometry are FAB, ESI (see above) and MALDI. In FAB, the sample dissolved in a non-volatile liquid matrix gets hit and ejected into the gas phase by a beam of neutral atoms or molecules. In MALDI, the sample is enclosed in a solid matrix of small organic molecules, that exhibit strong absorption at the laser wavelength being used. The laser induced excitation of the matrix molecules desorbs the sample. The performance of these methods is listed in Table 5. All of these techniques are characterized by the formation of stable gas-phase ions - as they have only low excess energy and pre-exist in solution - and by the absence of fragments.

Table 5: Characteristics of the various ionization methods used in the mass spectrometric analysis of peptides and proteins. [34]

Method:	**Detection limit / [pmol]:**	**Approximate MW limit / [Da]:**	**Precision: (in %)**	**Analyzer:**
FAB-MS	1-50	6'000	0.05	Magnetic or quadrupole
ESI-MS	0.01-5	>130'000	0.01	Magnetic or quadrupole
MALDI-MS	0.001-1	>300'000	0.05	Time-of-flight

As the resolution needed to separate different peaks in the isotopic cluster of a small peptide is lower than the resolution of most analyzers, the molecular weight that is measured (monoisotopic mass) corresponds to that calculated using the predominant isotope of each element. This is not so in the case of larger peptides and proteins. As the resolution required to resolve the isotopic cluster increases with the mass and charge of the ion and the resolution of the analyzer is limited, the various peaks in the isotopic cluster of proteins can combine and form a single peak that spreads over several masses. Thus, in such a case, the molecular weight determined (average mass) corresponds to that calculated using the chemical mass (avarage mass of an atom relative to the isotope abundances) of each element present in the protein.

The characteristic absence of fragment ions in the three mentioned ionization techniques, FAB, ESI, and MALDI, allows the analysis of complex mixtures without any previous separation. However, this is limited by the resolution of the analyzer being used if

peptides or proteins with similar molecular masses are present. Another factor that plays a role in the analysis of this type of mixture is the relative quantity of each component. If different peptides are present in very different quantities, a separation prior to the MS is required because of sensitivity problems. The currently most popular HPLC-MS interface technique for on-line separation and analysis of peptides and proteins is ESI because of its robustness and relative ease of operation. The high precision determination of a peptide or a protein molecular weight allows protein identification, detection of mutations within proteins, post-translational modifications, structure confirmation and the eventual correction of protein sequences derived from DNA sequences.

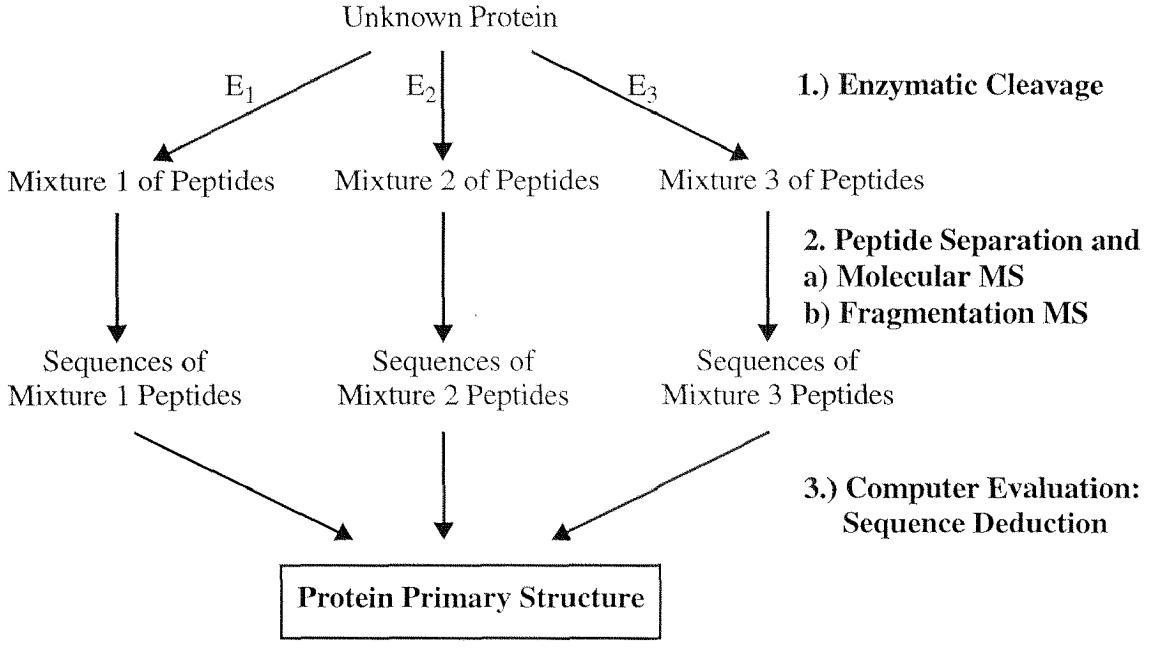


Figure 8: Strategy followed in protein identification and sequencing using mass spectrometry.

Mass spectrometry also allows the determination of peptide and protein sequences, especially when tandem (or MS^n) techniques are used. [45]-[51] Protein (peptide) identification by mass spectrometry consists of three (two) steps, as shown in Figure 8. In the first step, the protein is submitted to a specific proteolytic cleavage by an enzyme (i.e. trypsin, protease V8, etc.) or a reactant such as CNBr. In the second step, the resulting mixture of peptides is separated by HPLC and analyzed by mass spectrometry to determine their molecular mass. By using collision-induced dissociation (CID) or in-source fragmentation (ISF) it is possible to fragment the short peptides either within two mass analyzers or, in ISF, inside the ionization source. The resulting fragment ions give

information about the sequence of each peptide. In the final step, the mass spectra of the different protein digest mixtures and the fragmentation spectra of their peptides are analyzed with the help of computer software, which generates the peptide sequences (often with the help of databases). Knowledge of the cleavage behavior of the digestion enzymes used and by overlapping the peptides sequenced, the most probable primary structure of the initial protein can be deduced. [52]-[54]

1.7.2. Peptides and Proteins in ESI-MS

Large biomolecules examined by ESI-MS typically show a distribution of multiply charged species and no evidence of fragmentation, unless dissociation is induced during transport into the mass spectrometer by higher-energy collisions. The ESI mass spectra of proteins typically show a distinctive bell-shaped distribution of charge states in which adjacent peaks differ by one charge. A feature of ESI mass spectra for most proteins is that the average charge state increases in an approximately linear fashion with molecular weight. A good approximation is that for each 1000 Da mass there is about one additional charge present. [52]

It has been suggested that protonation occurs at basic residues (Arg, Lys, NH_2-terminus, His) for positive ions and deprotonation at acidic residues (Asp, Glu, Tyr, COOH-terminus) for negative ions. [55]-[57] Generally, the highest charged states observed for proteins are consistent with this explanation, but a few exceptions exist. Despite the attractiveness of explaining charge state by the presence of acidic or basic residues that are protonated or deprotonated in solution, pK_a data does not explain the production of negative ions from very acidic peptide and protein solutions at pH levels significantly lower than the pK_a values of the acidic residues. [58]-[60] LeBlanc at al. have suggested that gas-phase contributions can explain these results, since the protonation and deprotonation equilibria in the gas phase differ from those in solution. [61], [62]

To determine molecular mass from a bell-shaped mass spectrum of multiply charged molecules, the spectrum has to be deconvoluted. In other words, in each isotope-pattern, the monoisotopic mass peak and the following ^{13}C-containing isotope peak are separated by $1/z$ Da. Therefore, separation of the monoisotopic peak and its corresponding first isotope peak provides information on the charged state z of the molecule with the mass m, if the resolution of the analyzer is high enough. Assuming, the charges come from

protonation, then the molecular mass is the *m/z*-value multiplied by the number of charges *z* minus the mass of the *z* protons.

A simple, effective method to generate fragment ions is to increase the energy of the ions as they traverse the ESI atmospheric pressure/vacuum interface. This technique, originally described by Loo, Udseth, and Smith [63] and referred to by several names (e.g. in-source fragmentation, in-source collision activated dissociation (CAD), nozzle-skimmer dissociation), can be used to provide sequence information for peptides and proteins.

1.7.3. Fragmentation of Peptides

In order to generate structural data by mass spectrometry, the molecule that is studied must undergo fragmentation at one or several bonds so that the *m/z* values of the resulting fragments can be correlated with the assumed chemical structure. There are several methods for peptide fragmentation (e.g. ISF, CID). By the fragmentation of many known peptides and analysis by MS, various standard protocols have been developed for peptides. From a practical point of view, the fragments may be classified in either of two categories: (i) those derived from the cleavage of one or two bonds in the peptidic chain and (ii) those that also undergo a cleavage of the amino acid side chain. The nomenclature suggested by Roepstroff and Fohlman [64], [65] and later modified by Biemann [66] allows the labelling of the various fragments that are obtained and is discussed in the following.

The fragmentation processes undergone by protonated peptides are summarized in Figure 10 and the following: The cleavage of a bond in a peptide chain can occur in either of three types of bonds: Cα-C, C-N or N-Cα. These fragmentations yield six types of fragments that are labeled a_n, b_n, c_n when a positive charge is retained by the *N*-terminus and x_n, y_n, z_n respectively, when the positive charge is retained by the *C*-terminus. c_n and y_n are sometimes superscripted with two primes (c_n'', y_n'') to indicate the transfer of two extra hydrogen atoms (the hydrogen responsible for protonation and a second one derived from the other side of the peptide). The subscript *n* indicates the number of amino acids contained in the fragment. The mass difference between consecutive ions within a series allows one to determine the identity of the consecutive amino acids (see Table 6) and thus deduce the peptide sequence (with two exceptions: Leu/Ile, which are isomers, and Gln/Lys, which are isobars (same mass)). Normally, the

spectra show several incomplete series of ions that produce redundant data and make the spectrum very complex and difficult to interpret.

In addition to the above mentioned six types of fragmentation (see Figure 10), two other types of fragments found in most spectra result from the cleavage of at least two internal bonds in the peptidic chain. The first type is called an internal fragment as these fragments have lost the internal *N*- and *C*-terminal sides of the peptide fragment. [67] They are represented by a series of simple letters corresponding to the fragment sequence (e.g. $(a_n\text{-}z_m)_k$, where the internal fragment consisting of k amino acids is formed due to fragmentation at the bonds as if to form fragments a_n and z_m, see also Figure 10). Fortunately, these types of ions are often weakly abundant and as they rarely contain more than three or four amino acid residues, they appear among the low masses in the spectrum. These peaks confirm the sequence but are often more a nuisance than a help. Peptides containing proline are an exception to this as the proline imino group is included in a five-atom ring and thus has a higher proton affinity than the other amide bonds in the peptide. Hence the protonation and the cleavage of the proline amide bond are a favored process in yielding an internal fragment.

The second type of fragment that results from multiple cleavages of the peptidic chain appears among the low masses in the spectrum. These are the immonium ions I_n (see Figure 9) of the amino acids, where n is the position of the original amino acid in the peptide. Even though these fragments are rarely observed for all the peptide amino acids, those that appear yield information concerning the amino acid composition of the sample. A list of immonium ions commonly found in spectra is given in Table 6. [69], [70]

All the types of fragmentations discussed so far occur at both low and high energy fragmentation, even though the fragments obtained at low energy often lose small molecules such as H_2O or NH_3.

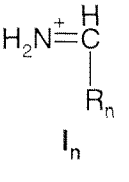
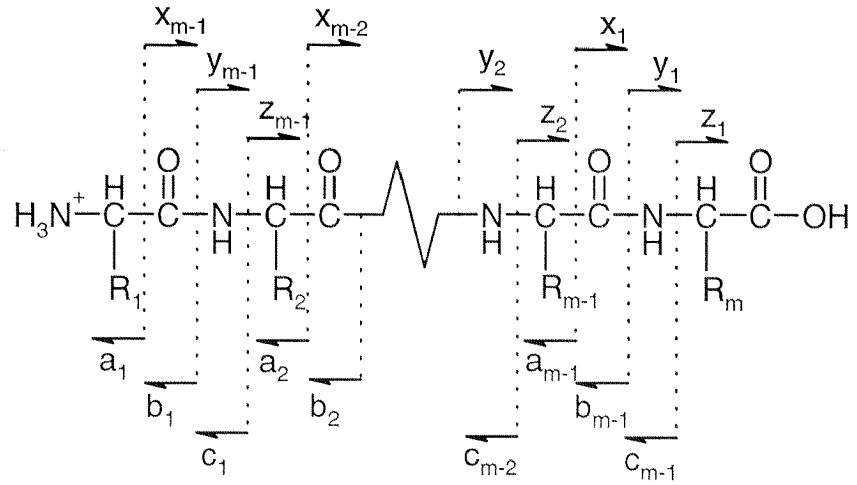
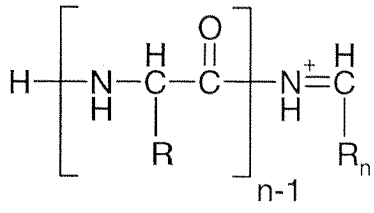


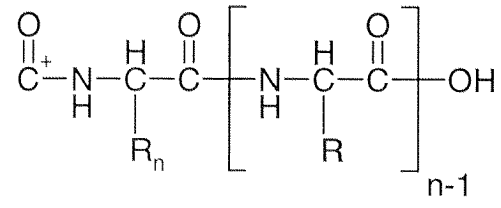
Figure 9: Immonium ion



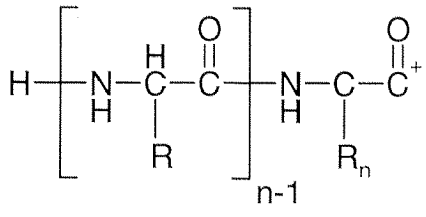
$[M+H]^+$



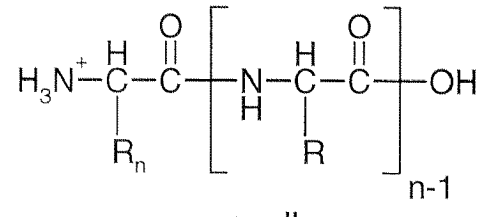
$\mathbf{a_n}$



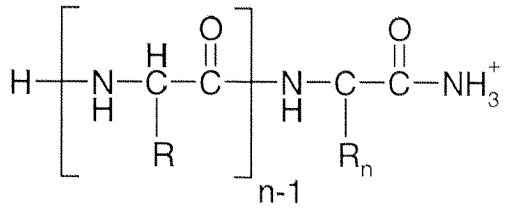
$\mathbf{x_n}$



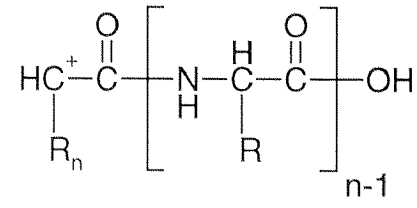
$\mathbf{b_n}$



$\mathbf{y_n}$ or $\mathbf{y''_n}$



$\mathbf{c_n}$ or $\mathbf{c''_n}$



$\mathbf{z_n}$

Figure 10: Fragmentation ions from protonated peptides. R_i resembles the substituent on the α-carbon atom (C_i^{α}) of the peptide.

Table 6: Residue masses and immonium ions of the amino acids.

Amino acid:	**Three letter code:**	**One letter code:**	**Residue mass:**[a]	**Immonium ion:**[b]
Alanine	Ala	A	71	44
Arginine	Arg	R	156	129
Asparagine	Asn	N	114	87
Aspartic acid	Asp	D	115	88
Cysteine	Cys	C	103	76
Glutamic acid	Glu	E	129	102
Glutamine	Gln	Q	128	101
Glycine	Gly	G	57	30
Histidine	His	H	137	110
Isoleucine	Ile	I	113	86
Leucine	Leu	L	113	86
Lysine	Lys	K	128	101[c]
Methionine	Met	M	131	104
Phenylalanine	Phe	F	147	120
Proline	Pro	P	97	70
Serine	Ser	S	87	60
Threonine	Thr	T	101	74
Tryptophan	Trp	W	186	159
Tyrosine	Tyr	Y	163	136
Valine	Val	V	99	72

[a] The amino acid residue mass is defined as the amino acid molecular mass minus the mass of water.

[b] The structure of an immonium ion is given in Figure 9.

[c] Cyclization of the immonium ion results in loss of ammonia, which yields an abundant *m/z* 84. [68]

In addition to the ions described, three new types of fragments that require the cleavage of the peptidic chain and of the amino acid lateral chain are observed only in high-energy spectra. These fragments are useful for distinguishing the isomers Leu and Ile. Figure 11 shows the mechanisms and the structures of the corresponding fragments d_n, v_n, and w_n. [71]-[73]

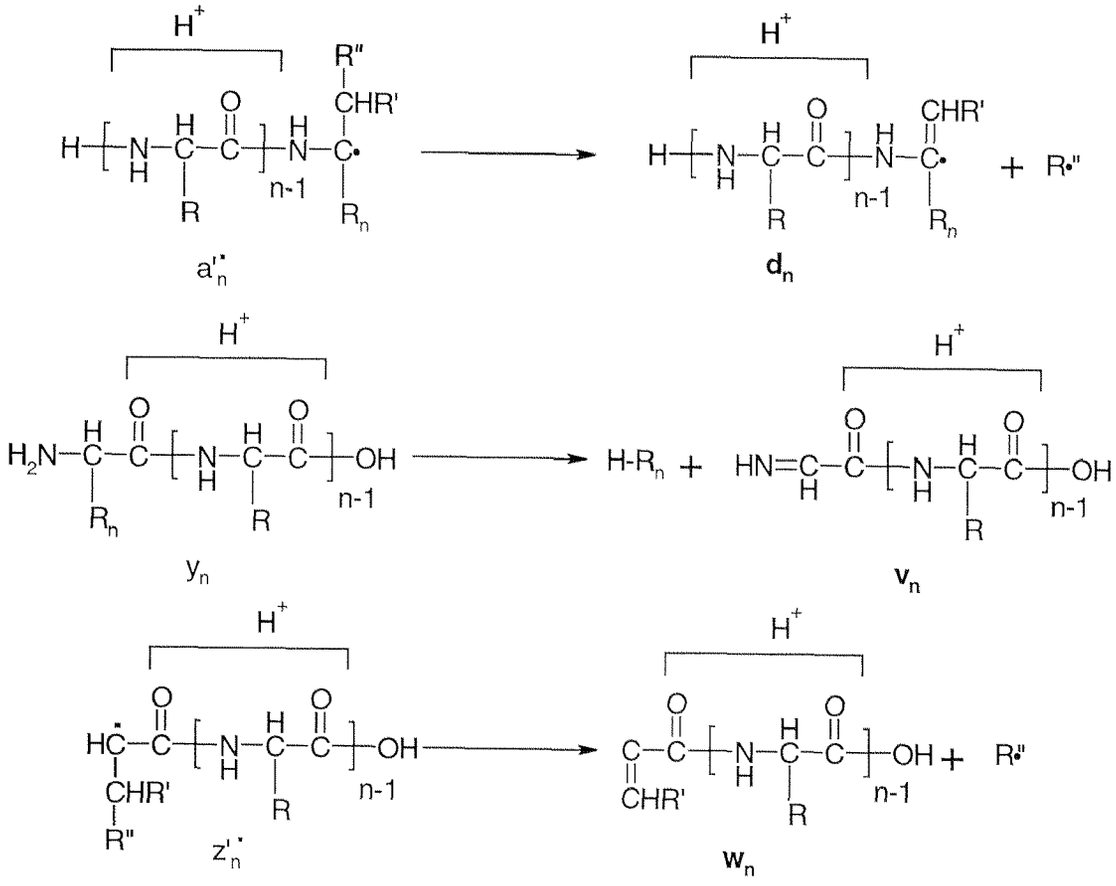


Figure 11: Fragmentation paths yielding the ions characteristic of amino acid lateral chains. Where the prime (') indicates an additional proton.

2. Origin of Life

The question of the origin of life is as old as humanity. Humans of every civilization have always been intrigued by their origins and the origin of living things in general. The hypotheses proposed to answer this question can be subdivided in three classes [74]: (i) A divine act by a creator that lies beyond the laws of science, (ii) a chance event that lies within the laws of physics and chemistry but is in no way predictable, and (iii) a deterministic event, where life is the result of the operation of natural laws on a certain physico-chemical system. This system (iii) evolves with time, is governed by physical principles, and eventually gives rise to living forms. The details need not be totally deterministic in every aspect, but the overall behavior follows a predictable path.

The first two approaches lie outside the realm of experimental science. Therefore, to undertake a scientific study on the origin of life, the third deterministic view is a prerequisite.

The question of where life began has two possible answers, either on Earth or somewhere else in the universe (panspermia hypothesis [75]). Depending on the answer, this question only sets restrictions on the conditions under which life was formed.

Rather more challenging is the question of "What is life?". Linus Pauling once wrote on this issue: "In connection with the origin of life, I should like to say that it is sometimes easier to study a subject than to define it." [76] These words explain nicely why the discussion of a broadly accepted definition of life still goes on, and probably will do so for some time.

The main definitions proposed are summarized and discussed in various articles. [77]-[81] A complete classification of these definitions is, however, beyond the framework of the present work. Some rather broadly accepted, popular definitions - which include life-as-it-is-now (cellular life), life-as-it-initially-was (protocellular life) and life-as-it-could-be (artificial or extraterestal life) - are given in Table 7.

Research on the origin of life is based on the elementary idea of chemical and molecular evolution[1] (see below). [85]-[88] According to this approach, life originated from inanimate matter via a spontaneous increase in molecular complexity and specificity.

[1] For some authors the term chemical evolution is used for the accumulation subprocess of molecular evolution, whereas others attribute chemical evolution to the accumulation period and molecular evolution to the pre-organization period. In the present work the first terminology will be used.

This process is believed to have been gradual and marked by successive stages of development. [78], [89]-[94]

Table 7: Different broadly accepted definitions of life, from the Exobiology project's (NASA), the molecular geneticist's, and the compartmentalist's view.

View:	**Definition:**
NASA definition:	*Life is a self-sustained chemical system capable of undergoing Darwinian evolution.* [82], [83]
Molecular geneticist's view:	*A system which is self-sustaining by utilizing external energy/nutrients owing to its internal process of component production and coupled to the medium via adaptive changes which persist during the time history of the system.* [81]
Compartmentalist's view:	*A system which is spatially defined by a semipermeable compartment of its own making and which is self-sustaining by transforming external energy/nutrients by its own process of components production.* [81]
'Is impossible' view:	*A complete definition, one that unambiguously separates non-life from life, would only be possible if the transition from the first to the second were discontinuous.* [79], [84]

2.1. How Long Did It Take?

The Earth is slightly more than 4,5 billion years old. For about the first half billion years, impacts occurred with objects large enough to evaporate the oceans and sterilize the surface. [95]-[98] Well-preserved microfossils of organisms date back about 3.5 billion years. [99]-[101] Other indirect evidence suggests that life might have already been present 3.8 billion years ago. [102] Life, therefore, must have originated on Earth[2] within a window of a few hundred million years that opened about 4 billion years ago.

The transition from the immediate post-impact world to a world including a 'minimal cell', can be divided into three phases [103]: (i) An accumulation period during which the supply of organic substrates accumulated (also through meteorites), (ii) a pre-organization period during which a hypothetical first replicating or reproducing system might have appeared, and (iii) a maturation period during which this first self-replicating

[2] The majority of workers in the origin of life field reject the hypothesis that life was transported to the Earth from somewhere else in the galaxy and take it for granted that life began *de novo* on early Earth. (Ockam's razor: Entities are not to be multiplied beyond necessity)

or self-reproducing[3] system gave rise to the 'minimal cell' by passing through the intermediates of the RNA-world and the appearance of enzymes (see Figure 12). Because the transition to life is a continuum, the position of the 'first form of life' is arbitrary according to the preferred definition of life.

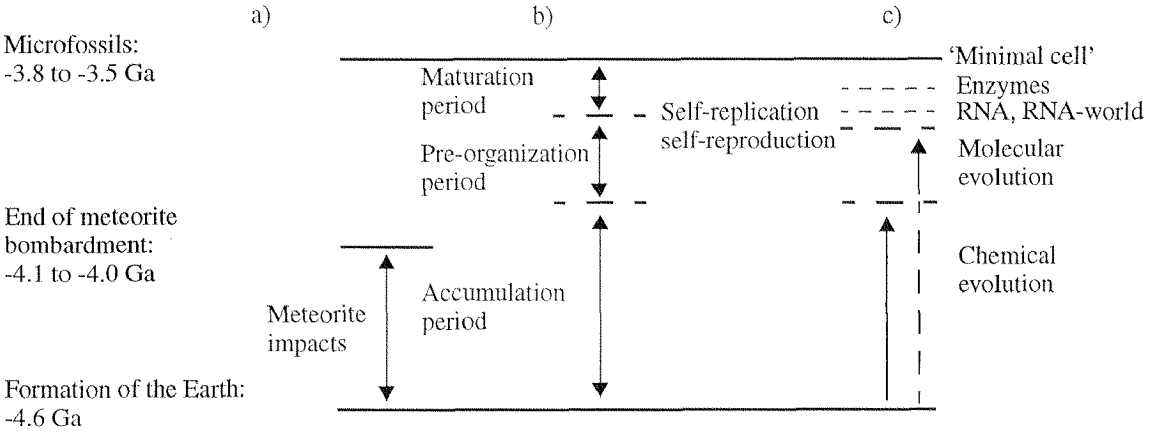


Figure 12: Transition from the post-impact world to a world including a 'minimal cell'. a) Environmental restrictions; b) Transition periods; c) Hypothetical intermediate stations.

2.2. Molecular Evolution

According to the molecular evolution scenario, the sequence of events started with the synthesis of simple organic compounds by various processes (chemical evolution). These simple organic compounds reacted to form polymers and other more complex materials, which in turn reacted, aggregated, and organized to form structures of greater and greater complexity, leading to something that could be called living. This idea was first expressed by Häckel [85] and later taken up by Oparin [86], [87] with further contributions made by Haldane [104], Urey [105], and Bernal [106].

2.2.1. Accumulation Period

The discussion of the Earth's early atmosphere in the context of molecular evolution is dominated by two views: (i) the early atmosphere was a reducing one, dominated by H_2, CH_4, H_2O and NH_3, as observed on the giant planets, Jupiter and Saturn. [87], [105] Organic compounds can be synthesized by lightning discharges in such an atmosphere; [107] (ii) the early atmospheric composition was largely produced by volcanic

[3] As proposed at the '1993 NATO Workshop on Self-Production of Supramolecular Structures' (Maratea, Italy, September 12-16, 1993) by P. L. Luisi, the term *self-replication* is limited to linear structures whose replication is based on the template chemistry of the nucleic acid type. The term *self-reproduction* is a more general one, valid for all other structures and systems.

outgassing, which is relatively oxidizing. The more oxidizing the atmosphere was, the less favorable was the synthesis of organic compounds on early Earth. [108], [109] Since the oldest known rock is dated at 3.8 Ga ($3.8*10^9$ years), there is no geological evidence concerning the conditions on Earth from the time of its formation to the time of deposition of these oldest known rocks. It is however clear, that the more reducing atmospheres are more favorable for the synthesis of organic compounds, both in terms of yield and the variety of compounds obtained.

A wide variety of energy sources has been proposed and used to synthesize organic compounds under prebiotic conditions since Miller's first experiment [107] using electric discharges in a reducing gas mixture. Besides sun light and chemical energy (e.g. pyrite [110]-[112] or thioesters [93], [113]), these include exotic energy sources such as cosmic rays, radioactivity, steam and hydrothermal vents, and volcanoes. [114] The use of any of these energy sources for prebiotic syntheses requires activation of molecules in a local area, followed by quenching of the activated mixture, and then protection of the synthesized organic compounds from further effects of the energy source. The quenching and protective steps are critical because the organic compounds will be destroyed if they are exposed continuously to the energy source.

Numerous compounds have been synthesized under so-called primitive Earth conditions, including the following: amino acids, purines and pyrimidines, sugars, fatty acids and phospholipids, and many others. [114], [115] It is, however, a matter of opinion as to what constitutes a plausible prebiotic synthesis. In some syntheses, the conditions are forced to such an extent (e.g. by the use of anhydrous solvents) or the concentrations are so high (e.g. 10 M formaldehyde), that the syntheses could not be expected to have occurred extensively (if at all) on primitive Earth.

There are still a number of biological compounds (e.g. porphyrins or riboflavin) for which adequate simulations of prebiotic syntheses do not yet exist, but it is probable that plausible prebiotic syntheses will be developed in the future. In other cases, important compounds may not have been synthesized prebiotically, because their occurrence in living systems is a result of intracellular biochemical evolution that occurred after the origin of life.

Apart from terrestrial prebiotic syntheses of organic compounds, there is an additional plausible source of organic matter. Namely large amounts of organic materials have been discovered in meteorites, comets, planetary atmospheres, cosmic dust, and interstellar

space. Comets seem to be the most promising source of exogenous organics. [94], [116] This has led some researchers to propose extraterrestrial sources for organic material on prebiotic Earth. [117]-[120]

Accumulation of such prebiotic organic compounds from either origin requires low temperatures on early Earth, since these organic compounds would have decomposed otherwise. [121]-[126] This does not exclude short-term, high-temperature processes, such as Darwin's "warm little pond"[4], but strongly suggests that temperatures prevailing on most of Earth could not have been much above 0°C during the accumulation period.

There are several sources of prebiotic organic molecules on early Earth. Each source is backed by some experimental data. A great deal remains uncertain about the accumulation period in the origin of life. However reasonable arguments suggest that the accumulation of an adequate supply of prebiotic organic molecules was not a major obstacle, once the Earth's environment had become cooler following the end of the heavy meteorite bombardment. [128]

The situation with regard to the evolution of a self-replicating or self-reproducing system is less satisfactory, because there are hardly any experimental data.

2.2.2. Pre-Organization Period

The previously described accumulation of small organic molecules was followed by an organization stage, where macromolecules were formed, which could provide a more systematic synthesis. There were also selection mechanisms that could direct the development. During this stage, functions which are crucial for living organisms, including catalytic activity, had to be developed through the self-organizing abilities of macromolecules. [127] This period is called the pre-organization period and resulted, according to the two main views, first in either (i) a self-replicating [129]-[137] or (ii) a self-reproducing system[5] (see Figure 13). [74], [110], [138]-[143]

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[4] On February 1st, 1871, Charles Darwin wrote a letter to his friend Hooker stating that "if (and oh, what a big if) we could conceive in some warm little pond with all sort of ammonia and phosphoric salts, light, heat, electricity, &c present, that a protein compound was chemically formed, ready to undergo still more complex changes, at the present day such matter would be instantly devoured, or absorbed, which would not have been the case before living creatures were formed." [94]

[5] Some authors use the term *metabolic system*, which is central to the metabolism-first hypothesis. [74], [138], [140]

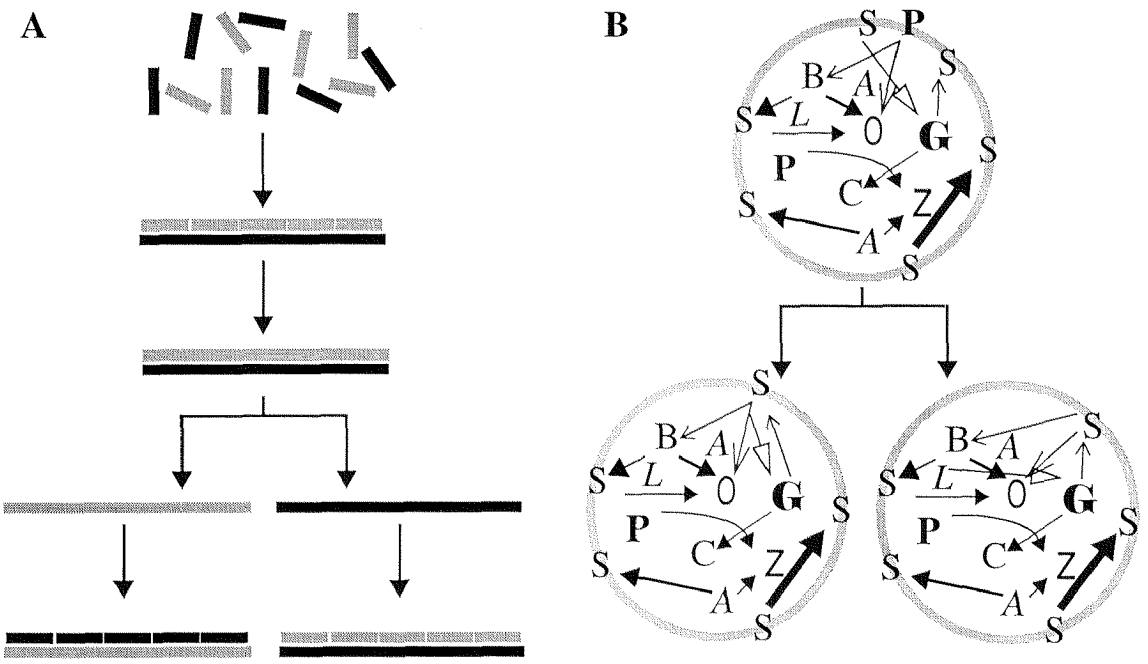

Figure 13: Schematic scheme of A) self-replication and B) self-reproduction. A) Monomers bind to a single-stranded template, oligomerize and form a double strand. This is separated in to two complementary single strands, each of which acts as a template for the formation of its complementary strand. B) An enclosed, externally fed catalytic set undergoes expansion and fission. If its catalytic network is effective its internal composition will tend to be more or less homeostatically preserved.

2.2.2.1. Self-Replication vs. Self-Reproduction

It is often claimed that the formation of macromolecules is a prerequisite for self-organizing systems.

In the self-replicating case, it is claimed that a template-forming, self-replicating macromolecule, such as RNA, would have to be at least 30-100 monomers long. [144] In principle, oligonucleotides can be oligomerized using activated nucleotides [145]-[149] and, in the presence of clay minerals, oligomers up to the 50mer have been obtained. [150]-[154] However, demonstrating self-replication using a single molecule in aqueous solution so far has failed because of various problems, one of them being product inhibition. [136] In addition, the general question has to be raised of whether there ever can be such a single molecule (template and replicator in one) with the antagonistic characteristics of conservation (demand for self-replication) and mutation (needed to undergo evolution and production of functional macromolecules to catalyze the syntheses of precursors in its surrounding). In the context of the self-replication

hypothesis, a self-replicating system consisting of more than one RNA macromolecule is often discussed, the so called "RNA-world", [155]-[158] as being either the result of a single self-replicating macromolecule or the start of a self-replication system itself. In this world, it is believed that RNA strands with catalytic activity (ribozymes [155], [159]-[163]) catalyzed the replication of their ensemble and the production of precursors such as nucleotides. According to the RNA-world hypothesis, such a system then gave rise to RNA directed peptide synthesis (i.e. cellular translation), and later on, more efficient proteic enzymes replaced the ribozymes[6]. There are many arguments for and against self-replication first. [164]-[166] It is, however, clear that at some point replication has to come in, either before or after a reproduction metabolism has been established.

For the case of self-reproduction, it is often argued that only macromolecular catalysts, such as enzymes, are able to catalyze anabolic reactions. [167], [168] Several syntheses of oligopeptides and proteins (proteinoids) have been published (see below). Only a few peptides or proteinoids synthesized in this way have exhibited weak catalytic activity. [91], [169]-[171] It seems likely that proteins, other than enzymes, with non-specific primary sequences, but determined amino acid compositions, can catalyze in the presence of certain metal ions more sophisticated reactions than simple hydrolysis. [172]-[174] In spite of some peptide self-replication experiments, [171], [175], [176] proteins and peptides do not generally show the characteristic of being able to template themselves. In a self-reproduction-first scenario, their formation always depends on the strong conditions of their producing events. Such conditions are, however, likely to be harmful for the products formed by such catalysts.

It is also conceivable that short peptides, other small molecules, and organo-metalic complexes might have brought about a self-reproducing, metabolic, and catalytic network[7] from which life eventually emerged. A two-dimensional metabolic start on pyrite surfaces, with autocatalysis and some lifelike features, such as membrane-building, has been proposed. [110]-[112], [177] From this, the template system would

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[6] It has to be mentioned at this point that in the literature catalytic activity often is misleadingly assigned to *ribozymes*, which do in fact show suicide inhibition but no turnover. The only reactions in which ribozymes have so far shown turnover, are hydrolysis and ligation.

[7] A *catalytic network* is a chemical reaction network, producing compounds that are catalysts for reactions in the network.

arise by a kind of natural development. In this picture, there is a true reproducing (metabolic and autotrophic[8]) start.

The capability of matter to organize itself into increasingly complex structures may be inherent in self-organizing systems or be driven by coupled reactions, where an energy liberating reaction drives an energy requiring one. Some self-organizing systems, which have been discussed theoretically in great detail, are dissipative structures [178] and hypercycles [132]. Experimental examples of such systems described so far in literature are not satisfying and try to assign such principles to some replicating systems. The precursors used however, are either complex, long oligomers of already specific sequences, [179]-[181] or synthetic model molecules, that only show the described characteristics in non-aqueous solutions. [182] An alternative scenario states that early information could be carried in the composition of molecular sets, propagated by mechanisms that involved the catalytic reproduction of the entire assembly. [74], [92], [140], [143], [183], [184] Thusfar, however, this scenario also lacks convincing experimental support.

Overlapping with the replication vs. reproduction discussion is the setting of the time point from which a compartment is needed to divert an evolving microsystem from the outside world.

2.2.2.2. Compartmentalization

Theoretically, the appearance of a microcompartment can be set at any time point in Figure 12: during the accumulation, the pre-organization, or the maturation period, before or after the first self-reproducing or self-replicating system evolved.

Although many different models of precellular systems have been suggested, the most significant ones seem to be liposomes (see Figure 14). [185]-[188] Such bilayer liposomal structures can easily self-assemble from a wide variety of lipidic molecules under both physiological and prebiotic conditions. [189], [190] Simple amphiphilic molecules such as fatty acids/soaps, alkyl phosphates, alkyl sulfates mixed with alkyl alcohols, glycerol monooleate, and oxidized cholesterol, assemble 'spontaneously' into liposomes under the right environmental conditions. [191]-[196] Bilayer-forming,

[8] *Autotrophs* are organisms that exploit the inorganic environment without recourse to compounds produced by other organisms. Their main carbon source is CO_2-fixation. In the context of the origin of life, *autotrophic* relates to CO_2-fixation and is not dependent on higher organic compounds from the prebiotic soup.

amphiphilic molecules have been formed under plausible prebiotic conditions, [192], [195]-[200] but as for most scenarios of chemical evolution, the problem is identifying an abundant source of precursors for the proposed prebiotic syntheses of such lipids. [194] The presence of lipids in the primitive environment is further supported by the existence of membrane-forming, nonpolar molecules detected in samples of the Murchison meteorite. [201], [202] Contemporary cell membranes incorporate phospholipids as the major component of the lipid bilayer, but they need not be required for early protocells.

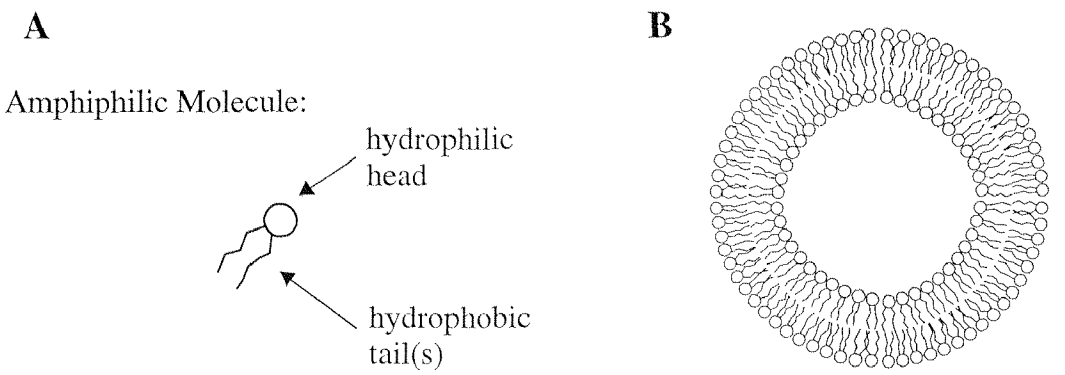

Figure 14: Amphiphilic molecules, such as membrane lipids or surfactants form liposomes (sometimes also referred to as lipid vesicles) under specific conditions. A) The lipid (surfactant) consists of a hydrophilic head and a hydrophobic tail. B) Such amphiphilic molecules self-assemble to spherical closed bilayers, called liposomes or vesicles (cross-section of small unilamellar vesicle).

The pre-organization period was probably strongly influenced by complex interactions between a large number of molecular components, held together by membranes. A decisive step towards the emergence of the first living systems is believed to have been the appearance of membrane-enclosed polymolecular systems. [86], [87], [187], [188], [203]-[212] By the interaction of oligomolecular or polymolecular systems with the lipid membrane, new physico-chemical properties probably appeared. It is known that different polypeptides can lead to major changes in the morphology and permeability of liposomes. [213]-[217]

A semipermeable prebiotic membrane would have clearly favored [218] (i) the cooperative interaction between different catalytic and/or replicative molecules, avoiding their dispersal, and providing the possibility of specific surface-chemistry processes; (ii) the creation of internal microenvironments substantially different from the exterior milieu maintained by, at least partially, selective transmembrane transport; and (iii) the

preferential accumulation and, eventually, differential multiplication of self-sustaining reproduction or replication systems.

2.2.3. Maturation Period

Once a self-replicating or self-reproducing system had evolved, the missing counterpart must have emerged sooner or later: either metabolic functions or the replication of coded information, respectively. In addition to this completion, the now replicating, metabolic, and self-reproducing system had to become more and more efficient in order to evolve rapidly to be able to adapt to different and rapidly changing environments.

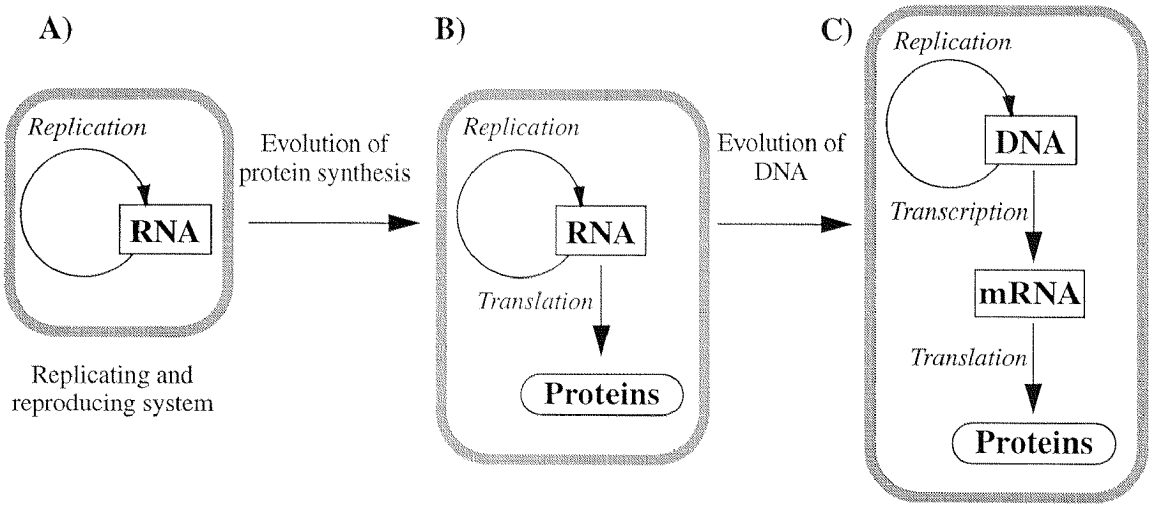

Figure 15: The hypothesis that RNA preceded DNA and proteins in evolution. A) represents the "RNA-world", where replicating RNA combines genetic, structural, and some catalytic functions (such as self-splicing); B) In the "RNA/protein world", RNA encodes genetic information for protein synthesis (translation); C) The "DNA/RNA/protein world" uses DNA as a stable information storage molecule which is transcribed into mRNA which is then translated into the corresponding protein.

There are two achievements that were very important in the maturation period to life: (i) gene-directed protein synthesis, and (ii) the introduction of DNA as an information carrier. Besides these, several other functions had to be developed in this late stage of prebiotic evolution: enzymatic production of the basic building blocks (amino acids, sugars, nitrogen bases, and lipids), systematic production of nucleotides and nucleic acid polymers, metabolic turnover mechanisms to store or retrieve energy, and mechanisms for active transport, (feedback) regulation, and signal transduction. [127]

It has been hypothesized that protein synthesis was a result of evolution using both replication and catalysis, strongly dependent on RNA molecules [155], [206]-[208] or

their nucleic acid-like precursors, [219]-[224] and probably also catalytic oligopeptides. [188], [225]-[228] Peptides were probably present on primitive Earth prior to the emergence of the ribosomal protein synthesis (see below) and catalytic peptides of nontranslational origin may also have existed in the primitive environment. [229]-[236] However, regardless of how many different catalytic polypeptides were formed abiotically on the primitive Earth, efficient protein synthesis needed translation coupled to a replicating mechanism to ensure the maintenance, stability and diversification of its components. [218]

It is generally accepted that RNA probably preceded DNA (see Figure 15). [155], [157], [206], [207] DNA was selected as a long, efficient, information-storing macromolecule to stabilize earlier RNA/protein world systems, having the possibility of error correction mechanisms that operate on DNA and permit large genomes. Chemically, it was probably not too difficult to produce DNA from RNA once the translational protein synthesis was present. It is believed that DNA emerged after the development of a reverse transcriptase and the enzymes to make the deoxyribonucleotide precursors from the ribonucleotide triphosphates. [237]

Another challenging question concerns the occurrence of additional functional proteins once the translation machinery had been developed. During the maturation period it was necessary to develop a large number of functions. However, the probability of producing a specific protein sequence consisting of 200 amino acids by chance (assuming random sequence formation) is close to zero (10^{-260}). The exon theory of genes, [209] which states that genes are assembled from small modules, proposes a resolution of this numerical paradox.[9] The organization of genes into introns and exons allows for the

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[9] The exon theory of genes suggests that the first genes were selected out of a group of genes corresponding to translated 20mer proteins. Out of the 10^{26} possible 20mers only a small fraction have relevant three-dimensional shapes, e.g. 10^6. Then the evolutionary path leading to a protein of 200 amino acids in length would be the selection of a first exon (coding for a peptide with weak catalytic activity) out of 10^6 possibilities, followed by the subsequent choice of a second exon (coding a peptide, that improves the activity of the first, when linked to it) again out of 10^6 shapes, then the choice of a third, and so forth. If done in order, the ten choices required to produce the 200 amino acid long final protein explore only 10^7 possibilities (rather than 10^{60} possibilities if a simultaneous choice were being made), far less than the 10^{260} possibilities contemplated originally. This argument, made by W. Gilbert, [209] shows that the numerology of evolution is not an insurmountable problem, and it implies further that evolution could have examined only a tiny fraction of the possible structures of proteins. There may be very many different three-dimensional configurations that can solve a given enzymatic problem with the same efficiency.

exchange of exons between genes (exon shuffling) via recombination events taking place in introns. If exons correspond to modules of protein function or structure, such recombination could arrange these modules to create proteins with novel functions and structures.

Despite the introns early vs. late debate,[10] exon shuffling might be an explanation for the evolution of functional proteins (see Figure 16). The processes of evolution were probably (i) the shuffling of small exons, which coded for short oligomers (15-20 amino acid residues long) with a defined secondary structure, as the first building blocks for new proteins; (ii) the loss of introns and hence the fusion of small exons to make larger ones; (iii) point mutations to optimize possible catalytic functions and substrate specificity of enzymes obtained in this way; and (iv) gene-duplication and divergence to retain the original genes which code for working enzymes. [209], [239], [243], [251]-[254]

From this point on, natural selection occurs if alternative alleles (forms of a gene) affect the fitness of the system or the survival and reproduction of an organism in a given environment (neo-Darwinian evolution).

As stated above, the transition to life was most likely a continuum and the position of the 'first form of life' is arbitrary depending to the definition of life preferred. However, the 'minimal cell' resulting from the maturation period was probably a DNA/protein organism with about 100-200 enzymes, with ca. 1000 bp/gene, enclosed by a lipid boundary. [128], [255]

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[10] Eucaryotes have discontinuous genes, which means that the DNA sequences coding for the amino acid sequences in the corresponding proteins are subdivided into exons by the interruption of introns. Intron sequences are removed after transcription and therefore not translated into protein sequences. Prokaryotes on the hand have continuous genes, which means that they do not have any introns. A debate about the origin of the introns arose soon after they were discovered. The extreme theories are the introns-early [238]-[244] and the introns-late [245]-[249] views. According to the introns-early theory the progenote [250] (first primordial cell, also called the 'last universal ancestor' or 'breakthrough organism') already had introns, which - for the case of prokaryotes - were lost during the evolutionary process. In the introns-late theory the progenotes did not have introns. Introns were inserted later on in evolution to the eucaryotic genome.

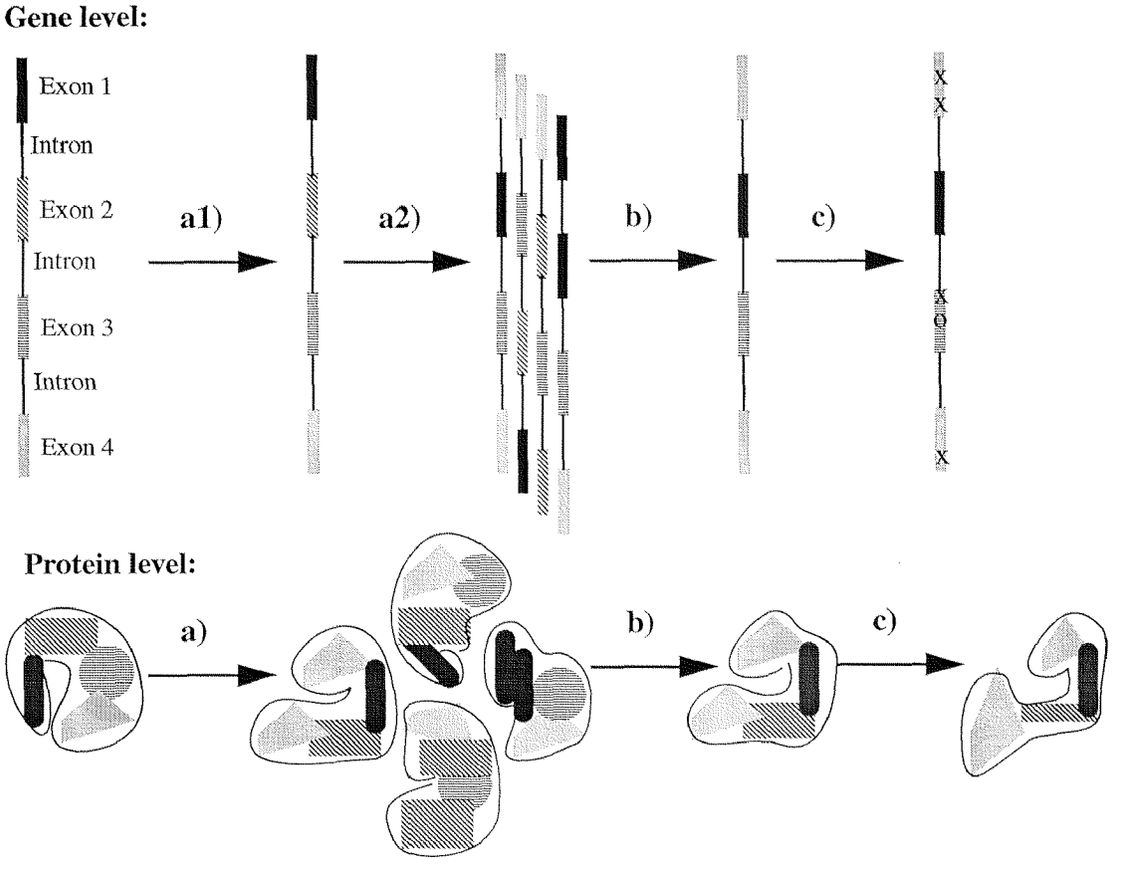

Figure 16: Evolution of functional proteins: a1) Gene-duplication leads to two copies of the same gene. a2) One of the two copies undergoes divergent evolution by exon shuffling. The protein products of these new genes correspond to newly constructed proteins, consisting of the exon product building blocks with defined secondary structure. b) Selection favors the organism with the protein having a new function which has a positive effect on reproduction. c) Point mutation leads to an optimized enzyme.

2.3. Unsolved Problems

An attempt has been made in this chapter to put parts of the puzzle together by briefly summarizing and ordering the main elements of current knowledge in the field of research on the origin of life. The resulting sketch, a plausible picture of the transition to life is, however, becoming more and more blurred the closer one zooms in on it. There are many missing pieces. [257] This section aims at listing the principal remaining problems (see Table 8) in the origin of life, which must be fitted into the puzzle in the future.

As mentioned in section 2.2.1., there is still a debate ongoing as to whether the primitive atmosphere was oxidizing or reducing. To clarify this point, more detailed studies on plausible chemical reactions under such conditions are needed. Such investigations

would shed more light on the often discussed, but never presented, list of prebiotic molecules.

Table 8: There are many problems remaining to be solved in the origin of life. This table lists the main missing parts in the transition to life.

Problem:	**Description:**
Primitive Earth's environment:	*It is still unclear how reducing the early atmosphere was, what type of chemical reactions were common under these conditions and what type of molecules belong to the 'prebiotic list'.*
Ribonucleotide formation:	*Feasible prebiotic pathways to ribonucleotides and their activated forms, that have prebiotic potential, are still lacking.*
Syntheses of lipids:	*Despite published prebiotic syntheses for amphiphilic lipids and surfactants, the yields and an abundant source of precursors in the proposed reactions are problematic. Additional studies on the prebiotic synthesis of lipids and surfactants capable of forming semipermeable membranes are required.*
Origin of homochirality:	*The amino acid monomers in proteins have only the L-configuration, while the ribose and 2-deoxyribose monomers in RNA and DNA are exclusively of the D-configuration, as are glucose monomers in glycogen, starch, and cellulose. Natural lipids in biological membranes are mostly chiral and only one enantiomer can be found in natural systems (i.e. L-glycerol 3-phosphate moiety of the phosphoglycerides). To date, the origin of this absolute homochiral purity is still unclear.*
Enzymes/RNA/DNA:	*Self-organization contains several challenges, such as the accumulation of activated monomers for polymerization, the demonstration of a self-replicating or self-reproducing system of prebiotic relevance, and experiments which verify some of the enormous number of theories proposed to explain the origin of translation and the genome.*

One type of molecule of great prebiotic relevance are ribonucleotides. Although possible prebiotic syntheses of purines, pyrimidines, [115] and ribose-2,4-diphosphoribose [256] have been reported, there is no efficient prebiotic synthesis known today that couples ribose, base and phosphate. Much remains to be done in research into feasible pathways

leading to activated ribonucleotides which eventually are able to condensate to RNA oligomers.

An abundant source of long-chain hydrocarbon components of prebiotic membranes is also lacking: Concentrations in meteorites and yields in the proposed prebiotic syntheses of amphiphilic compounds are very low. [192], [195]-[200] Therefore, additional studies on the prebiotic synthesis of lipids and surfactants capable of forming semipermeable membranes are required.

Another unsolved problem arises from the fact that all the essential biopolymers and aggregates associated with life are made up of chiral monomer units characterized both by unique homochiralities (i.e. L-amino acids in proteins,[11] D-sugars in RNA and DNA, components of the plasma membrane, such as lecithins) as well as by absolute chiral purity (i.e. no 'unnatural' enantiomeric D-amino acid or L-sugar monomer units are found in these biopolymers). [259]-[263] Despite many hypotheses supported by few experimental results, the questions how, when, and why absolute homochirality appeared are still under intense debate. [264]-[269]

Quite a lot of problems pertain to the self-organization stage, between the end of the chemical evolution and the appearance of the first cell: (i) the polymerization reaction of nucleotides and amino acids requires activated monomers, which have short life-times in aqueous solution and can not easily accumulate; (ii) the hypothetical emergence of self-reproducing or self-replicating systems from a mixture of simple prebiotic molecules has never been demonstrated experimentally; (iii) the origin of translation and the genome has many theoretical explanations, but none of the proposed theories has any experimental support.

In spite of these great difficulties, the large number of competing theories, the wealth of knowledge and the sizable body of experimental data acquired in this field to date is remarkable. Although many unsolved problems remain, the interdisciplinary research that has resulted from the interest mediated by questions of our origins is a great, unique event per se.

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[11] There are some rare exceptions. [258]

3. Peptide Bond Formation

Synthesis of a peptide bond from its constituent free amino acids or peptide fragments is an energetically unfavorable process because of the considerable increase of free energy involved. The values of ΔG^{o}_{298} for the synthesis of dipeptides in aqueous solution range between +10 and +20 kJ/mol. [270]-[273] In a purely formal sense, one might interpret the endergonic process of peptide bond formation from ionized amino acid residues as the sum of (i) the energy-releasing step of peptide bond synthesis from nonionized amino acids and (ii) the energy-consuming step of the proton-transfer. [274], [275] However, the values for the free enthalpy for the formation of the dipeptide from uncharged reactants are about -20 kJ/mol, [271], [276] and the proton-transfers for each ionized amino acid to its neutral form are about +30 kJ/mol (see Figure 17). [273] These calculations confirm that for peptide synthesis in an aqueous medium, the endergonic conversion of the charged reactants into the nonionized forms represents the essential thermodynamic barrier. Therefore, energy must be added to the aqueous system in order to get amide bond formation through condensation of amino acids or peptides. In principle, energy supply can either be produced through simply heating the system or by activation of the carboxyl group of the amino acid or peptide to be condensed. The temperatures for the first, however, at which condensation occurs, exceed the limits considered safe for many peptides. In spite of this, thermal condensation is an effective way to condense amino acids to thermal proteins, also called proteinoids (see below).

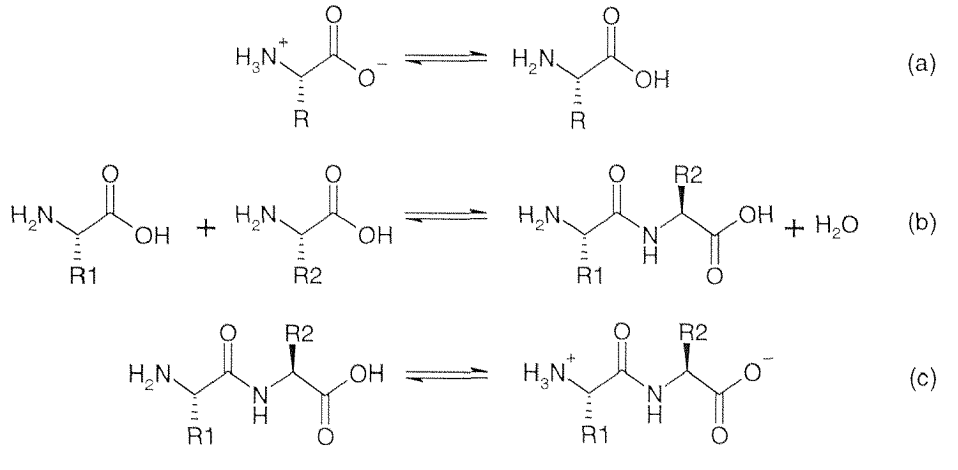


Figure 17: Formal steps required for the peptide bond formation and their free energy changes (ΔG^{o}_{298}): (a) Proton-transfer for each amino acid (from left to right ca. +30 kJ/mol); (b) Peptide bond formation (ca. -20 kJ/mol); (c) Proton-transfer in the resulting neutral dipeptide to the ionized species (ca. -30 kJ/mol). [276]

The following paragraphs deal mainly with amino acid and peptide condensations in aqueous solution with respect to a prebiotic scenario. A detailed discussion of many challenging problems in peptide synthesis, such as side chain protection and epimerization, is beyond the framework of the present work.

In order to convert carboxylic acids into acylating agents, their hydroxyl group must be replaced by an electron-withdrawing substituent (X) to enhance the polarization of the carbonyl group and the electrophilicity of its carbon atom. Thus, nucleophilic attack by the amino group of the amino acid to be acylated is greatly facilitated. In an aqueous system, however, water and hydroxide anions compete with the amine for the activated *C*-terminus, so that conditions have to be well chosen in order to reduce these secondary reactions.

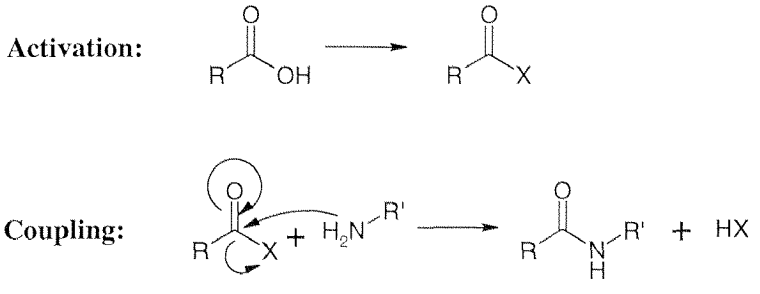
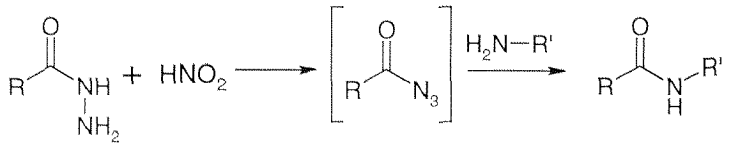
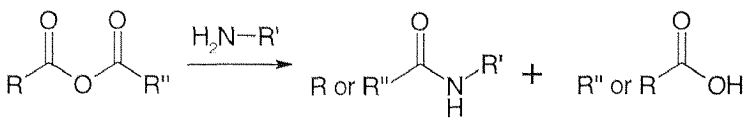
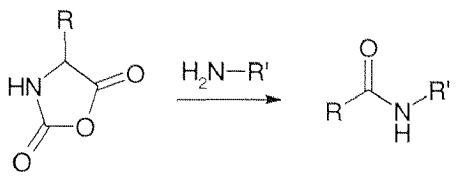
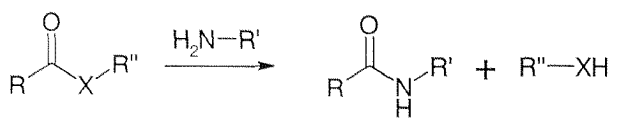
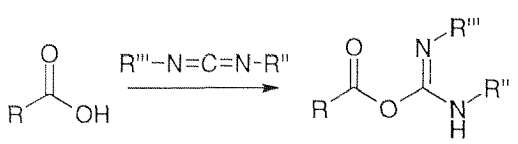
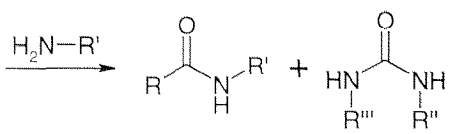


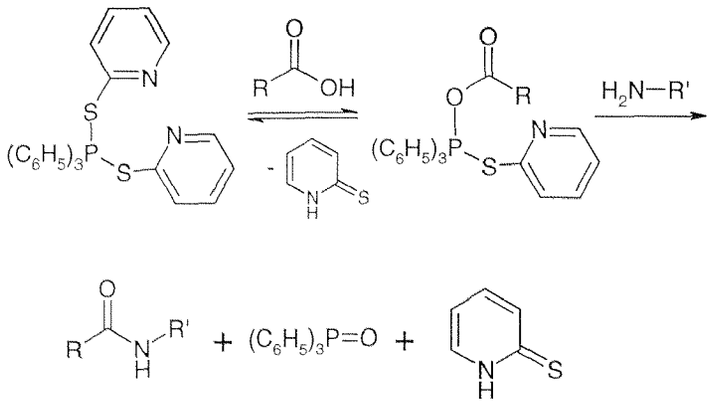
Figure 18: Activation and coupling of amino acids and peptides to form peptide bonds.

Practically all of the techniques which have been used for constructing peptide bonds are of the type outlined in Figure 18. A great variety of leaving groups and reactions for attaching them to the electrophilic carbon atom of the carboxyl component have been investigated. [274] The coupling methods can be divided into classical methods, such as the azide, anhydride, active ester, and carbodiimide types, and newer methods, such as those used in solid-phase synthesis and fragment condensation strategies. [277]-[280] Table 9 lists the main coupling methods known today.

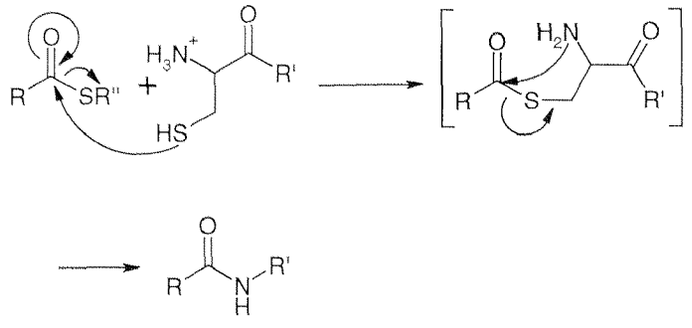
Table 9: Some of the main coupling methods and their reaction scheme with practical relevance today.

Method:	**Reaction Scheme:**
Azide Method: [281]	
Anhydride Method: [280]	 R = R": Symmetrical anhydride R ≠ R": Mixed anhydride
	 N-Carboxyanhydride (NCA)
Active Ester Method: [280]	 X = O, S, Se
Carbodiimide Method: [282]	 

Mukaiyama Method:
[283]



Kent Method: [284]



4. Prebiotic Peptide Condensation

Suggestions for nontemplated prebiotic polypeptide synthesis envision either (i) direct formation from hydrogen cyanide (HCN) polymers and aminoacetonitrile, or (ii) polymerization of amino acids in an aqueous environment by the application of heat under drying conditions, or with chemical activation (see Figure 19).

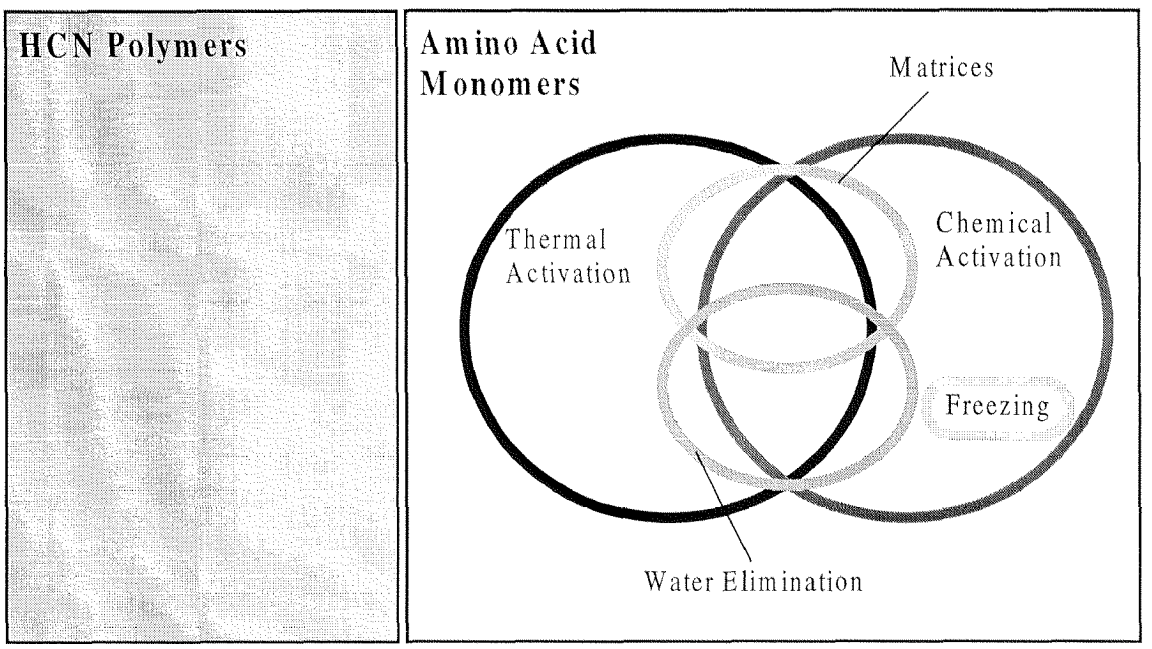


Figure 19: Prebiotic peptide formation starting from HCN polymers or amino acid monomers: Heteropolypeptides can be formed by HCN polymerization and their subsequent transformation into polyglycine, where different side chains are inserted. Amino acids can be condensed by heating in the solid state or using chemical activation in aqueous solutions. Concentration enhances polycondensation and can be achieved by freezing, interactions with matrices, or elimination of water.

4.1. Polypeptides from HCN Polymers

The occurrence of HCN on early Earth is strongly supported by its facile formation under a variety of prebiotic conditions, [285] and HCN polymers may have been among the earliest naturally occurring macromolecules on Earth, since HCN polymerizes spontaneously at low temperatures in the presence of a base such as ammonia or another amine. [286] Although the mechanisms have not yet been solved in detail, there is evidence for direct transformation of such polymers into heteropeptides by chemical modification of the side chains (see Figure 20). [287]-[290] The following α-amino acids were detected, when HCN, [291]-[295] its trimer (aminomalononitrile), [296], [297] or its tetramer (diaminomaleonitrile) [296], [298] were first oligomerized under prebiotic

conditions and then hydrolyzed for analysis: Gly, Ala, Asp, Ser, Glu, Leu, Ile, Arg, Val, Lys, Thr, and His. At present it is clear that higher oligomers of HCN contain a complex variety of functional units. This complexity is apperent in the diverse mixture of biological molecules in addition to amino acids that are obtained upon hydrolysis of the oligomers. [285]

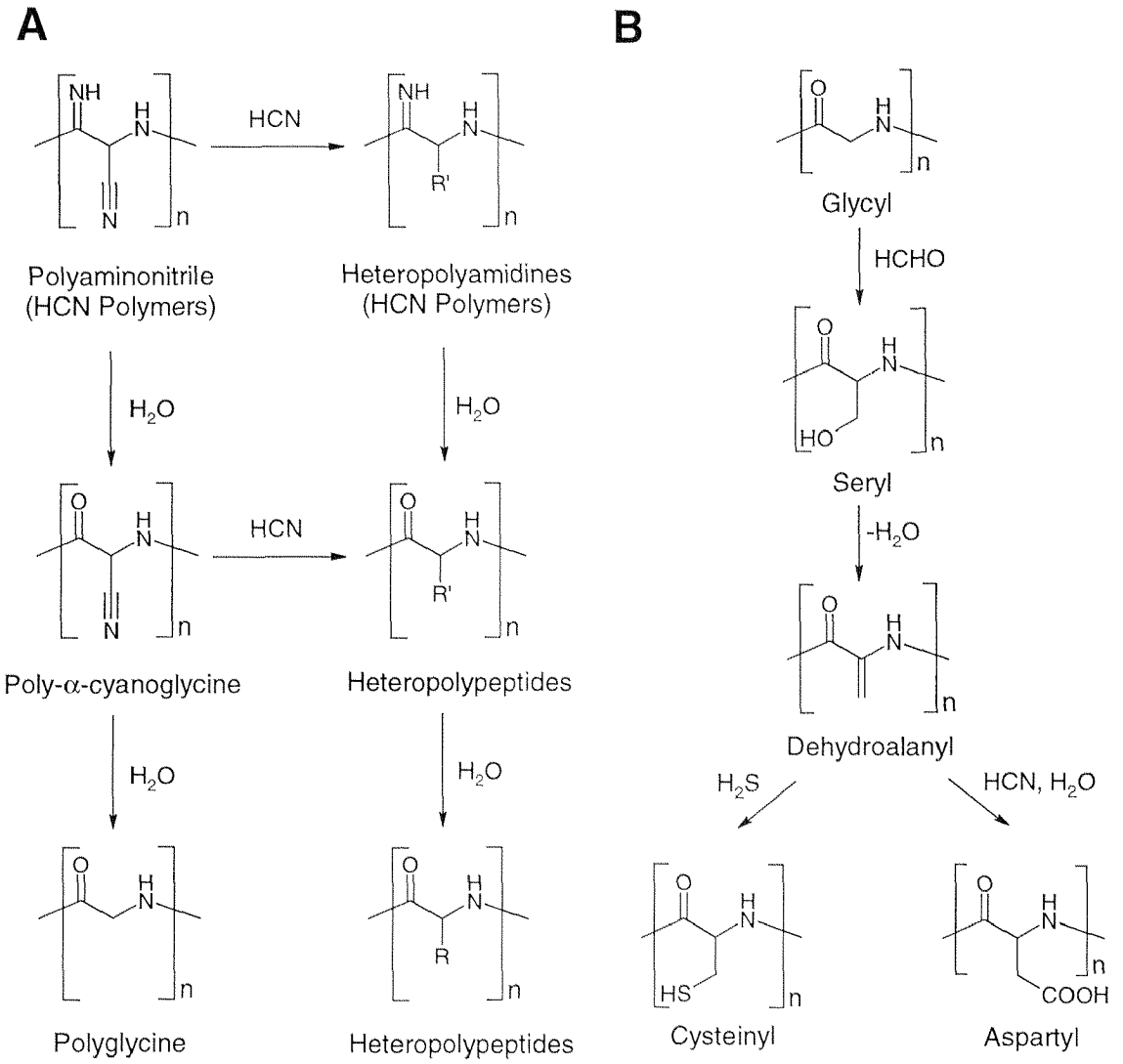


Figure 20: Direct formation of heteropolypeptides from (A) HCN polymers and (B) polyglycine. [305], [306]

In the presence of ammonia and formaldehyde, hydrogen cyanide is converted into aminoacetonitrile. [299] By mixing aminoacetonitrile with clay minerals and heating, oligoglycines have been obtained. [300]-[303] A protein can formally be considered as a stereoselectively polysubstituted polyglycine. The synthesis of a protein might therefore be visualized as occurring through progressive substitution of preformed polyglycine.

[300] It was shown that specific chemical modification at glycyl residues results in heteropeptides consisting of Gly, Ala, Ser, Thr, Asp, and Glu (see Figure 20). [304]
Despite what has been said above, an obstacle is the relatively high concentration of cyanide which would have been needed on prebiotic Earth (0.01 M or greater in the absence of UV radiation) in order to achieve oligomerization. [285] The most effective means of concentration seems to be the formation of an eutectic phase by freezing, since evaporation would lead to the loss of volatile HCN. [296]

4.2. Polypeptides from Amino Acids

It is highly likely that amino acids were present on primitive Earth. Various amino acids have been synthesized under prebiotic conditions using different sources of energy such as electric discharges, [307] UV light, [308] shock-waves, [309] X-rays, [310] and heat. [311] They have also been found in carbonaceous chondrites and meteorites. [312], [313]
As mentioned above, the condensation of amino acids and peptides is fairly endergonic. Energy has to be added in some way in order to form oligomers starting from monomers. In principle, one can think of two types of energy, either (i) of a physical nature (i.e. heat or pressure) or (ii) a chemical nature (chemical activation).

4.2.1. Physical Activation

There are three physical activation scenarios in the prebiotic polycondensation of amino acids which have been discussed and evaluated in the literature so far: (i) thermal condensations of amino acids in the absence of water, (ii) hydrothermal condensations at high pressure and temperature, simulating submarine hydrothermal vents, and (iii) heating-wetting cycles at lower temperatures which might have occurred in lagoons.

4.2.1.1. Dry State Thermal Condensation

The thermal condensation of amino acids in the dry state is the only prebiotic method which has yielded polymers of amino acids containing all the amino acids common to contemporary proteins, with molecular weights of a few thousand, and possessing modest catalytic activities (hydrolysis, decarboxylation, amination, deamination, and oxidoreduction). [314]
In the dry state, the 20 proteinogenic amino acids respond differently to heat. The average amino acid will quickly form a black, carbonlike substance when heated above

100°C. [315] However, this is not the case for all amino acids. Aspartic acid polymerizes to polyaspartylimide (**1**), when heated to high temperatures (120-200°C). If subsequently treated in water under alkaline conditions, the polymer undergoes internal ring opening leading to polyaspartic acid. [316], [317] Glutamic acid does not polymerize but rather cyclizes to form hot liquid pyroglutamic acid (**2**). [318] Similarly, the basic amino acid lysine is converted into its seven-membered lactam (**3**) by heating; the latter is not zwitterionic and therefore melts easily. Prolonged heating results in formation of thermal polylysine. It was observed that in such polymers the number of ε-linked lysine residues is larger than α-linked ones. [319] When proline is heated in a dry state, it forms a clear melted liquid with physical and chemical properties similar to pyroglutamic acid. [315]

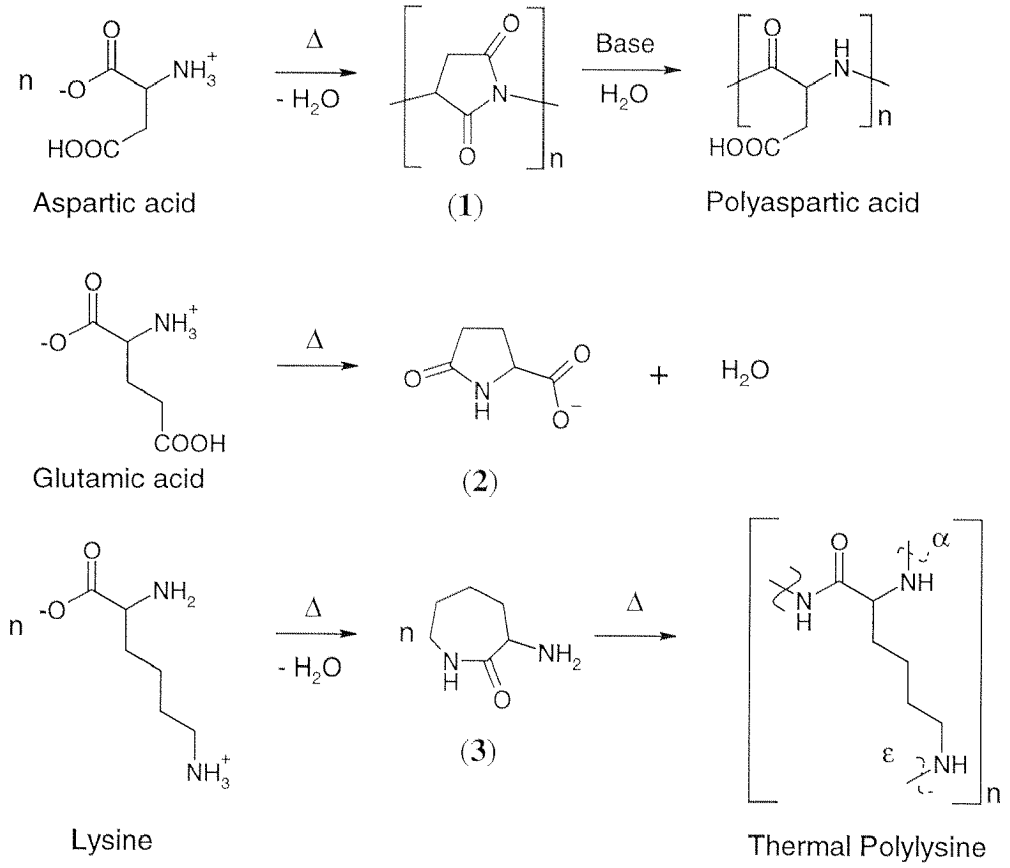


Figure 21: Thermal condensation of aspartic, glutamic acid and lysine.

When various other amino acids are mixed with Glu, Asp and/or Lys and heated, copolymers are obtained in high yields. [320]-[322] It was shown that proteinoids containing eighteen amino acids common to proteins can be obtained by heating the appropriate amino acid mixtures under melted conditions. [323], [324] However, the

resulting polycondensates are often only around 50% peptidic. The amino acid units are often epimerized, while the α, β, γ and ε functions of the amino acids lead to frequent crosslinking. [325]-[328]

4.2.1.2. Submarine Hydrothermal Condensation

Submarine hydrothermal vents have been recognized as a possible energy source for prebiotic synthesis. In such systems, products that are formed in the hot vents can re-enter the vents after being quenched in cold surrounding water. [329] Thus, hydrothermal vents in the sea could have provided an environment where oligomers and polymers were synthesized and selected for with the help of high temperatures and pressures. For instance, if two amino acids form a peptide bond in the hot area of the vent and the peptide is thereafter ejected into the cool surrounding area, the peptide bond can survive and maybe even reenter the vent for a second peptide-bond-forming-cycle, and so forth. [330]

Condensation of amino acids by dehydration has been achieved by treating a solid mixture of amino acids and inorganic materials under hydrothermal hot high pressure environmental conditions (58.6 MPa, 270°C), as observed in the dynamics of plate tectonics. Dipeptides, such as H-GlyGly-OH and H-PhePhe-OH resulted in 1-2% yield in the aqueous fractions, starting from Gly and Phe, respectively. [331]

The circulation of a 100 mM glycine solution in a constructed flow reactor that simulated a submarine hydrothermal system (high-pressure high-temperature chamber: 110-350°C, 23.0 MPa and low temperature chamber 0°C, 24.0 MPa), led to oligomers up to the size of a trimer (about 3% yield based on starting Gly). When divalent ions, such as copper, were added to prevent the hydrolysis of the tetramer under acidic conditions, the oligoglycine was even elongated to a hexamer (about 0.006% yield based on initial Gly). [330], [332] It has been claimed that hydrothermal oligomerization is not limited to Gly, [332] but such condensations have yet to be demonstrated for other amino acids.

4.2.1.3. Heating-Wetting Cycles

Dry-wet cycles have been proposed as possible processes that could have assisted the oligomerization of amino acids into oligomers. Heating helps to overcome the energy barrier of condensation and evaporation leads to concentration of the reactants. The primitive environment was constantly undergoing periodic changes because of the

succession of night and day, tidal cycles in lagoons, seasonal changes, and so on. The evaporating-pond model provides a geologically plausible model in that cyclic changes in humidity and temperature, often in the presence of inorganic condensing agents such as salts, silica, alumina and clays, may have yielded significant amounts of oligopeptides.

If mixtures of Asp, Glu and another proteinous amino acid are slurried in water and heated at 65 to 85°C (temperatures generated by solar radiation in deserts today) [333] and periodically reslurried in water, they oligomerize without any dehydating agents being present. [334] Similar results were obtained when amino acid mixtures were evaporated to complete dryness by microwave heating, reslurried in the starting mixture and evaporated again several times over. Polypeptides with molecular weights of at least 1000 to 4000 daltons, similar to proteinoids, were obtained in this way. [335]

In other experiments, amino acids such as Gly, Ala, Pro, Val, and Leu were condensed in heating-wetting cycles at 85°C, in the presence of salts (NaCl, $CuCl_2$), silica, alumina, or clay minerals (hectorite). [336]-[342] Dipeptides and tripeptides with different amino acid compositions resulted after 7 cycles in yields of up to 1% of the initial amino acids. [343]-[345] In some cases, the presence of other amino acids and dipeptides enhanced the formation of other homopeptides. [343], [346] In similar studies with clay minerals, oligomers up to the 6mer have been observed for Gly. [346]-[348]

4.2.2. Chemical Activation

An alternative method for thermal condensation is the use of condensing agents to chemically activate amino acids and peptides. The direct use of activated amino acids as starting materials is also possible.

As discussed above, peptide chemistry offers a wide variety of condensing agents and activating groups in organic solvents, but only a few of them fulfill the criteria of being prebiotically relevant and capable of surviving long enough to support peptide condensation in an aqueous environment.

4.2.2.1. Prebiotic Condensing Agents

Prebiotic condensing agents for peptide formation can be divided into two classes: (i) cyanide-containing compounds, such as cyanamide, carbodiimides like (**4**), dicyanamide

(**5**), dicyandiamide (or cyanoguanidine) (**6**), and the hydrogen-cyanide-tetramer (or diaminomaleonitrile) (**7**), and (ii) condensed phosphates, like cyclic polyphosphates.

Of all the cyanide containing condensing agents, cyanamide is the simplest. Its structure is $NC\text{-}NH_2$ and it has been applied in the synthesis of di- and trimers of Ile and Leu with total yields in the range of 7-17%, [349], [350] and di-, tri-, and tetramers of Gly and Phe have been obtained in yields between 5 and 66%. [350]-[352] All these reactions have been performed under slightly acidic to neutral conditions (pH 3-7). Studies have shown that cyanamide can be synthesized by UV irradiation of solutions containing HCN or ammonium cyanide. [353], [354] It has also been detected in interstellar space. [355] However, the availability of cyanamide on primitive Earth is very doubtful, since it dimerizes very rapidly into dicyandiamide (**6**). [356] The mechanism for the cyanamide-mediated formation of a peptide is probably similar to that suggested for the carbodiimide-mediated reaction, [357], [358] since cyanamide and carbodiimide are tautomers. [356]

Carbodiimides, R-N=C=N-R', are commonly used in organic media. They can be used in water given a careful choice of the substitutents R and R'. Peptides of nonrandom composition have been obtained by incubating an equimolar mixture of His, Arg, Asn, Thr, Ser, Glu, Pro, Gly, Ala, Val, Met, Ile, Leu, Tyr, Phe, and 0.5 molar of one of the *N*-acyl amino acids (to prevent cyclization of the dipeptide) and the water-soluble 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) (**4**). [359] In the presence of EDAC, polymerization of Asp at room temperature can produce up to 6mers of oligoaspartic acid. If the condensation is performed in the presence of the clay mineral hydroxyapatite, the length of the oligomers resulting from successive feeding experiments was drastically increased (>30mers). [154] Similar results were obtained for β-glutamic acid, where oligomerization is not very temperature dependent between 4-50°C, but proceeds very efficiently at -20°C under eutectic conditions. [360]-[362] These freezing conditions are, however, not applicable to monomeric α-amino acids. [360] The problem of these systems is that both of the carboxyl groups of the amino acid can be activated, so that branching occurs. [154] The condensation of Glu with EDAC is not very successful; [361] nor does its trimer oligomerize efficiently, unless the clay mineral hydroxyapatite is added. [362]

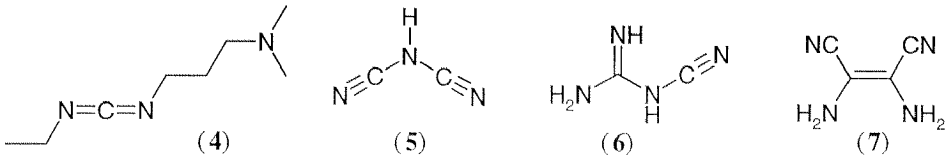


Figure 22: Molecular structures of EDAC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) (**4**), dicyanamide (**5**), dicyandiamide (or cyanoguanidine) (**6**), and hydrogen-cyanide-teramer (**7**).

The mechanism (see Figure 23) of the carbodiimide-induced condensation starts with the direct attack by the carboxyl group on the carbodiimide to form an *O*-acylisourea (**8**). The free amino group of another amino acid attacks this activated species to form the amide bond. In the case of α-amino acids, if the carboxyl group of the dipeptide is activated, efficient cyclization to give a diketopiperazine ensues, thus inhibiting oligomerization. This is the reason why β-amino acids are more efficiently oligomerized than α-amino acids by carbodiimides. [361]

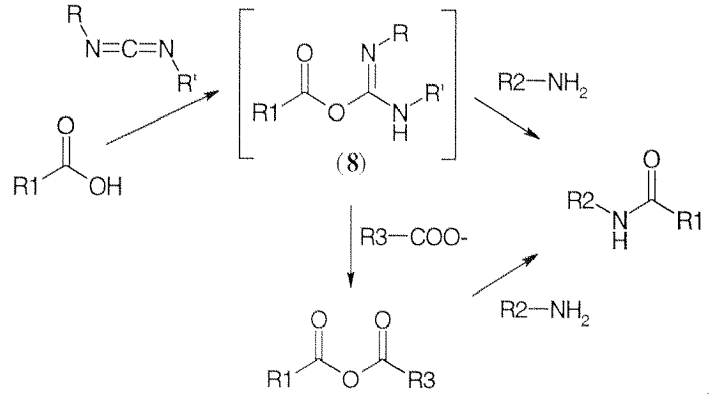


Figure 23: Mechanism of the carbodiimide-induced reaction. The condensation proceeds over the activated *O*-acylisourea form (**8**). [363]

Another condensing agent that presumably reacts according to a similar mechanism is dicyanamide (**5**). It is in equilibrium with tautomeric carbodiimides, that are themselves protonated. Nucleophilic attack by the carboxyl group of an amino acid on these protonated species, results in an activated amino acid. Subsequent attack by the amino group of a second amino acid gives the peptide and cyanourea. Low pH (1-3) and using a five to tenfold excess of dicyanamide dipeptides can be obtained in around 1% yield when starting from amino acid solutions of Gly, Ala, Leu, Ile or Phe. [364]-[370]

Dicyandiamide or cyanoguanidine (**6**) is the dimer of cyanamide. It is easily synthesized prebiotically by UV irradiation of an aqueous HCN solution. [353] Equimolar solutions of Ala and dicyandiamide at pH 2 have led to the formation of up to the trimer in low yields (1%). [368], [370]

The polymerization of HCN in solution by UV irradiation at a pH close to the pK-value of HCN (9.5), leads easily to the HCN-tetramer (cis-diaminomaleonitrile) (**7**). The behavior of this compound as a condensing agent has been studied for the synthesis of diglycine. [371] Yields of up to 5% have been obtained at pH 9 and 85°C. The proposed mechanism is shown in Figure 24. Since cyanide is presumably the electron acceptor, a neutral to alkaline pH is preferable. [356]

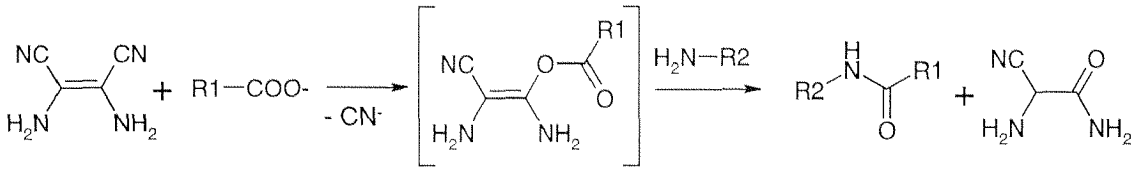


Figure 24: Mechanism for the condensation of amino acids using the HCN-tetramer.

Several kinds of condensed phosphates can be distinguished: linear, cyclic polyphosphates, and the branched phosphoanhydrides. It has been demonstrated, that volcanic activity can produce water-soluble phosphates and polyphosphates. [372] The branched phosphoanhydrides are extremely reactive, with very short hydrolytic half-lives, so that their accumulation on the primitive Earth is very unlikely. [356]

Trimetaphosphate (**9**) gives very high yields (up to 40%) of dipeptides when incubated at slightly alkaline pH, low temperature, and with low concentrations of Gly, or Ala. For Ser no dimers are observed, but the major product is the *O*-phospho-serine (4%). [373]-[377] When starting with glycylglycine, the yield of tetrapeptide formed is about 2%. [378] If divalent ions such as magnesium are added, the yield can go up to 14%, although hexapeptides are also formed (1.4%). [379] It is believed that the divalent cation coordinates with the phosphoanhydride and partially withdraws negative charge from the oxygen atoms, causing a larger positive charge on the phosphorous atom. This effect facilitates attack by nucleophiles and forms more stabile intermediates (see Figure 25). [356], [379]

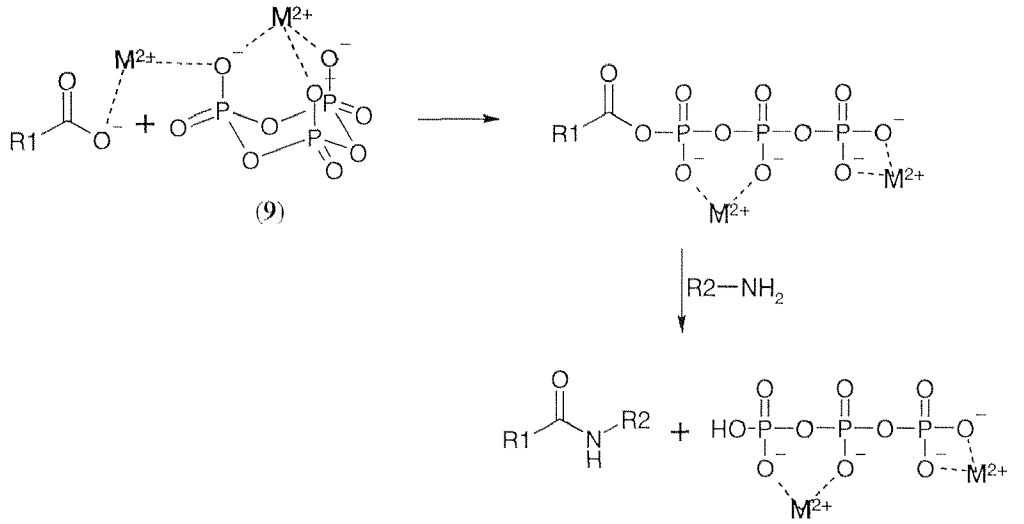


Figure 25: A possible reaction mechanism for the condensation with trimetaphosphate (**9**) in the presence of divalent cations. [379]

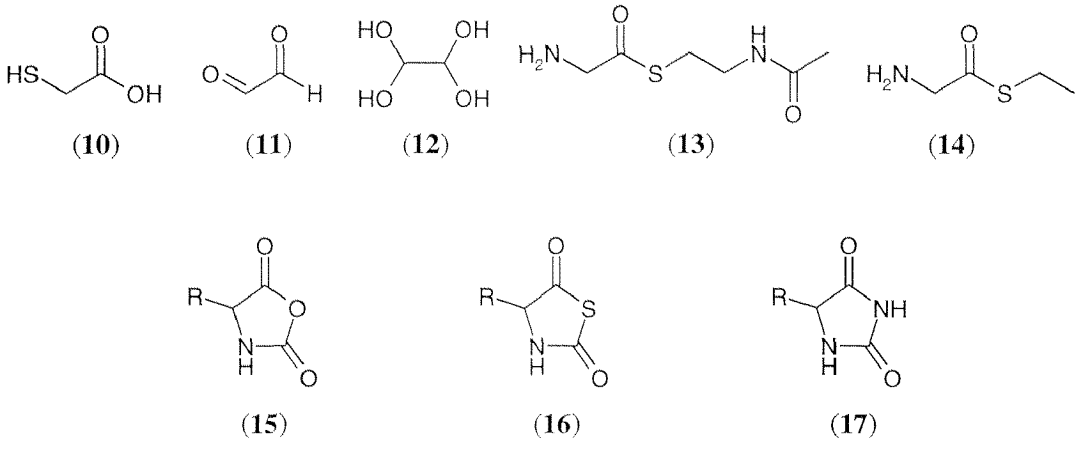
4.2.2.2. Activation Groups

Activating groups for amino acids with possible prebiotic relevance are esters (including thioesters) and anhydrides (including *N*-carboxyanhydrides, their thio equivalents and phosphoanhydrides). For some of these compounds, plausible prebiotic chemical pathways for their formation have been demonstrated, for others this has not yet been done. This paragraph will mainly discuss the capability of such activated amino acids to oligomerize in aqueous solutions.

The aqueous polymerization of *p*-nitrophenyl esters of proteinaceous amino acids is quite efficient in the presence of sodium hydrogen carbonate. [380] The reason for this seems to be the intermediary formation of *N*-carboxyanhydrides (see below), probably via the carbamate. [381] For Leu, oligoleucines up to a polymerization degree of 10 with a total yield of 77% are obtained in this way. When equimolar mixtures of p-nitrophenylesters of Ala, Leu, Val, β-Ala, and α-amino butyric acid were subjected to oligomerization, the precipitate was somewhat enriched in proteinaceous amino acids. [381] It has also been observed, that the presence of CO_2 accelerates the polycondensation of Gly-OEt and Ala-OEt at 55°C via carbamate formation. [381], [382]

Thioesters are more reactive than regular esters. Some prebiotic chemical pathways which lead to amino acid thioesters have been described. One exploits the driving force

of pyrite (FeS_2) formation from FeS and H_2S for reductive acetylation of amino acids with mercaptoacetic acid (**10**). [383] Another is the internal redox reaction of an α-ketoaldehyde (**11**) or glyoxal (**12**) in the presence of a thiol and ammonia for the amino acid thioester formation. [384], [385] Aminoacyl thioesters have been shown to form of peptides by autocondensation. [386]-[388] If *S*-glycyl-*N*-acetylcysteamine (**13**) or *S*-glycyl-ethanethiol (**14**) were incubated in aqueous solutions at pH 9.5 and ambient temperature, high yields of di- (46%), tri- (10%) and tetraglycine (2%) resulted after 4 days. Most peptide synthesis products probably occur by direct condensation of the glycine thioesters. It is, however, possible that intermediates formed by reaction of the glycine thioesters with the buffers (like glycine imidazolide, glycine phosphoanhydride, and glycine carboxyanhydride) may sometimes be significant. [389]



(**10**) (**11**) (**12**) (**13**) (**14**)

(**15**) (**16**) (**17**)

Figure 26: Molecular structures of mercaptoacetic acid (**10**), α-ketoaldehyd (**11**), glyoxal (**12**), *S*-glycyl-*N*-acetylcysteamine (**13**), *S*-glycyl-ethanethiol (**14**), *N*-carboxyanhydride amino acid (NCA) or Leuch's anhydride (**15**), 2-thio-NCA-amino acid (NTA) (**16**), and 2-aza-NCA-amino acid (hydantoïn) (**17**).

Even though linear anhydrides are often intermediates in the coupling of amino acids with various condensing agents, only their cyclic *N*-carboxyanhydride (NCA) form has been extensively used with respect to a prebiotic scenario. The activation by NCA is extremely interesting because of recent claims that NCA-amino acids (**15**) and the 2-thio (NTA) (**16**) and 2-aza (hydantoïn) (**17**) analogs are prebiotic compounds. [390]-[394]

The hydrolysis of NCAs is a reaction that was carefully investigated in several labs. [395]-[400] The reaction of NCA with water may take two extreme courses: (i) the formation of polypeptides at high NCA/H_2O molecular ratios (>10) and (ii) complete hydrolysis of the NCA at low NCA/H_2O ratios (<0.0001). Intermediate NCA/H_2O ratios

favor the formation of oligopeptides. [401] The controlled synthesis of peptides in an aqueous solution was systematically investigated using the reaction of NCAs of glycine, alanine, α-aminobutyric acid, and phenylalanine with other amino acids and the di- and trimer of Gly. [402], [403] A further extensive study of the mechanism of the reaction of amino acid NCAs was undertaken later. [404]-[406] Although additional secondary reactions were observed in these studies, it was concluded that a narrow range of experimental conditions exists in which NCAs can be usefully employed in aqueous solution for the synthesis of peptides, producing yields of about 90%. Optimal conditions for such condensations require careful control of reaction parameters such as pH, rate of mixing, temperature, choice of buffer, etc. [407]

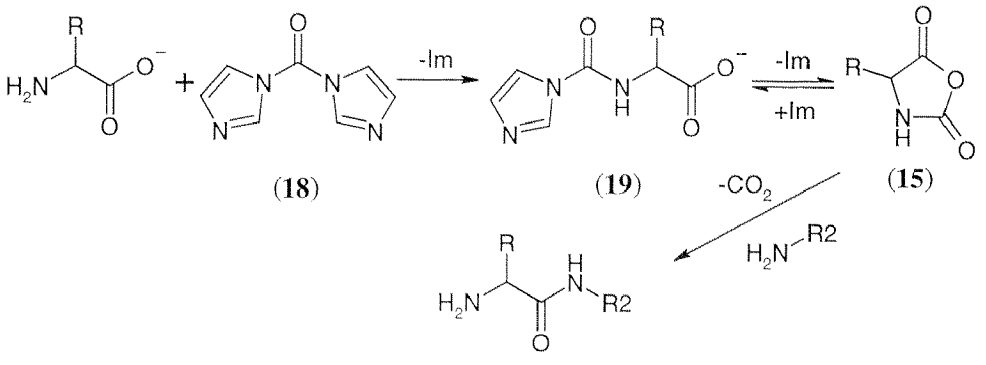


Figure 27: The mechanism of CDI-induced oligomerization of α-amino acids. The amino group reacts with the CDI (**18**) to give a *N*-[imidazolyl-(1)-carbonyl]-amino acid (**19**) which subsequently cyclizes to form an *N*-carboxyanhydride (NCA) (**15**). [390] NCA hydrolyzes spontaneously in aqueous solutions to give carbamic acid derivatives. Decarboxylation of the carbamic acids generates free amino acids whose amino groups can attack the carbonyl group of another NCA molecule, resulting in the formation of a peptide bond. [361], [401]

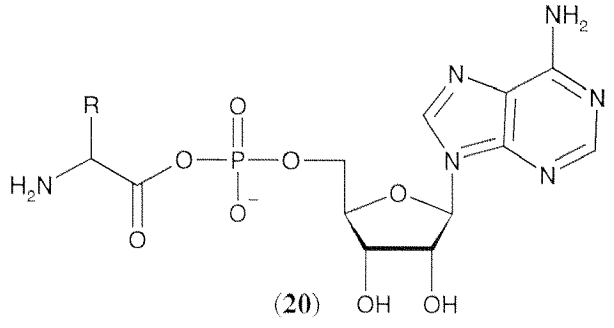
Even though the prebiotic relevance of *N,N'*-carbonyldiimidazole (**18**) (CDI) is controversial, [361], [390], [408] it has often been used efficiently as a condensing agent for the *in situ* formation of prebiotically relevant NCA-amino acids. [390] When CDI is added to a buffered solution of Gly, Ala, or Leu at pH 7 and 0°C, a slow reaction leads to the formation of 70-80% of polymeric material with polymerization degrees of up to 5, 6, and 8, respectively (see Figure 27). [390], [381], [409] When Phe was used instead of Gly or Ala, a 13% total yield of oligophenylalanine was detected immediately after the addition of CDI. [390] Ser and Thr fail to oligomerize to peptides under similar conditions. His oligomerizes up to a 9mer in excellent yield (85-90%) at 30°C, although via a slightly different mechanism. [410], [411] Much more efficient is the NCA-

oligomerization of Asp and Glu. [361], [412] In a typical reaction at 16°C, Asp can oligomerize up to the 13mer with a total yield of oligomers of about 75%. Glu results in up to the 18mer and a total yield of at least 90%. Under similar conditions, *O*-phospho-serine gives a 9mer, also in 90% yield. All these condensations of negatively charged NCA-amino acids are catalyzed by divalent ions, such as Mg^{2+}, Ca^{2+}, and Mn^{2+}. [412] This observation led to NCA condensation experiments in the presence of positively charged clay mineral surfaces. By sequential feedings of freshly prepared NCA-amino acids solutions at low concentrations to clay minerals (hydroxyapatite and illite), it was possible to obtain oligomers up to 50 monomers in length for Glu, 27 for Asp, and 13 in the case of *O*-phospho-serine. [154], [413] Similar results were obtained for γ-carboxyglutamic acid. [414] The 'polymerization on the rocks' scenario can be extended to positively charged amino acids. Using the feeding technique, oligomerization of Arg on illite led up to the 12mer, whereas in the absence of the mineral, the longest detectable oligomer was the 6mer. Interestingly, FeS_2 also favors the oligomerization of Arg, even though it does not bind oligoarginine. [362]

N-Carboxyanhydrides are good candidates for selective oligomerization of α-amino acids. Ring-closure to the 5-membered ring of NCA in the case of α-amino acids is kinetically favored compared to the 6-membered ring of β-amino acids and for larger rings it is not effective (Baldwin-rule). Selective oligomerization was shown with an aqueous mixture of proteinaceous amino acids (Gly, Ala, Glu, Val) and non-proteinaceous amino acids (isovaline, α-amino butyric acid, γ-amino butyric acid, β-alanine), that was treated with CDI. The polycondensate, obtained in 55% yield, was enriched in monosubstituted α-amino acids. [381] Another unusual feature of CDI-induced oligomerization is that only the monomeric amino acids but not the oligomers are activated. Consequently, oligomers can extend only by reacting with activated monomers and not by ligating with each other. Therefore, carboxyl-activated dipeptides are not generated and the formation of 2,5-diketopiperazine (cyclo-dipeptide) does not occur to a significant extent, which is often a problem when other condensing agents such as carbodiimides are used. [361]

It is assumed that aminoacyl adenylates (**20**) were synthesized spontaneously in the prebiotic ocean from amino acids and ATP, [356] which itself might have been synthesized spontaneously in an aqueous medium equilibrated with the prebiotic atmosphere. [415] The aminoacyl adenylates which are precursors of polypeptides in

contemporary living systems [416] are highly reactive and undergo spontaneous formation of peptides at slightly alkaline pH. Alanyladenylate at pH 10 produces tetraalanine in total yields of 60%. [417] Prolyladenylate even yields traces of hexaproline at pH 8.5. [418] Non-random polymers were obtained in 5% yield when adenylates of 16 different amino acids were incubated together. [419] When alanyladenylate was incubated for 24h in the presence of clay minerals, such as montmorillonite, the degree of polymerization has been reported to increase drastically to over 50. [416], [420] However, montmorillonite-mediated polymerization of alanyladenylate in other laboratories has not lead to large oligopeptides in quantitative yields. [421], [422]



(**20**)

Figure 28: Molecular structure of aminoacyl adenylate (**20**).

4.2.3. Matrices

It is assumed that the concentration of each amino acid type in the primordial soup was less than 1 mM and that the concentrations of their activated forms would most likely have been even lower. [115] Matrices are often an efficient tool to overcome the competing hydrolysis reaction in aqueous condensation experiments of amino acids at low concentrations. Matrices accumulate the activated monomers on their surfaces by specific or non-specific interactions and locally increase their concentration, which eventually leads to longer oligomers. Plausible prebiotic matrices include clays and other minerals, [89], [106], [110], [423] and surfactant aggregates such as micelles and liposomes. [74], [104], [424]-[426]

4.2.3.1. Clay and Other Minerals

Reaction of volcanic rock with water results in the dissolution of aluminum and silicon compounds, which then react with each other to form the alumina silicates that constitute

clays. Monmorillonite clay for example, consists of aluminosilicate in which Fe(II), Fe(III) and Mg(II) are substituted for some of the Al(III), and some of the Si(IV) is substituted by Al(III). Because the oxygen content does not change, the substitution of a higher valent metal ion by a lower valent one generates a negatively charged sheet. This negative charge is balanced by cations, such as Na^{+}, Ca^{2+}, and Mg^{2+}. Cationic organic compounds bind to such clay surfaces by the displacement of exchangeable metal ions. Divalent metal ions are especially helpful in enhancing the binding of anionic organics, since they are capable of simultaneously binding the clay surface and the negatively-charge compound. [427]

As described above, charged amino acids with and without various condensing agents and heating and wetting cycles, [336]-[342] EDAC or CDI activated charged amino acids, [154], [362], [413], [414] and aminoacyl adenylates [416], [420] have been condensed in the presence of such clay minerals. In such systems the length of the oligomers produced is often clearly enhanced.

Multiple-feeding experiments with freshly activated amino acids at low concentrations have been performed in some cases. [154], [362], [413], [414] After each feeding cycle and an appropriate incubation time, the clay mineral was centrifuged to the bottom of the tube and the aqueous supernatant removed. An aliquot of freshly activated amino acid was then added again for the next incubation round and so forth. With 50 feeding rounds, oligomers with polymerization degrees of up to 50 were obtained. [154], [413] Binding studies have shown that higher oligomers (e.g. 7mer produced in the first round) are almost completely adsorbed to the clay mineral surface. Therefore, long oligomers remain in the tube and elongate with each additional feeding round. At the end of the experiment, they are washed from the clay mineral surface with high salt concentrations (ion-exchange). [413], [428] It is important to emphasize that clay minerals such as illite, montmorillonite, and hydroxyapatite act only as solid-phase supports, but there is no evidence that they chemically catalyze the oligomerization. The only exception to this is FeS_2 in the case of NCA-arginine condensation, where no binding occurs. [362]

Despite the fact that multiple-feeding protocols involving clay and other minerals promote efficient polycondensation and yield high oligomers, the chemical and physico-chemical properties of the support must be tailored to the different types of amino acids. Therefore, the possibility of co-oligomerization by this mineral method seems very limited.

4.2.3.2. Surfactant Aggregates

Surfactants are surface active, amphiphilic substances. Phospholipids constitute a subgroup of surfactants and usually occur in biological membranes. Amphiphiles, consisting of hydrophilic "heads" and hydrophobic "tails" can associate into a variety of supramolecular structures in aqueous solution. These surfactant aggregates may transform from one into another when the solution conditions are changed, for example when the surfactant concentration, the electrolyte concentration, temperature, or the pH are changed. [429] Surfactants tend to concentrate as a monolayer at the air-water interface and reduce the surface tension of the air-water interface. Any excess surfactant that is not accommodated at the interface migrates to the bulk solution. If it is present there at higher concentrations than the critical aggregation concentration (CAC), it forms aggregates such as micelles or liposomes (also called lipid vesicles or simply vesicles) (see Figure 29). [430] The driving force behind this aggregation is the hydrophobic effect. [431]

Spherical micelles, shown in Figure 29A, are the smallest and simplest form of lipid aggregates. The polar headgroups are located mostly at the surface, and the core has a relatively hydrophobic surrounding. However, it must be noted that surfactant molecules in micelles are in dynamic motion. [432] Therefore, micelles have a relatively disorganized structure, with water molecules penetrating into the core and some alkyl chains exposed at the micellar surface. Micelles are typically formed from a small number of surfactants (50-100) and the size of the aggregate is limited (radius: 2-5 nm).

Membrane lipids associate to form bilayers in aqueous solution. These structures can be converted by: simple stirring, peeling, [433]-[435] sonication, [436], [437] cosurfactants, [438]-[440] organic solvents, [441], [442] or extrusion [443], [444] (depending on the surfactant used), into closed, self-sealing, water-filled liposomes, that are bounded by only a single bilayer, the so called unilamellar vesicles (UV) (Figure 29B), or a few concentric bilayers, the multilamellar vesicles (MLV). Some non-concentric multimembrane liposomes may even be multivesicular vesicles (MVV). Liposome sizes may vary from diameters as small as 20 nm (SV, small vesicle), 50 nm to 1 μm (LV, large vesicle), up to larger than 1 μm (GV, giant vesicle). [445] Liposomes are in equilibrium with the monomer surfactants in solution. For some lipids the kinetics of such processes are slow and the free monomer concentration is very low, so that the liposomes can be

separated from the solution in which they reside (i.e. by dialysis, gel filtration chromatography or centrifugation). In this case, liposomes with differing internal and external environments can be readily prepared. [446]-[448]

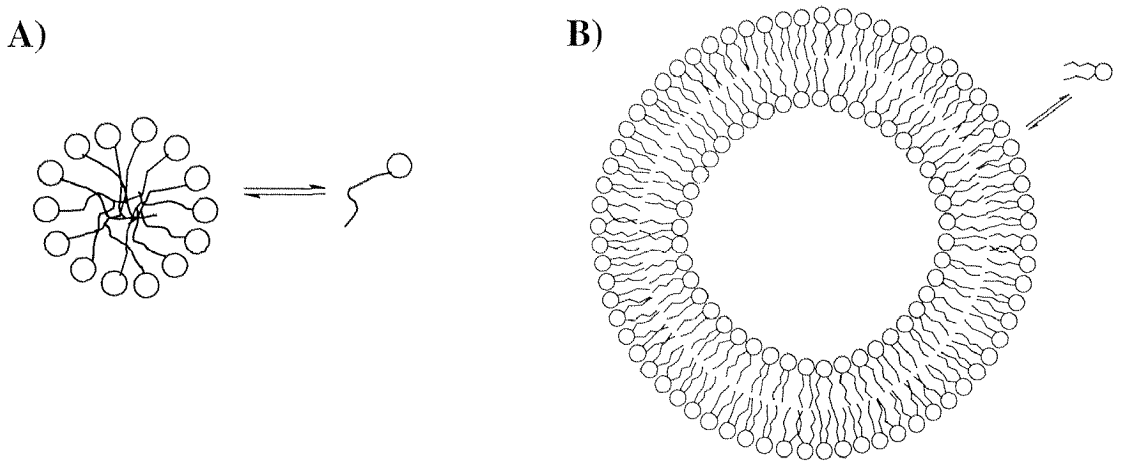


Figure 29: Examples of amphiphilic aggregate structures in water: A): Molecules with charged or large head groups tend to form spherical micelles with a hydrophobic core, which are in rapid equilibrium with the monomer in solution. B): Liposome formed by small regions of bilayers closing back on themselves to form a hollow spherical structure in which the interaqueous compartment is isolated from the surrounding solution. The surfactants in the membrane are generally in slow equilibrium with the monomer in solution.

From a prebiotic point of view ("origin of life"), liposomes can also be looked at as a type of (empty) protocell (see above). They also deserve attention for their extraordinary high mechanical stability, which is reflected by their huge size range (20 nm to 10 μm) and dynamic spherical shapes.

Lipid bilayers are practically impermeable to most polar substances (with some exceptions like water and glycerol), but charged substances can adhere to the membrane (i.e. through Coulomb interactions). Hydrophobic substances can be solubilized inside the membrane interfaces or the hydrocarbon core (i.e. hydrophobic interactions). Therefore, liposomal membranes can encapsulate many different substances either at the surface or in the enclosed water pool (i.e. entrap polar molecules or molecules of high molecular masses) (see Figure 30). Lipid composition and concentration, liposome size, and number of lamellae can affect this ability. [449] It is also a well known fact that lipophilic and amphiphilic proteins are solubilized in bilayers of biological membranes. [450], [451]

Surfactant aggregates can be used as matrices for the polycondensation of amino acids and peptides. In the literature, three types of polycondensation experiments have been

presented: with either (i) micellar aggregates (including reversed micelles), (ii) mono- or multilayers at the water-air interface or (iii) bilayer membranes.

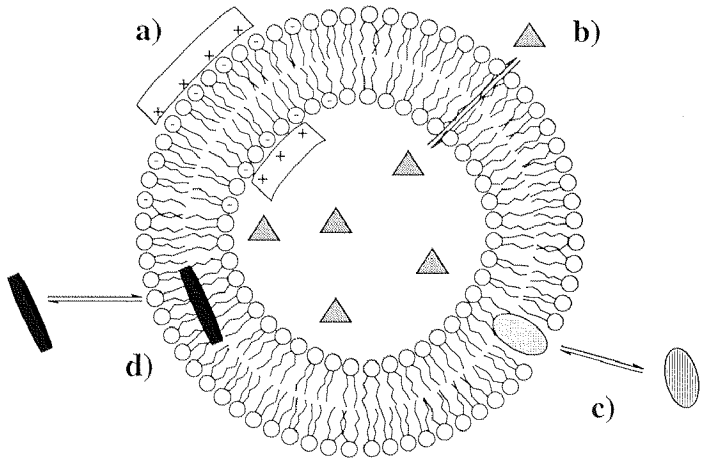


Figure 30: Possible sites of solute solubilization in liposomes and possible transformations: a) Electrostatic interactions (Coulomb forces) can lead to adhesion of oppositely charged (macro-) molecules to the headgroup-surface of charged liposomes. b) Dialysis or gel filtration chromatography allows the separation of liposomes from the solution in which they were prepared. With this procedure, hydrophilic molecules can be easily entrapped inside liposomes. c) Amphiphilic molecules participate in the bilayer as cosurfactants, although an excess can lead to mixed micelle formation. d) Substances which are hardly soluble in water incorporate into the hydrophobic core of lipid bilayers due to hydrophobic interactions.

In the aqueous phase of reversed micelles in benzene, alanyl adenylate is reported to oligomerize into polyalanines in yields of up to 95%, with some of them having the length of 42 amino acid units. [452] Cationic micelles of cetyltrimethyl ammonium bromide (CTAB) (**21**) can catalyze the condensation of NCA-activated, negatively charged amino acids at low concentrations (5-10 mM). *O*-Phospho-serine NCA's at pH 8 and 16°C led to oligomers up to the 8mer in the presence of cationic CTAB micelles, whereas in the reference system (absence of micelles) the trimer was the highest oligomer observed. For Asp and Glu, up to 10 and 15mers have been obtained in the micellar system, whereas the reference only led to oligomers up to the 5 and 6mer, respectively. It is assumed, that the negatively charged monomers are probably concentrated close to the cationic surface of the micelles, due to electrostatic interactions. The polyanionic oligomers, once formed, attach to the surface with somewhat more affinity since this increases with their length. The N-terminus of an oligomer is, therefore, in an environment enriched in activated monomers and

consequently the chance that an oligomer will be extended increases with its length. [453]

When a long-chain thioester of glycine, thioglycine *S*-dodecyl ester hydrobromide, is treated with water and weak bases, it undergoes condensation to oligoglycine *S*-dodecyl ester and diketopiperazine (7:3) in quantitative yields. Treatment with an alkaline solution leads to oligoglycines with an average degree of polycondensation of 10. The authors claim the reaction occurs in micelles formed by the glycine thioester (see Figure 31 A). [454]

The condensation in mono- and multilayers of hexadecyl or octadecyl amino acid esters of Gly and Ala at the water-air interface leads to oligoamino acids at room temperature at pH values of 6 to 8 (see Figure 31 B). If the pH in the aqueous subphase is increased to 10 and higher, hydrolysis of the long-chain ester is the main reaction and no oligomerization is observed. It was also found that an optimum packing for esters in the monolayer exists with respect to oligomerization. [455] Oriented polypeptide films have also been prepared by self-condensation of esters of non-natural long-chain amino acids such as methyl 2-aminooctadecanoate (**22**), docosanyl 2-aminooctadecanoate (**23**), methyl 2-aminohexacosanoate (**24**), and docosanyl 2-aminohexacosanoate (**25**). All of the four long-chain α-amino acid esters form monolayers at the water-air interface and self-condense to oligopeptides at slightly alkaline pH. In contrast to (**22**) and (**24**), the long-chain amino acid esters (**23**) and (**25**) form monolayers, in which the hydrophobic ester units are built into the membranes (Figure 31 C). By sonication in water under acidic conditions, (**23**), (**24**), and (**25**) form liposomes. Only the liposomes of (**24**) are stable in alkaline solutions. They condense to oligopeptides in the bilayer membrane (see Figure 31 D). [456]

The glycerophospholipid, glycyl-*O*-(*O*-1,2-ditetradecyl-*sn*-glycerophospho)-seryl-glycine propargyl (**26**), also forms liposomes at pH 8.50 if ultrasonicated at 0°C. This type of liposome suspension forms oligomers up to 7mers in length (21 amino acids of the tripeptide head group via aminolysis of the propargyl ester) when incubated at 25 to 50°C. [457], [458]

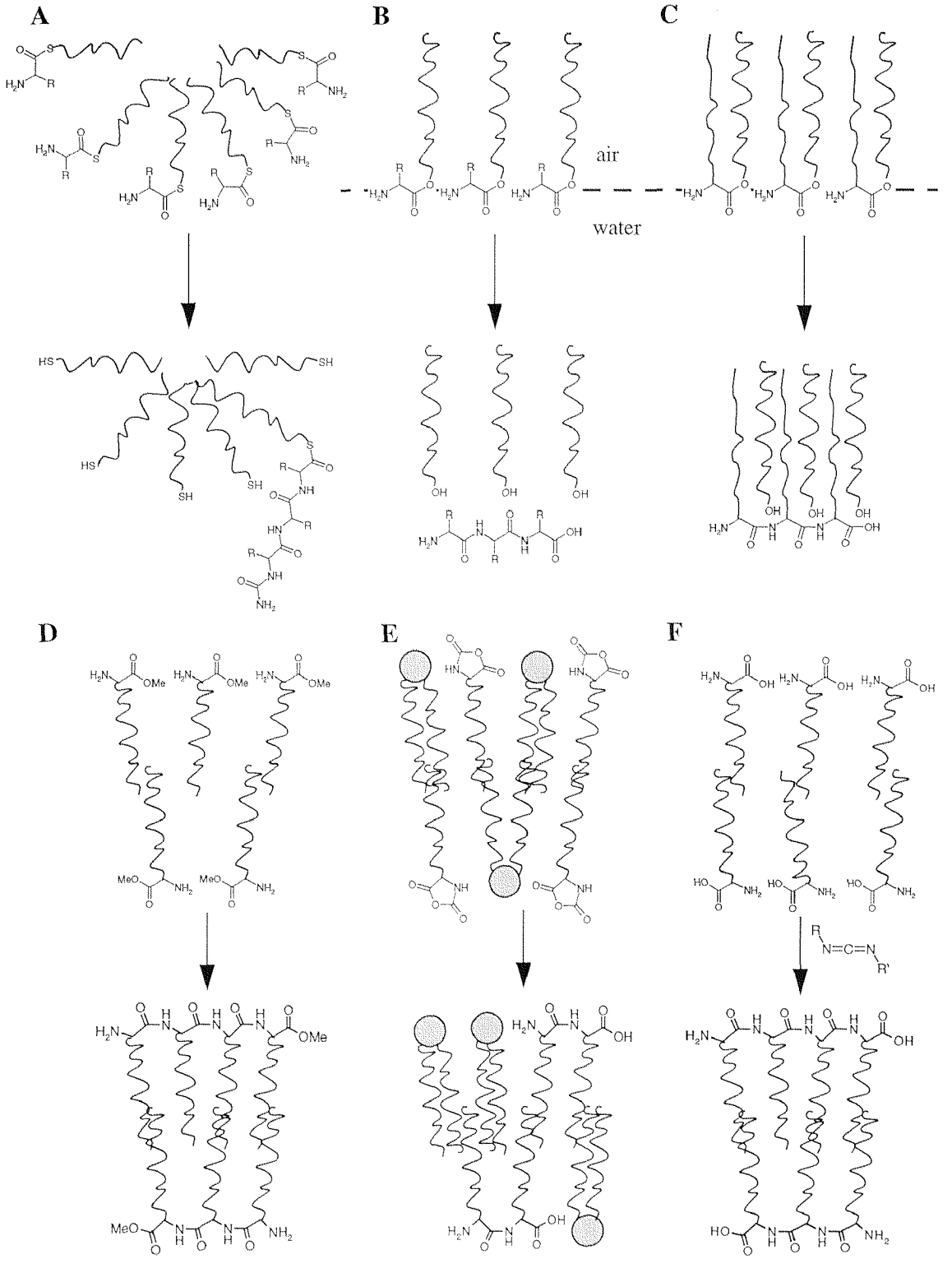


Figure 31: Schematic representation of the major polycondensations which have been published in the literature using surfactant aggregates as matrices.

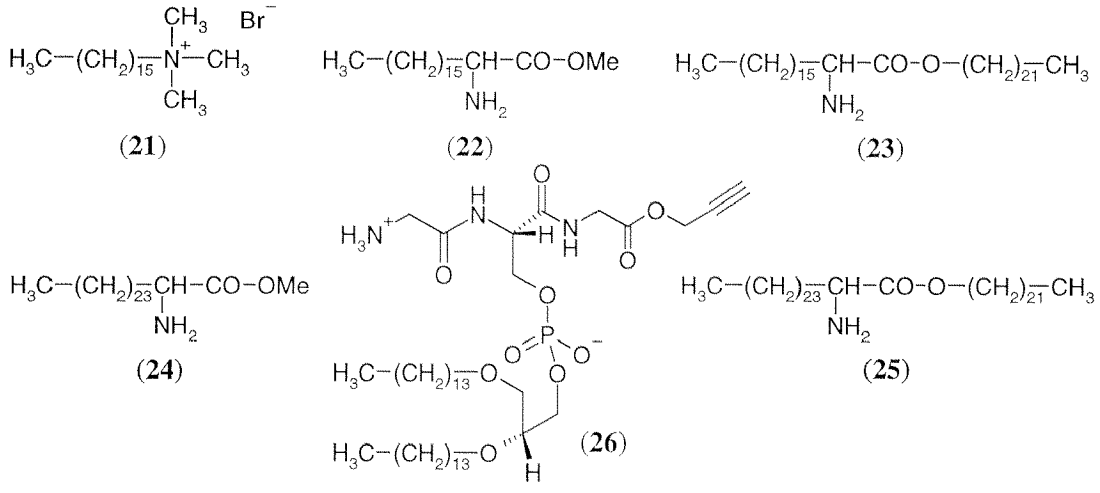


Figure 32: Molecular structures of cetyltrimethyl ammonium bromide (CTAB) (**21**), methyl 2-aminooctadecanoate (**22**), docosanyl 2-aminooctadecanoate (**23**), methyl 2-aminohexacosanoate (**24**), docosanyl 2-aminohexacosanoate (**25**), and glycyl-*O*-(*O*-1,2-ditetradecyl-*sn*-glycerophospho)-seryl-glycine propargyl (**26**).

The polycondensation reactions in oriented monolayers, micelles, and liposomal bilayers occur only as a consequence of the high packing density of the reactive groups and their orientation in the bilayers. No polycondensation is detected in solution below the CAC, where no aggregates are present. [456]

N-Carboxyanhydrides with hydrophobic side chains of γ-dodecyl-L-glutamate (**27**) and γ-benzyl-D-glutmate (**28**) have been oligomerized in bilayer membranes of large unilamellar 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) (**29**)/dicetyl phosphate (DCP) (**30**) liposomes (see Figure 31 E). From these studies, it is not clear to what extent the oligomerization in the presence of liposomes is enhanced compared to the reference system with no liposomes present. However, the results indicate that in the membrane-free aqueous phase the oligomerization of the hydrophobic NCAs promotes the formation of β-structured oligomers, while in the lipid bilayer membrane α-helical structures predominate. Further, it was observed that the water permeability of the liposomal membrane is increased in the presence of oligomerization products. [459], [460]

Amino acid dicarboxylic amphiphiles which contain either cysteine (**31**) or homocysteine (**32**) form liposomes if extensively sonicated above 60°C. Treatment of these liposomes with the water-soluble carbodiimide-type condensing agent, 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate (**33**), during

and after sonication at room temperature leads to oligopeptides up to the decamer at the liposomal surface (see Figure 31 F). As mentioned above, these peptide liposomes also exhibit an increased membrane permeability compared to the precursor liposomes. [461]

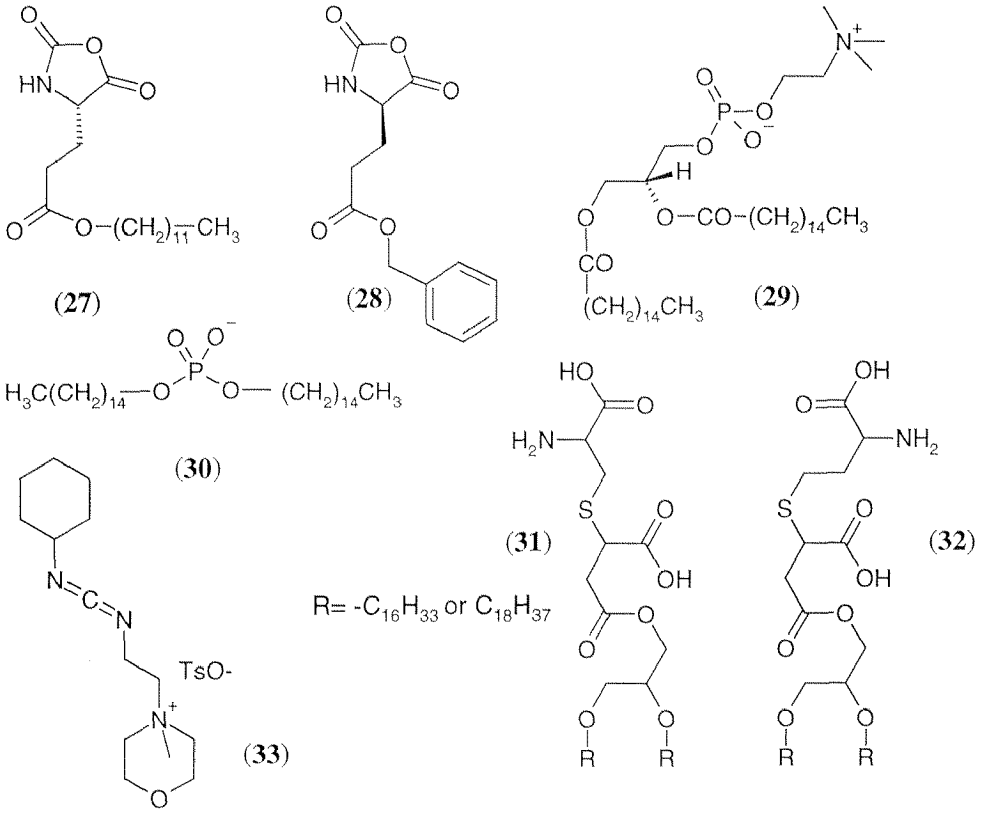


Figure 33: Molecular structures of γ-dodecyl-L-glutamate (**27**), γ-benzyl-D-glutmate (**28**), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) (**29**), dicetyl phosphate (DCP) (**30**), cysteine dicarboxylic amphiphile (**31**), homocysteine dicarboxylic amphiphile (**32**), and 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate (**33**).

4.3. Concluding Remarks on Prebiotic Peptide Condensation

Clearly much work has been done in the field of prebiotic peptide condensation. Nevertheless, the problem of the prebiotic formation of functional macromolecules is far from solved.

Experiments on peptide formation *via* HCN polymers gave small quantities of short peptides in complex mixtures of other compounds produced via side reactions.

One may propose that oligopeptides on the primitive Earth formed directly from amino acids, since they can be assumed to have been present in a prebiotic environment in concentrations in the millimolar range. Attempts to condense them in aqueous solutions has been achieved many times in different ways. Neat thermal condensation or heating-

wetting cycles of amino acids in solution has indeed produced peptides with molecular weights of up to a few thousand daltons, but the resulting dark and hardly soluble mass is rich in crosslinked, branched products. Furthermore, the amino acids are often epimerized under these conditions, and the peptidic content of the product is only about 50%. Experiments simulating submarine hydrothermal vents have yielded hexapeptides from Gly, but products higher than dimer are not detected with other amino acids. At this point it must be mentioned that the production of oligoglycines, which can be synthesized in many different ways compatible with prebiotic scenarios, is not representative for the formation of peptides with other amino acids, as is often suggested. Chemical condensing agents that are believed to have been present on primitive Earth in high enough concentrations include dicyanamide, cyanamide dimer, probably carbodiimides, and trimetaphosphate. The first two fail to produce oligomers longer than the trimer. Carbodiimides generally inhibit the formation of higher oligomers because of diketopiperazine formation from dipeptides. Phosphates have yielded some hexapeptides, but again using Gly as a model system which is not representative. Synthesis of polypeptides from amino acids that have already been chemically activated has also been investigated, but this approach has questionable their prebiotic relevance. Plausible chemical pathways for the synthesis of activated amino acids in a primitive environment have to be demonstrated experimentally. The only two activation groups reported in the literature that fulfill this criteria so far are thioesters and anhydrides. Aminoacyl adenylate fails, despite its efficiency, because a pathway to ribonucleotides is still lacking. Thioesters are efficient but have only been shown to function for the polymerization of Gly. The only anhydride that as been explored to any great extent with respect to prebiotic condensation is the cyclic *N*-carboxyanhydride. There are several plausible pathways for its formation on primitive Earth. The NCA condensation shows some selectivity for α-amino acids over β-amino acids, and the condensation of NCA-amino acids produces good yields of oligomers when starting monomer concentrations are high. Despite the fact NCA-amino acid polycondensation is efficient in yielding large oligomers by multiple-feeding to clay and other minerals, different kinds of minerals with different chemical and physico-chemical properties are needed for different types of amino acids. Therefore, the possibility of co-oligomerization by the clay mineral method seems very limited and will probably not work at all for hydrophobic amino acids. Rather promising are the results obtained using surfactant aggregates as matrices for

peptide oligomerization. Apart from the experiments done with NCA-Asp and NCA-Glu and positively charged micelles, where electrostatic interactions are important, no results have yet been published (the present work excluded) on the liposome-assisted condensation of any of the 20 biological amino acids or their peptides in aqueous solution. Therefore, it was one of the aims of the present work to shed some light on that field using both a condensing agent and *in situ* produced NCA-amino acids in the presence of liposomes. The focus in the future on selectivity and stereoselectivity of such reactions seems to be of special interest with respect to the origin of life and homochirality.

5. Results and Discussion

As we have seen above, the formation of macromolecules and in particular functional macromolecules is still one of the unsolved problems in the origin of life field of research. Macromolecules would have been necessary for any system that possessed the characteristics of life today, namely, metabolism, reproduction, transcription and translation of genes, diversification through mutation of the genetic code, and construction from one or more cells which have substructures and biochemical reactions occurring within them that are efficiently catalyzed by enzymes.

Assuming that the origin of life was a deterministic event - a result of the operation of natural laws on a physico-chemical system - one might imagine possible pathways leading to the synthesis of macromolecules within the framework of the currently widely accepted scenario of chemical and molecular evolution. One can further assume that increasing complexity of the prebiotic environment by various chemical reactions between molecules in the prebiotic library on Earth would have led to molecules of higher molecular masses, some of which (i.e. short peptides or organo-metallic complexes) would possess weak catalytic properties. These catalysts would have allowed more efficient reactions to take place, and thereby speed up molecular evolution. The interaction of molecules in this large pool of educts, products, and catalysts, might have led to complex networks of chemical reactions. If the mean concentration of the catalytic molecules was maintained at a certain level for a longer time period, such a metabolic system might have become more and more efficient in producing components of its chemical subnets. Since efficiency in catalysis of biochemical reactions seems to be related to increasing molecular mass of the catalyst, [462]-[464] these metabolic networks of catalysts probably culminated in mRNA-based translational production of gene encoded proteins - the essential macromolecules of life today - which themselves are involved in the reproduction of such genetic systems.

In order to restrict the numerology problem of the primary structure of oligopeptides, and to enhance the probability of an oligopeptide being formed over and over again to maintain high concentrations in a prebiotic environment, there must have been ways for efficient, selective peptide condensation and accumulation that also operated at low concentrations.

I strongly believe that the appearance of compartments such as liposomes early on was essential for the transition to life. The spherical shape of liposomes is in the range of living cells today and they enclose a pool of water. Their permeability barrier properties can be influenced by changes in temperature or interactions with certain substances (i.e. peptides). Their different solubilization sites and binding capabilities make liposomes unique compartments. Using liposomes with these properties for dividing the huge batch of the prebiotic soup into much smaller microcompartments, interesting properties would have emerged. For example, molecules synthesized at the liposomal membrane or inside the interior aqueous pool will be physically or even chemically associated with the liposome and differentiate the microenvironment from its surrounding. Because this can occur multiple times in close proximity, molecular evolution could exploit several, parallel tracks of developement by microcompartimentalization.

In this work, two complementary polycondensations of amino acids in the presence of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) (**34**) bilayers as matrices were investigated and compared to an aqueous reference system. In the first approach, the classic polycondensation method of *N*-carboxyanhydride (NCA) derivatives of hydrophobic amino acids was examined. In the second approach, the hydrophobic condensing agent EEDQ (2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline), which exhibits an affinity for the hydrophobic membrane of lipid bilayers, was used to condense Trp-containing dipeptides. These polycondensations are promoted by a selection process based on a hydrophobic interaction between the bilayer and peptides. POPC membranes can also exploit electrostatic interactions if positively or negatively charged cosurfactants are added to them. Mixed liposomes with variable charge densities were prepared using the positively charged dimethyl didodecylammonium bromide (DDAB) (**35**) or the negatively charged 1,2-dioleoyl-*sn*-glycero-3-phosphate (DOPA) (**36**). Such charged membranes were used to bind oppositely charged amino acids or peptides, making polycondensation of mixtures of amino acids and peptides based on both hydrophobic and electrostatic interactions possible. The synthesis of oligopeptides consisting of polar and hydrophobic amino acid residues in the same chain will be presented.

In the last part of this study, it will be shown that in the case of NCA-amino acid condensation, the stereochemical interaction between the liposome matrix and the monomer had no particular influence on the product distribution. However, the NCA

condensation of an amino acid racemate itself showed some interesting features, namely preferential formation of homochiral oligomers at higher degrees of oligomerization. Their representation in the stereoisomer distribution of higher n-mers is several times higher than expected based on random distribution.

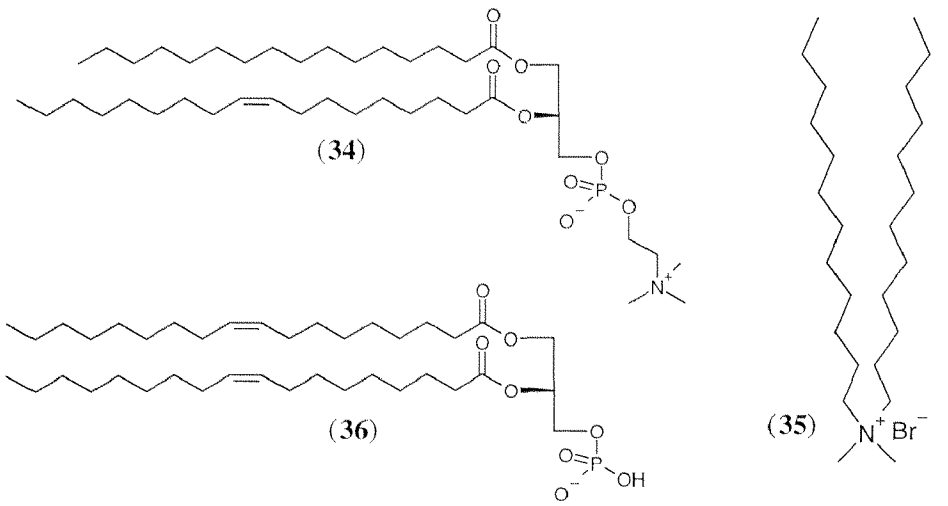


Figure 34: Molecular structures of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) (**34**), dimethyl didodecylammonium bromide (DDAB) (**35**), and 1,2-dioleoyl-*sn*-glycero-3-phosphate (DOPA) (**36**).

5.1. Amino Acid and Peptide Condensation on POPC Liposomes

As we have seen above, anhydrides of amino acids readily undergo condensation in aqueous solution. The main competing reaction is hydrolysis. In the following, the results of condensations with *in situ* formed NCA-amino acids are discussed first; the condensation of dipeptides using the condensing agent EEDQ (which forms a peptide anhydride as intermediate) is considered subsequently. Comparisons of the condensation of NCA-amino acids and peptides in the presence of POPC liposomes with an aqueous reference system are emphasized.

5.1.1. CDI-Induced Condensation of Amino Acids

A review of the literature on the condensation reactions of NCA-amino acids in aqueous solution was presented in paragraph 4.2.2. The current section deals with the effect of liposomes on the CDI-induced condensation of hydrophobic amino acids[1].

[1] All amino acids were in the L-form if not otherwise specified.

If Trp solutions in 0.4 M imidazole buffer (pH 6.80) were incubated with a 2.5-fold excess of CDI at 0°C for 2 min, yields of up to 95% of NCA-Trp (ESI^+ m/z: 231.1) and *N*-[imidazolyl-(1)-carbonyl]tryptophan (ESI^+ m/z: 299.1) were obtained. Figure 35 shows the time dependent decrease of activated NCA-Trp (□) which undergoes oligomerization and *N*-[imidazolyl-(1)-carbonyl]tryptophan (●) which subsequently cyclizes to form an NCA-Trp, once condensation of NCA-Trp has already started. Both are also partially hydrolyzed. The mechanism for the CDI-induced condensation is shown in Figure 27.

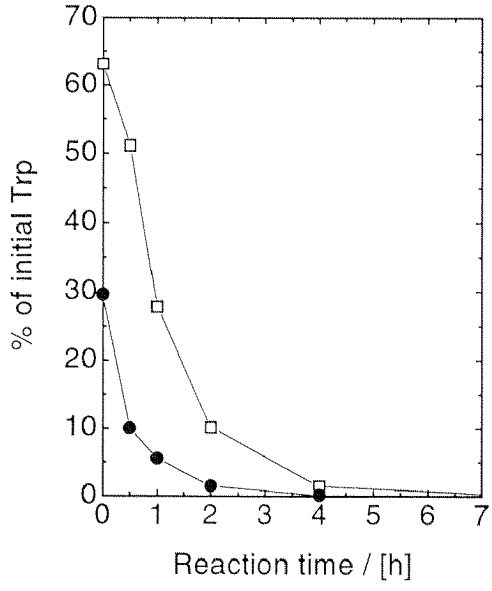


Figure 35: The concentration of activated tryptophan species, NCA-Trp (□) and *N*-[imidazolyl-(1)-carbonyl]tryptophan (●) formed by incubation of Trp with CDI, decrease with time as *N*-[imidazolyl-(1)-carbonyl]tryptophan is transformed into NCA-Trp and the condensation reaction of NCA-Trp proceeds. A 20 mM Trp solution in a 0.4 M imidazole/HCl buffer, pH 6.80, was incubated for 2 min at 0°C with a 2.5-fold excess of CDI and mixed 1:1 (v/v) with a 100 nm extruded 20 mM POPC liposome suspension in 0.4 M imidazole/HCl buffer, pH 6.80. The first sample was taken immediately (t = 0h). The remaining solution was further incubated at room temperature. Samples were taken at the appropriate times and analyzed by LC-MS. The major secondary reaction is the hydrolysis of the two intermediates.

5.1.1.1. Single-Feeding Experiments

If stock solutions of NCA-Trp were mixed 1:1 (v/v) with 0.4 M imidazole/HCl buffer (pH 6.80) at room temperature and incubated for 12 hours, oligo-Trps up to the 8mer (but never higher) were obtained as shown in Figure 36A. The precipitates, which were usually observed when no liposomes were present and at Trp concentrations higher than 10 mM, were solubilized by adding one volume equivalent of acetonitrile (ACN) before injection into the HPLC. In this case, oligomers no longer than the 8mer were detected.

However, if the NCA-stock solution was mixed with a 100 nm extruded POPC liposome suspension in 0.4 M imidazole/HCl buffer, pH 6.80, much higher oligomers were obtained. In the presence of liposomes the highest oligomers formed exceeded by far the maximal observed length of 8 monomer units - as observed in the buffer reference system (see Figure 37).

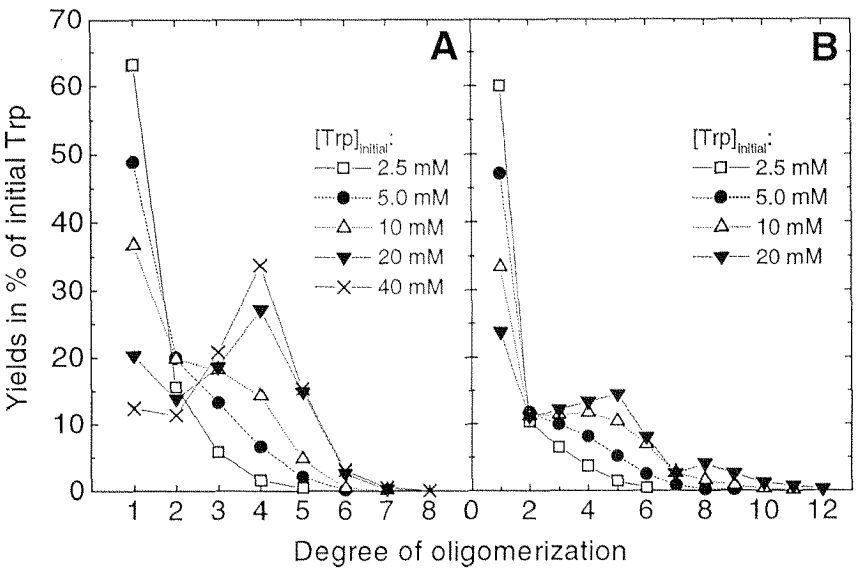


Figure 36: The effect of NCA-Trp concentration on the condensation is shown for *in situ* formed solutions of 2.5, 5.0, 10, 20, and 40 mM NCA-Trp in 0.4 M imidazole/HCl, pH 6.80 (A); and 2.5, 5.0, 10, and 20 mM NCA-Trp in the presence of 100 nm extruded 25 mM POPC liposomes in 0.4 M imidazole/HCl, pH 6.80 (B). The reaction samples were incubated on a shaker for 12 hours at room temperature prior to analysis by HPLC.

Higher concentrations of Trp in the starting solution increased the yields of higher oligomers as well as the oligomerization degree of the longest oligomer detected (see Figure 36 B). In contrast to the reference condensation (buffer only), no precipitation of condensation products was observed up to the maximal possible NCA-Trp concentration of 40 mM (limit of Trp solubility in the starting solution) in the liposome system. Between a concentration of 0 and 10 mM POPC, increasing lipid concentration seemed to improve the elongation of the oligo-Trps formed, whereas concentrations above 10 mM of POPC did not have much influence on the product distribution or their length. Also, varying liposome size (50 nm, 100 nm, and 400 nm extruded) from a 20 mM POPC in 0.4 M imidazole/HCl buffer (pH 6.80) suspension did not affect the products formed. No significant difference was observed when DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine) was used instead of POPC. A slight pH dependence was observed

around the optimum of pH 6.80, so that this pH value was chosen for all following NCA-amino acid condensation experiments.

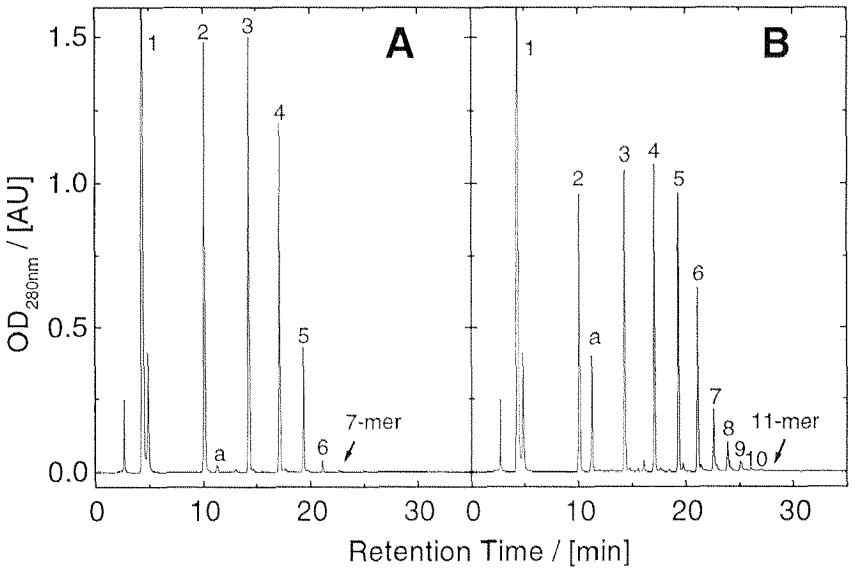


Figure 37: The two NCA-Trp oligomerization chromatograms in the absence (A) and presence of liposomes (B) are compared. A 40 mM NCA-Trp solution in 0.4 M imidazole/HCl, pH 6.80, was mixed 1:1 (v/v) and incubated for 24 hours at room temperature with either (B) a 100 nm extruded 20 mM POPC liposome suspension in 0.4 M imidazole/HCl, pH 6.80; or (A) 0.4 M imidazole/HCl as reference. Numbers indicate the oligomerization degree n of the oligo-Trps, whereas (a) stands for the 2,5-diketopiprazine (cyclo-dipeptide) of Trp.

Experiments with amino acids other than Trp were informative. A similar, but less pronounced effect of extruded POPC liposomes on the condensation of NCA-Phe was observed; a weak but marginally insignificant influence of the lipid membrane was found in the case of His, Met, Leu, Val, and Ile (see Table 10). Since the aromatic amino acid side chains seemed to favor longer oligomers in the presence of lipid bilayers, the condensation of NCA-Tyr would have been of particular interest, too. This experiment failed, however, due to the low solubility of Tyr under the conditions used.

The effect of longer oligomer product formation in the presence of POPC liposomes is most pronounced for amino acids having the lowest whole-residue free energies of transfer from water to the POPC bilayer interface ΔG_{wif} and to *n*-octanol ΔG_{woct} (see Table 10). These two whole-residue free energy scales of bilayer-interface and octanol hydrophobicities are strongly correlated, but the bilayer values are generally about one-half of those for octanol for all 20 naturally occurring amino acids. Some amino acids, however, show deviation from this behavior. For example, the β-branched hydrophobes, Val and Ile, are much less hydrophobic, whereas the aromatics Trp, Phe, and Tyr are

more hydrophobic towards the bilayer than expected. This suggests that the affinity of the aromatic NCA-amino acid and the efficient binding of its oligomers (in particular the 4mer and higher) to the liposomal surface might be responsible for longer oligomer formation with Trp and Phe in the liposome system.

Table 10: Since yields for oligopeptides formed from amino acids with no chromophoric side group are difficult to determine, the maximal degrees of oligomerization detected are given for the different NCA-amino acid condensations in the absence ($n_{max,Ref}$) and presence ($n_{max,POPC}$) of 100 nm extruded POPC liposomes. For all amino acids (except His), an *in situ* prepared 40 mM NCA-amino acid solution in 0.4 M imidazole/HCl, pH 6.80, was incubated 1:1 (v/v) either with 0.4 M imidazole/HCl, pH 6.80 (Ref), or 100 nm extruded 40 mM POPC liposomes in a 0.4 M imidazole/HCl buffer of pH 6.80 (POPC) for 12 hours at room temperature. ΔG_{wif} and ΔG_{woct} are whole-residue free energies of transfer from water to the POPC bilayer interface (wif) and to *n*-octanol (woct), respectively. These values were determined experimentally [465], [466] by examining the partitioning of two peptide families into large unilamellar liposome membranes formed from POPC. One family consisted of the complete set of acetyl-TrpLeu-X-LeuLeu-OH peptides with X being any of the natural amino acids and the other peptide family of the homologous series of acetyl-TrpLeu$_m$-OH peptides with m=1-6. [467] His^0: uncharged, His^+: charged His.

Amino Acid:	$n_{max,Ref}$:	$n_{max,POPC}$:		ΔG_{wif}: (in kJ/mol)	ΔG_{woct}: (in kJ/mol)
Trp	6	12		-7.75 ± 0.25	-8.75 ± 0.46
Phe	7	9		-4.73 ± 0.21	-7.16 ± 0.46
Tyr	-	-		-3.94 ± 0.25	-2.97 ± 0.46
His[a]	10	11	His^0:	0.71 ± 0.25	0.46 ± 0.46
			His^+:	4.02 ± 0.50	9.76 ± 0.46
Met	9	10		-0.96 ± 0.25	-2.81 ± 0.46
Leu	10	10		-2.34 ± 0.17	-5.23 ± 0.46
Val	8	8		0.29 ± 0.21	-1.93 ± 0.46
Ile	6	6		-1.30 ± 0.25	-4.69 ± 0.46

a. NCA-His was also formed *in situ*, but this time a 40 mM His solution in 50 mM imidazole/HCl, pH 6.80, was incubated with a 2-fold excess of CDI for 20 min at 0°C and thereafter incubated 1:1 (v/v) either with 50 mM imidazole/HCl, pH 6.80 (Ref), or 100 nm extruded 50 mM POPC liposomes in a 50 mM imidazole/HCl buffer of pH 6.80 (POPC) for 168 hours at room temperature.

Partitioning of peptides into lipid bilayers, which are often considered to be 'non-polar' phases, is quite complex. Figure 38 shows that the fluid bilayer can be divided (vertical lines) into two interfacial regions and one hydrocarbon core region. This separation is based upon the distribution of the hydration water of the headgroups. The hydrocarbon

region and the combined interfacial regions are each about 30 Å in width, so that the bilayer interfaces account for about 50% of the total thickness of the lipid membrane. The bilayer is not simply a thin, apolar, and spherical microphase separated on the inside and outside by an aqueous phase. It is better described as a hydrocarbon core embedded between two regions of particular importance for interfacial partitioning that consist of a complex mixture of water and phosphocholine, glyceryl, carbonyl, and methylene groups. These interfacial regions are especially rich in possibilities for non-covalent interactions with peptides. [466]

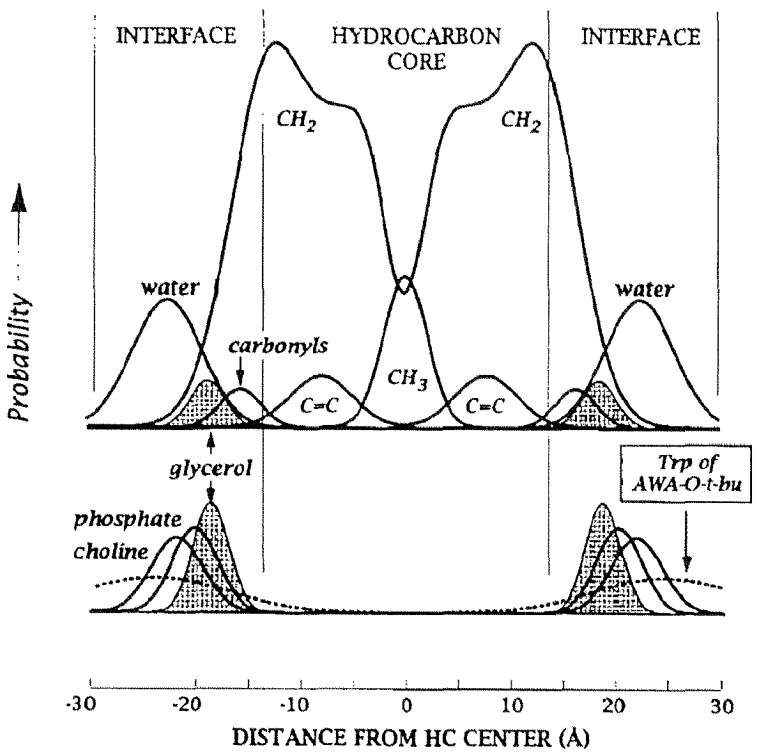


Figure 38: The structure of a DOPC bilayer determined by the joint refinement of X-ray and neutron diffraction data from [468]. In the graph, the time-averaged distributions of the principal structural groups of the lipid, constructed from Gaussian distributions, whose areas equal the number of structural groups represented by the Gaussian, are projected onto an axis normal to the bilayer plane. The distributions therefore represent the probability of finding a structural group at a particular location. Also shown is the distribution of the Trp-containing H-AlaTrpAla-*O*-*t*-butyl (AWA-O-t-bu) partitioned into the bilayer from [469]. The figure was adapted and modified from [466].

Included in Figure 38 is the transbilayer distribution of the Trp residue of the small peptide H-AlaTrpAla-*O*-*t*-butyl, as determined by neutron diffraction. [469] Similar results have been obtained and published from NMR studies on tryptophan analogues, such as indole, 3-methylindole, N-methylindole and indene, and large multilamellar POPC liposomes or other phosphocholine lipids. [470], [471] The interfacial location of

Trp is believed to be due to the balance of the hydrophobic effect that tends to drive it out of water, complex electrostatic interactions that favor residing in the hydrated headgroup region, and cohesive repulsion that keeps it out of the hydrocarbon core. In this scenario, dipolar, quadrupolar, H-bonding, and π-cation interactions act together to determine the precise disposition of Trp within the lipid membrane interface. [470]-[472] Interesting in this context is also the fact that Trp residues in membrane proteins are found largely in the same region as the phosphocholine group of the membrane lipids and have important implications for the assembly and stability of these proteins. [473] Other recently published data show that equilibration between the inner and outer surface of POPC membranes through the flip-flop mechanism (transbilayer diffusion) of tryptophan analogues like *N*-acetyl-tryptophan-octylamid or tryptophan-octylester is very rapid (1-2 min) when these are added to a liposome suspension (from the outside). [474] This result suggests that NCA-Trp as well as the short oligo-Trps (i.e. the dimer or trimer) may show similar behavior. If NCA-Trp is added from the outside to liposomes it probably readily diffuses across the POPC bilayer via the flip-flop mechanism and therefore oligomerizes not only on the outer surface of the liposomes, but also on the inner surface.

All these arguments make the binding of NCA-Trp and the oligo-Trps to the POPC liposome membrane interfacial surfaces (on the inside as well as on the outside) very plausible. Binding prior to the CDI-induced condensation reaction leads to a slightly increased local concentration of NCA-Trp at the liposomal surfaces compared to the aqueous bulk. Liposomes therefore promote the NCA-Trp condensation to oligomers because of physical accumulation on their surface (see Figure 36). The short oligo-Trps remain bound to the membrane surface after their formation, since the higher number of tryptophan side chains increases their affinity to the liposome interfacial region. For the higher and practically water insoluble oligomers, the elongation steps take place exclusively at the membrane surface. The final products are very hydrophobic oligo-Trps of high degrees of oligomerization that are solubilized by the POPC liposomes and therefore prevented from precipitation. For comparison, in the case of the reference system where no liposomes are present, oligomers higher than H-Trp_4-OH tend to aggregate and precipitate so that no longer oligomers than the 8mer can be obtained (see Figure 36A). The same seems to be true for Phe, but not for the other amino acids which have, at best, a weakaffinity for the lipid membrane.

An exception seems to be the CDI-induced condensation of His. At pH 6.80, His and its derivatives are expected to be partially protonated at the π-imidazole nitrogen (pK_a ~ 5-8). [475] His^0 has positive ΔG_{wif}- and ΔG_{woct}-values, and His^+ exhibits the most positive values of all natural amino acids. One would therefore expect His to show low affinity POPC membranes. Nevertheless, Figure 39 shows a slightly enhanced oligomerization efficiency in the presence of liposomes.

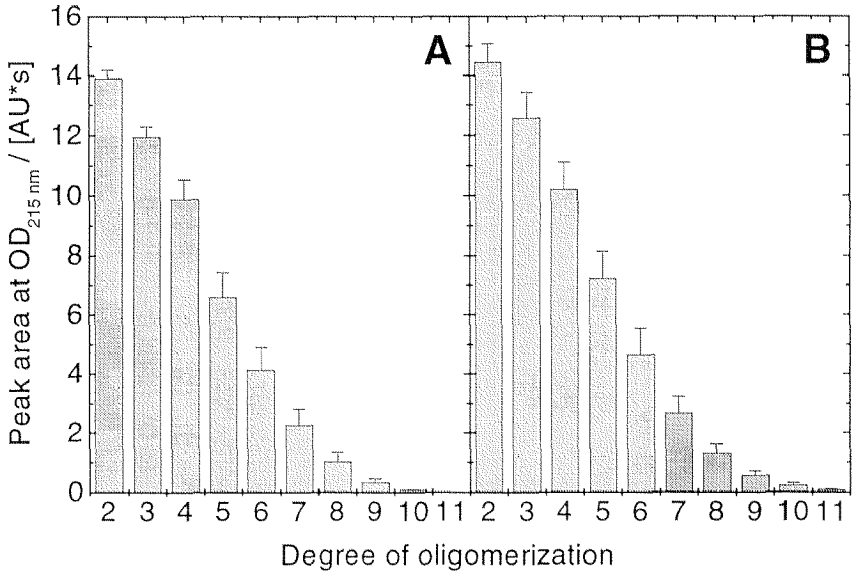


Figure 39: CDI-induced oligomerization of His in the absence (A) and presence (B) of POPC liposomes. A 40 mM His solution in 50 mM imidazole/HCl, pH 6.80, was incubated with a 2-fold excess of CDI for 20 min at 0°C. The resulting solution was incubated 1:1 (v/v) either with 50 mM imidazole/HCl, pH 6.80 (A), or 100 nm extruded 50 mM POPC liposomes in a 50 mM imidazole/HCl buffer, pH 6.80 (B) for 168 hours at room temperature. The columns are mean values of three measurements with standard deviation error bars.

As mentioned before, it must be kept in mind that the CDI-induced condensation of His is a special case. The different experimental conditions reported in Table 10 might already indicate that His condensation takes place via a different mechanism than is the case for the other amino acids. When CDI is incubated with His in an imidazole buffer, a complex mixture of intermediates is obtained. In time, they transform into 7-carboxy-imidazo-[1,5*c*]-tetrahydropyrimidin-5-one (**37**). If His is incubated with two equivalents of CDI at 0°C for 20 min, (**37**) is formed in yields of about 80-90%. [410], [411] The half-life of this transformation at 0°C is about 14 hours. After longer times, (**37**) begins to transform into histidylhistidine and at room temperature it forms oligohistidines. The half-life of conversion of (**37**) at 30°C is about 60 hours. [411]

Looking at the molecular structure of (**37**), it seems plausible that this compound has a higher affinity than His (or NCA-His would) to POPC membranes. Its rigidity and extended electronic π-system probably decreases the values of ΔG_{wif} and ΔG_{woct}, due to additional favorable interactions in the interface regions.

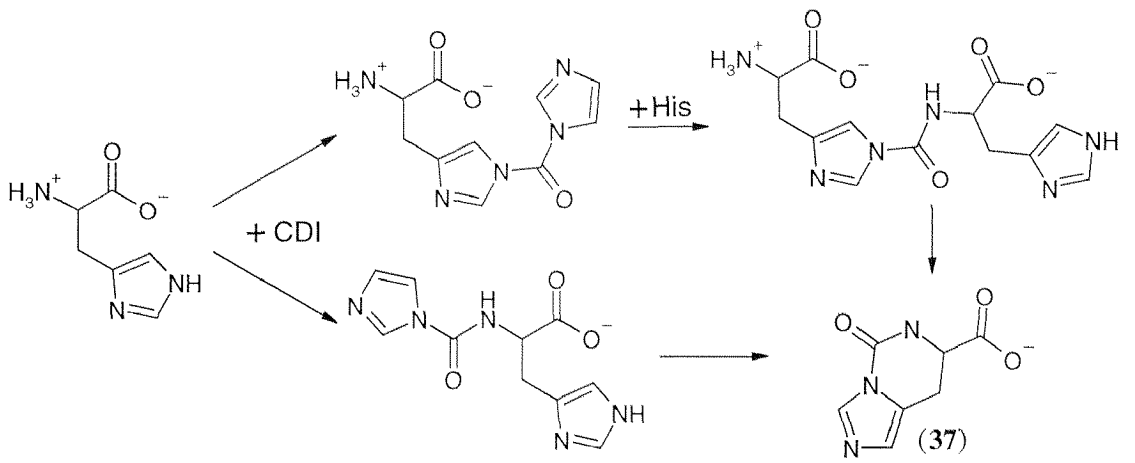


Figure 40: When His is incubated with CDI, 7-carboxy-imidazo-[1,5*c*]-tetrahydropyrimidin-5-one (**37**) is formed via several intermediates, adapted from [411].

5.1.1.2. Multiple-Feeding Experiments

Inspired by the results of multiple-feeding experiments of polar NCA-amino acids to clay and other minerals (section 4.2.3.1.), CDI-induced condensation experiments in the presence of liposomes were extended. For multiple-feeding experiments in the presence of liposomes Trp was chosen, since it seemed to be the most promising candidate.

The NCA-Trp condensation mechanism in aqueous solution proceeds through nucleophilic attack of an *N*-terminal amine, either of Trp or one of its oligomers, on the 5-carbonyl of the NCA-ring. The 2-carbonyl is found exclusively in the CO_2 released. [476] The elongation of oligo-Trp can therefore be looked at as a step by step addition of Trp to the *N*-terminus of the oligomer (see Figure 41). It is to be expected that the degree of oligomerization of the products obtained, when NCA-Trp added subsequently in batches to a reaction suspension containing liposomes (multiple-feeding), will be much higher than for a single-feeding experiment. With each feeding step the length of the possible candidates for the next elongation increases, consequently leading to longer oligomers.

That this is indeed the case for NCA-Trp is shown in Figure 42. The second feeding of NCA-Trp to a liposome suspension that had already been incubated for 12 hours with a

first feeding of NCA-Trp did improve the amount and length of higher Trp-oligomers produced. Another feeding further increased the yields of higher oligomers formed, but more then 3 feedings did not improve yields further. In the reference system without liposomes, traces of the 8mer remained the highest oligomer produced, independent of the number of feedings made (data not shown).

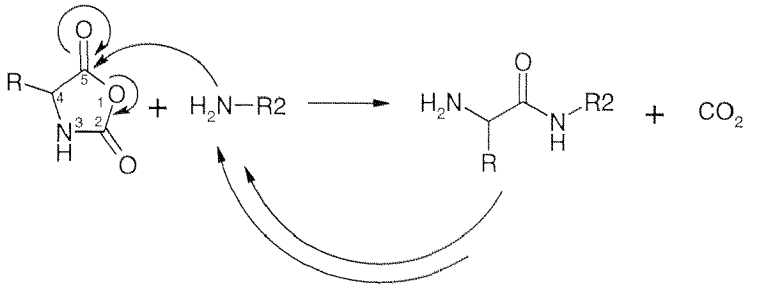


Figure 41: In an aqueous solution some of the NCA-amino acid molecules are hydrolyzed. The *N*-terminus of the resulting amino acid attacks the 5-carbonyl of an other NCA-amino acid and forms the dipeptide. This peptide attacks the electrophile 5-carbonyl of a further NCA-amino acid and so forth. Peptide chains elongate by this mechanism in a step by step addition of single amino acids to the *N*-terminus.

When multiple-feeding POPC samples were stored without shaking for a couple of hours after the reaction had ended, precipitation was often observed. The reason for this observation might be the high hydrophobicity of the oligo-Trps formed in such feeding experiments. Increasing concentrations of oligomers with high degrees of oligomerization are obviously difficult to solubilize, even in the presence of POPC liposomes.

If the concentration ratio of POPC to added NCA-Trp is very large, solubilization of the relatively small amount of NCA-Trp and oligo-Trps by the liposome suspension is highly favorable. The large lipid membrane surface offered to the NCA-Trp decreases the local NCA-Trp concentration at the bilayer surface (dilution), so that high yields of oligo-Trps will not be observed. On the other hand, when the POPC to NCA-Trp ratio is very low, the capacity for solubilizing oligo-Trp formed in the reaction will be limited, negatively influening the yields. Accordingly, there must be an optimum for POPC to NCA-Trp ratios at which the yields for oligo-Trp formation are maximized. Since higher concentrations in NCA-Trp increase the yields of oligomers as well as the degree of the highest oligomer formed (Figure 36B), the POPC or the NCA-Trp concentration at the optimum POPC/NCA-Trp ratio should be set to the maximum possible experimental concentration in order to get the highest oligomers.

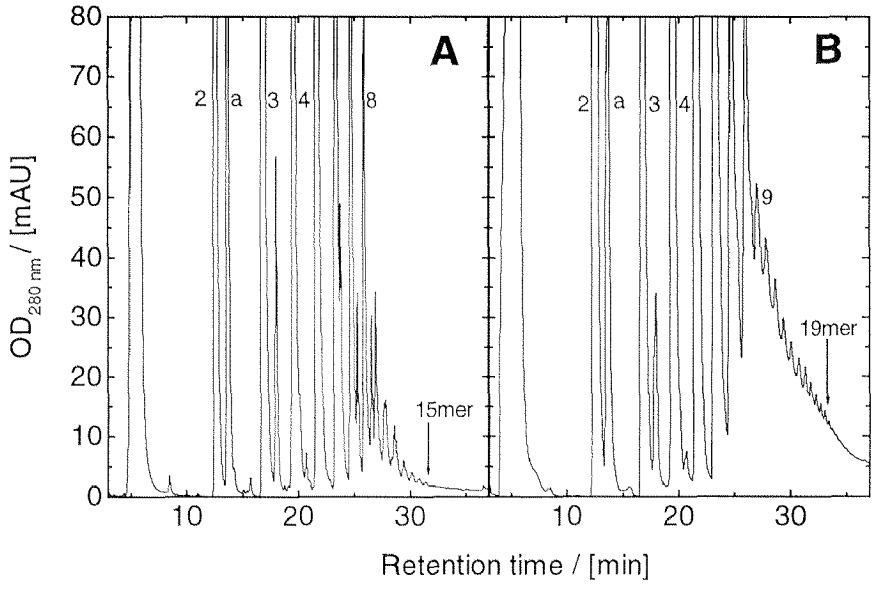


Figure 42: The influence of multiple NCA-Trp feeding in the presence of POPC liposomes on the oligo-Trp formation is shown. A freshly prepared 60 mM NCA-Trp solution in 0.4 M imidazole/ HCl buffer, pH 6.80, was mixed 1:3 (v/v) with a 100 nm extruded 100 mM POPC liposome suspension in 0.4 M imidazole/HCl, pH 6.80, and incubated for 12 hours at room temperature (A). After this time, the same volume of a freshly prepared 60 mM NCA-Trp was added to the reaction mixture (second feeding) and incubated for another 12 hours (B). 25 µl of each suspension were injected into the HPLC for analysis. With positive ESI it was possible to identify up to the 8mer (A) and 9mer (B), respectively. The assignment of the higher oligomers was done by extrapolation.

The purpose of the three experiments shown in Figure 43 was to push the multiple-feeding type of experiment with the POPC system to its limit. The POPC concentration was kept at the highest possible level at which reasonable HPLC separation could be guaranteed. The initial Trp solution for *in situ* formation of NCA-Trp was successively increased from 20 up to 80 mM, the latter concentration being the solubility limit of Trp. Three feedings were applied at 4 hour intervals. The highest oligo-Trp, a 29mer, was found at the very limit of the experimental conditions (Figure 43C). However, under such extreme conditions, the liposome suspension became very unstable after the third feeding and precipitated soon after shaking was stopped. It was therefore of great interest to know whether the liposomes remain stable after condensation at the more moderate conditions presented in Figure 43B. For this reason, dynamic light scattering (DLS) was performed on the POPC suspension before and after condensation. The hydrodynamic radii after (before) condensation were 59.1±0.6 nm (57.4±0.6) at 60°, 56.3±0.5 nm (55.9±0.9) at 90°, and 55.3±0.2 nm (54.7±0.6) at 120°. The slight increase in the radius might be attributed to the Trp-oligomers bound to the POPC membrane. The low

scattering angle dependency of the hydrodynamic radii for different scattering angles emphasizes that under these conditions, the liposomes remain monodisperse and stable.

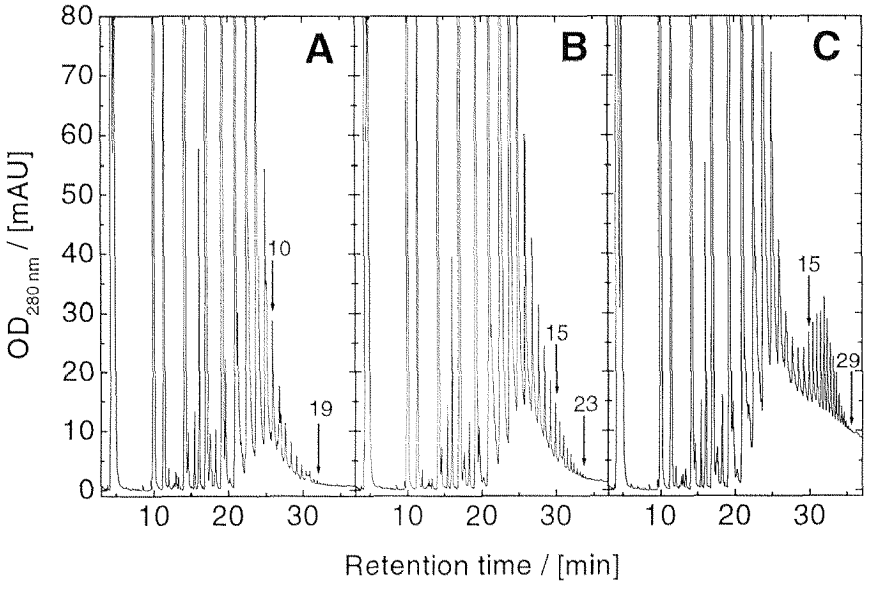


Figure 43: The NCA-Trp condensation in the presence of POPC liposomes was pushed towards its limit. Three feedings of 200 µl at 4 hours time intervals of a freshly prepared 20 mM (A), 40 mM (B), or 80 mM (C) NCA-Trp solution in 0.4 M imidazole/HCl, pH 6.80, were mixed with initially 600 µl of a 100 nm extruded 100 mM POPC liposome suspension in a 0.4 M imidazole/HCl buffer of pH 6.80, and incubated for a total of 12 hours at room temperature. The local maximum between 13mer and the 29mer in (C) might be an artifact originating from the injection of precipitated sample. The yields in % of total Trp added for oligomers higher than the 10mer are (A) 1.8%, (B) 2.6%, and (C) 6.4%.

The condensation experiments discussed so far, have dealt with activated hydrophobic amino acids that exhibit an affinity to POPC bilayer membranes and are therefore preferentially oligomerized on them compared to the aqueous reference system with no liposomes. In the following paragraph, the liposome-assisted, selective oligomerization of hydrophobic dipeptides will be discussed, making use of the lipophilic condensing agent 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) (**38**).

5.1.2. EEDQ-Induced Condensation of Dipeptides

Since the hydrophobic condensing agent EEDQ was first introduced in the literature, [477] it has attracted considerable attention. It is a potent central-nervous system depressant, [478] as it is an alkylating agent which produces an irreversible blockade of various membrane bound receptors in the brain, such as α- and β-adrenergic, [479]-[483] dopaminergic, [484]-[486] and muscarinic receptors. [487], [488] Irreversible receptor blockade by EEDQ occurs in two steps. In the first, EEDQ specifically reacts with a

carboxylic group in the receptor protein and forms a mixed anhydride. In the second step, this reactive intermediate can react with any nucleophilic group in its vicinity to form an irreversible bond. [489] EEDQ was also found to promote the coupling of acyl amino acids and amino acid esters in high yields. [477] In addition, it has proved to be a valuable reagent in condensation reactions, comparable to *N,N'*-dicyclohexylcarbodiimide (DCC) in efficiency [490]-[492] and even less epimerization in model systems. [493] In more recent publications, this reagent has been shown to be useful for coupling hydrophobic amines to carboxylic acids, potentially including those buried in a cellular membrane. [494]-[497] This characteristic in particular makes (**38**) an interesting condensing agent for investigating whether liposomes can induce selective oligomerization of dipeptides[2].

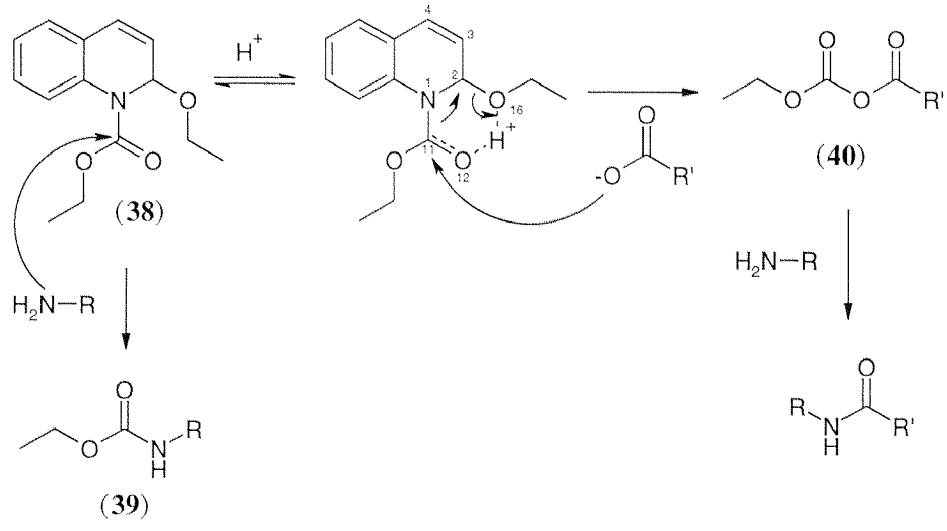


Figure 44: 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) (**38**) is a hydrophobic condensing agent with a low pK_a of 4.22, due to the postulated stabilization *via* the cyclic conformation. [498] The formation of the mixed anhydride (**40**), which is the reactive intermediate in the peptide bond formation, proceeds through this protonated EEDQ. The main secondary reaction is the carbamate (**39**) formation.

5.1.2.1. Reaction Mechanism of EEDQ

EEDQ is a white crystalline material. Its high basicity (pK_a = 4.22) and hence high reactivity is suggested to originate from the assistance of O(16) in the protonation of the carbonyl O(12) (see Figure 44). [498] In EEDQ-induced condensation reactions of amino acids in organic solvents, EEDQ is not entirely inert towards the nucleophilic attack of amines, but the rate of urethane (or carbamate) (**39**) formation is low compared

[2] All the dipeptides used were in the all L-form (H-L-aa1-L-aa2-OH).

with the reaction of EEDQ with carboxylic acids. [279] The latter reaction starts with the attack of the carboxylate anion on the carbonyl C(11) which leads to the formation of a carbonic acid mixed anhydride (**40**) by releasing ethanol and quinoline. [498] The anhydride is then attacked by an amine, leading to the desired peptide bond. An earlier proposed mechanism [477] that is often still found in text books on peptide synthesis, [277], [279], [499] starts with the displacement of ethanol by the carboxylate anion in the 2-position, followed by the intermolecular break down to yield the mixed anhydride. However, there are several arguments that make the initially described mechanism *via* the protonated species more likely. [498]

There are several possible side reactions described in the literature which might occur when condensing dipeptides with EEDQ. The first is carbamate formation, which has already been discussed. This reaction is expected to be pH-dependent in aqueous solution. The optimal pH should be below the pK_a of the dipeptide's amine group (< 7.8), [475] which should be protonated in order to suppress the carbamate formation. The optimal pH is, however, should also be above the pK_a of the *C*-terminal carbonic acid (> 3.6), [475] which must be deprotonated in order to form the anhydride with EEDQ. If the pH chosen is too low, the amine group of the peptide will be completely protonated and attack on the mixed anhydride by the *N*-terminal amine will be very slow. Hydrolysis of the anhydride, which dominates at very high and at very low pH values, will then compete with peptide bond formation. Another limiting factor is the rapid hydrolysis of EEDQ at room temperature and pH values below 5 ($t_{1/2}$ < 10 min); this is not a problem above pH 7 ($t_{1/2}$ > 1 h). [498]

The second side reaction complicating many peptide coupling reactions is the formation of 5(4*H*)-oxazolones (also called 2-oxazoline-5-ones or azlactones) (**41**). It is known that activated acylamino acids and peptides often isomerize during coupling because they generate the easily epimerized 2-alkyl-5(4*H*)-oxazolones (**41 a**). [280], [500], [501] In contrast to (**41 a**), 2-alkoxy-5(4*H*)-oxazolones (**41 b**) can often be safely employed in peptide synthesis without significant epimerization, depending on the nature of the substituent R. [502], [503] Both 2-alkyl- and 2-alkoxy-5(4*H*)-oxazolones react with amines to form peptide bonds. The latter does not possess an inherently higher chiral stability than the first, but is more reactive with respect to the addition of amine nucleophiles. This addition competes with epimerization over time. [504], [505] In Figure 45 these aspects are taken into account for the mixed anhydride (**40**) produced in

an EEDQ-induced dipeptide condensation (R1 = -CHR'-NHR''). Consecutive reactions of the oxazolone tautomer (**42**) derived from the carbamates and peptides with EEDQ during condensation can lead to further by-products, as shown in Figure 46. [361], [506]-[509]

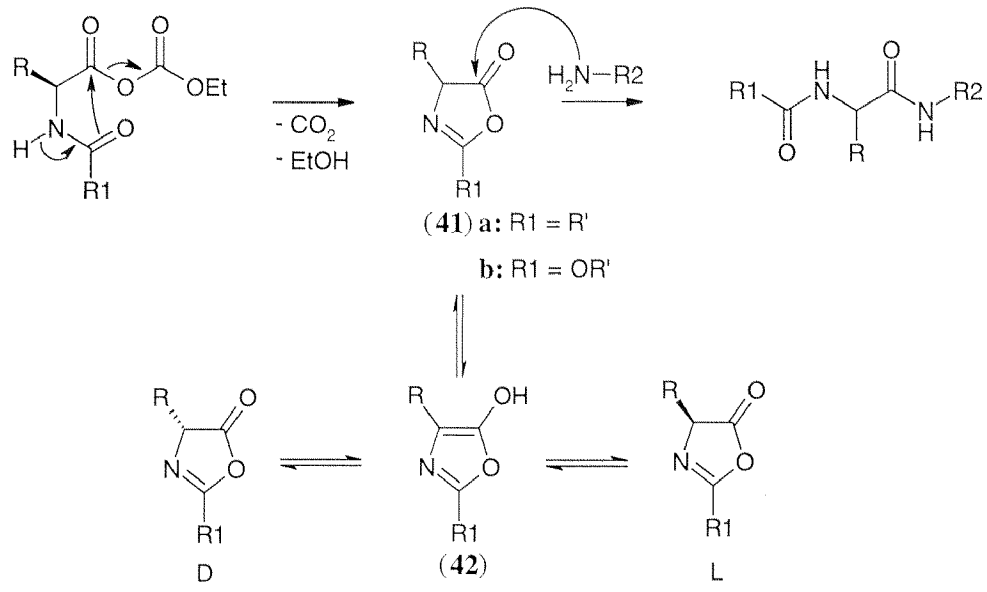


Figure 45: The formation of 5(4*H*)-oxazolones (**41**) from activated acylamino acids and peptides can lead to enantiomerization *via* the tautomer (**42**).

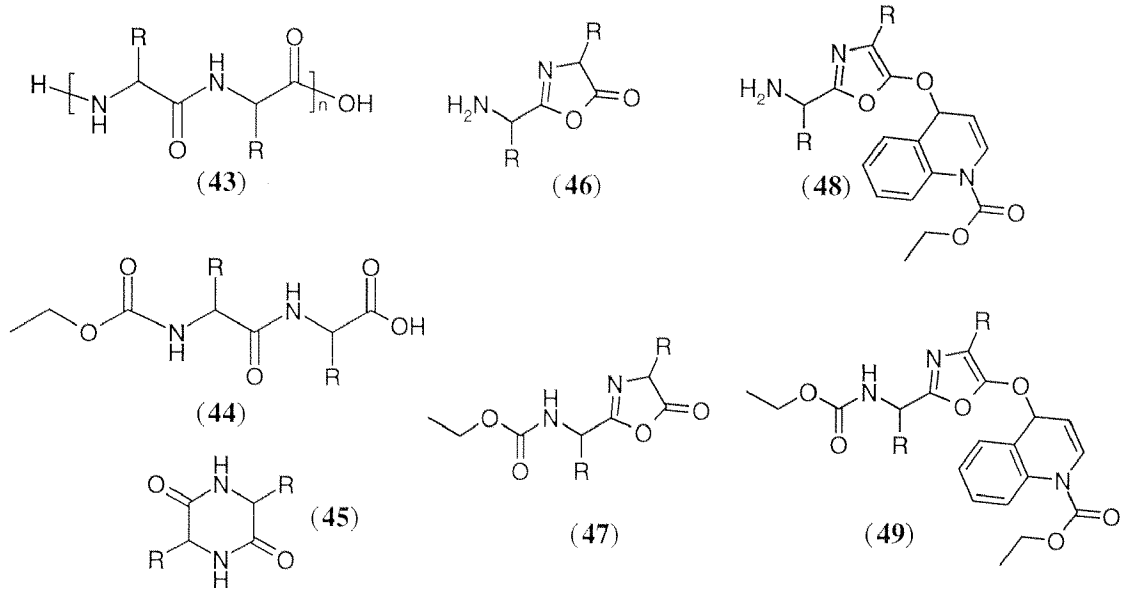


Figure 46: The desired oligopeptides (**43**) and the major unwanted by-products: peptidyl cabamates (**44**), 2,5-diketopiperazines (**45**), 5(4*H*)-oxazolones (**46**) and their carbamates (**47**) of the dipeptide, and their derivatives with EEDQ (**48**) and (**49**). These products are expected for the EEDQ-induced condensation of dipeptides in aqueous solution. Due to the lack of information in the literature on the reaction behavior of EEDQ with amino acids and peptides in water, the references are for reactions in organic solvents. [361], [506]-[509]

5.1.2.2. Finding Optimal Conditions

Our primary goal was to determine whether POPC liposomes enhance the EEDQ-induced oligomerization of hydrophobic dipeptides. For this reason, 10 mM H-TrpTrp-OH was incubated with 10 mM EEDQ in the presence and absence of a 100 nm extruded 25 mM POPC aqueous liposome suspension of pH 8.50. HPLC chromatograms of samples taken after time intervals of 20 hours and monitored at 215 nm did not change significantly at reaction times longer than 40 hours. During this time, the pH of the samples dropped about 0.50 - 0.70 units, and the polydispersity of the lipid aggregates in the POPC system increased drastically as juged by DLS and EM analysis (data not shown).

Figure 47 shows a comparison of the POPC and the reference systems after incubation for 142 hours on a shaker at room temperature. The huge difference between the products formed in the two systems is apparent. The presence of lipid membranes increased the number of the different types of products formed, as well as the degree of oligomerization observed in the oligopeptides and their derivatives. In the reference system, carbamate formation was the main reaction taking place. Only a small fraction of dipeptide was activated to the anhydride by EEDQ. The bulk of the mixed anhydride cyclized intramolecularly to the diketopiperazine, and the rest, if not hydrolyzed, condensed with another dipeptide to yield the tetrapeptide which turned almost completely into the carbamate. On the other hand, with the exception of (**47**) and (**46**) in part, all the predicted products shown in Figure 46 were formed in relatively high yields by the POPC system. Detailed analysis by mass spectrometry revealed that the octapeptide H-Trp_8-OH and its derivatives were the highest oligomers obtained. Multiple peaks with the same m/z values as 5(4*H*)-oxazolones, or products derived from them, suggest that epimerization is extensive under the conditions used.

Similar results were obtained with H-PhePhe-OH, where up to the decamer in derivative (**49**) was detected (data not shown). Based on the results obtained for condensation with NCA-Phe and NCA-Trp, this analogy in the behavior of H-PhePhe-OH and H-TrpTrp-OH in the presence of the lipid membranes was expected.

Despite the clear enhancement of oligomerization by the liposomes, the complex product mixture was not satisfying at all. First, the initial aim was to oligomerize dipeptides to yield oligopeptides and not their derivatives. Second, epimerization should be prevented. Third, it was not our intention to transform the liposomes into other aggregates or even to destroy them completely, as indicated from DLS and freeze-fracture electron microscopy (ff-EM) pictures. It

was clear that the conditions had to be changed and optimized. Several parameters were varied individually in order to find better conditions.

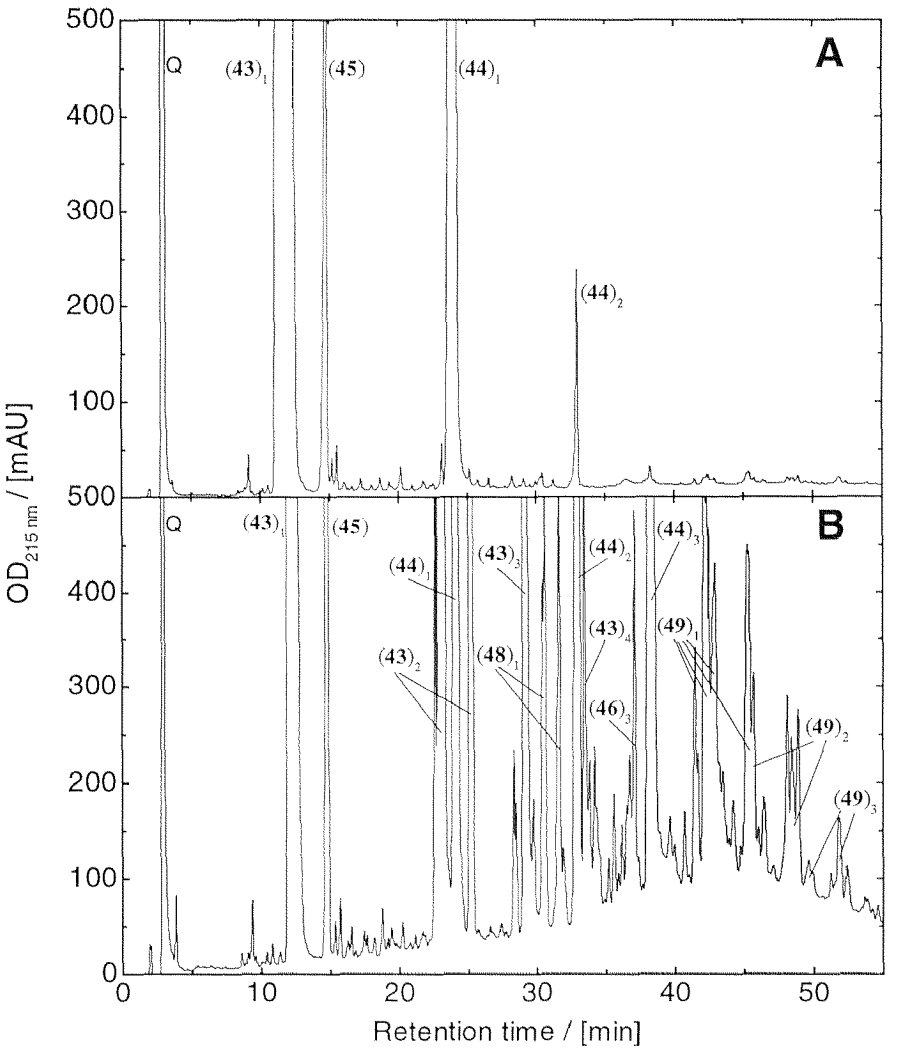


Figure 47: The EEDQ-induced condensation of H-TrpTrp-OH in the absence of liposomes (A) is compared with condensation in the presence of POPC liposomes (B). The chromatograms are the UV-traces at 215 nm of 100 µl of injected sample after 142 hours incubation at room temperature. 1 ml of an aqueous 10 mM H-TrpTrp-OH at pH 8.50 (A) or a 10 mM H-TrpTrp-OH, 100 nm extruded 25 mM POPC liposome suspension at pH 8.50 (B) were incubated with 12 µl of a 0.824 M EEDQ stock solution in ACN. The resulting reaction sample was 10 mM in EEDQ. Q stands for quinoline and the numbers in parentheses correspond to the molecular structures in Figure 46 and indicate the type of derivative, whereas the subscript is the number of dipeptides in the chain of the derivative.

Since carbamates were one of the main by-products, a reduction of the reaction pH was necessary to suppress their formation. At the same time less 5(4*H*)-oxazolone is expected at lower pH, as its formation is base catalyzed. In addition, the affinity of hydrophobic dipeptides to neutral lipid membranes will increase as the pH value approaches their isoelectric point. It seemed further appropriate to use buffered solutions for greater

control of the reaction conditions in the future. However, due to the nature of the condensation reaction induced by EEDQ, potential buffers were restricted to those having neither amine or carboxylate groups. Preliminary experiments with citrate showed that coupling with amines occurs in the presence of EEDQ, as expected. In order to eliminate derivatives (**48**) and (**49**) which are due to overreaction, the initial EEDQ concentration was reduced relative to the amount of dipeptide.

Experiments performed at pH 5.90 in 50 mM phosphate buffer with 10 mM H-TrpTrp-OH and 4 mM EEDQ turned out to reduce the by-products in an effective way. However, ff-EM and DLS still displayed liposome transformation to polydisperse suspensions, as indicated by the hydrodynamic radii after (and before) condensation at 60°: 308.7±24.2 nm (53.91±0.70), 90°: 364.3±23.3 nm (53.49±0.56), and 120°: 471.4±58.0 nm (53.91±0.74). A further reduction of the EEDQ concentration to 2 mM improved the situation, but did not solve the problem entirely: 60°: 153.6±10.9 nm, 90°: 127.7±14.3 nm, and 120°: 123.1±22.7 nm.

The solubility capacity of the POPC membrane is limited and both the hydrophobic dipeptide and EEDQ bind to the liposomes through the hydrophobic effect, as do their products. If the concentration of molecules bound to the membrane is too high compared to the lipid bilayer present, the result will be aggregation and destruction of the liposomes. Dialysis experiments on 10 mM H-TrpTrp-OH, 100 nm extruded 25 mM POPC liposomes and 50 mM phosphate buffer at pH 5.90 revealed an association constant K_a of 39.5±7.2 M^{-1}, which is equivalent to a dipeptide concentration of ca. 50 times higher at the POPC membrane interface than in aqueous buffer, i.e. 5 POPC lipids solubilize one molecule of H-TrpTrp-OH.

In consideration of these facts, it seemed reasonable to reduce the concentration of the dipeptide to prevent destruction of the liposomes. Using 5 mM H-TrpTrp-OH and the latter conditions, ff-EM and DLS indicated no major changes of the liposomes during condensation (60°: 55.59±0.60 (54.70±0.84), 90°: 53.49±0.41 (55.37±0.38), 120°: 52.51±0.58 (54.24±0.51)), and dialysis confirmed that the membrane was quite saturated at the concentration of 10 mM, since K_a was now increased (58.28±0.96 M^{-1}). Under these conditions, the membrane is 70 times more concentrated in H-TrpTrp-OH and 8 to 9 POPC lipids are present per bound dipeptide.

In summary, the optimized EEDQ-induced condensation conditions are 5 mM dipeptide concentration and 2 mM initial EEDQ at a pH of 5.90. Figure 48 shows the

chromatograms measured at 290 nm, of the EEDQ-induced condensation of 5 mM H-TrpTrp-OH at pH 5.90 in the absence and presence of 100 nm extruded 25 mM POPC liposomes. Compared to the UV-traces in Figure 47, product diversity is clearly reduced to the main products, Trp-oligomers, and small amounts of diketopiperazine and oligomer carbamates. The other by-products obtained previously at pH 8.50 in unpleasantly high yields are largely absent. A closer evaluation, however, showed that they still make up about 10% of the H-TrpTrp-OH that has reacted.

The appearance of two peaks corresponding to H-Trp_4-OH in the chromatogram B of the POPC system (labelled $(\mathbf{43})_2$) needs explanation. At a first glance one might attribute it to epimerization during coupling, but a closer look shows that this is not the case, since the multiple peaks of the corresponding carbamate and higher oligomers are missing. They would have to be present if stereoisomerization took place during tetramer formation. Another explanation attributes the extra peek to the symmetrical anhydride of H-TrpTrp-OH, but longer incubation times did not reduce either of the two peaks. Two peaks corresponding to tetramer, which was observed exclusively for the tetramer in this type of experiment, seemed to be an artifact of the separation in the presence of liposomes. In Figure 42 a similar effect was observed for H-Trp_3-OH using the same column.

A detailed analysis of the results shown in Figure 48 allowed several conclusions to be made: In the POPC (reference) system, 43% (1%) of the 2mM EEDQ converted 33% (<1%) of the initial 5 mM H-TrpTrp-OH into oligopeptides (760 µM (13 µM) H-Trp_4-OH, 58 µM (-) H-Trp_6-OH, and 2 µM (-) H-Trp_8-OH) and 25% (63%) of the EEDQ converted 10% (25%) of the initial H-TrpTrp-OH into carbamates or diketopiperazine (360 µM (1230 µM) EtO-CO-Trp_2-OH, 52 µM (4 µM) EtO-CO-Trp_4-OH, 6 µM (-) EtO-CO-Trp_6-OH, and 21 µM (12 µM) Cyclo(-Trp_2-)). The remaining EEDQ was either hydrolyzed or formed other minor by-products with about 5% (1%) of the initial H-TrpTrp-OH. In total, 49% (26%) of the H-TrpTrp-OH initially present, which makes up about 80% of the dipeptides bound to the liposomes at the beginning of the reaction, and all the EEDQ had reacted after 26 hours.

The enhanced oligomerization reaction of H-TrpTrp-OH in the presence of POPC liposomes can be explained with arguments based on the ΔG_{wif}-values given in Table 10. Compared to the aqueous phase, the hydrophobic H-TrpTrp-OH and its oligomers accumulate at the liposome interface by a factor of 70 or more. The lipophilic

condensing agent EEDQ behaves in a similar fashion. [494] Since both compounds are locally concentrated on the lipid bilayer (see Figure 38), they react with each other more efficiently than in the dilute aqueous reference system when no liposomes are present. Thus, the enhancement of oligomerization by the liposomes can be described as physical catalysis. The presence of the hydrophobic surface allows the reaction partners to bind, accumulate (come into close contact) and then react with each other.

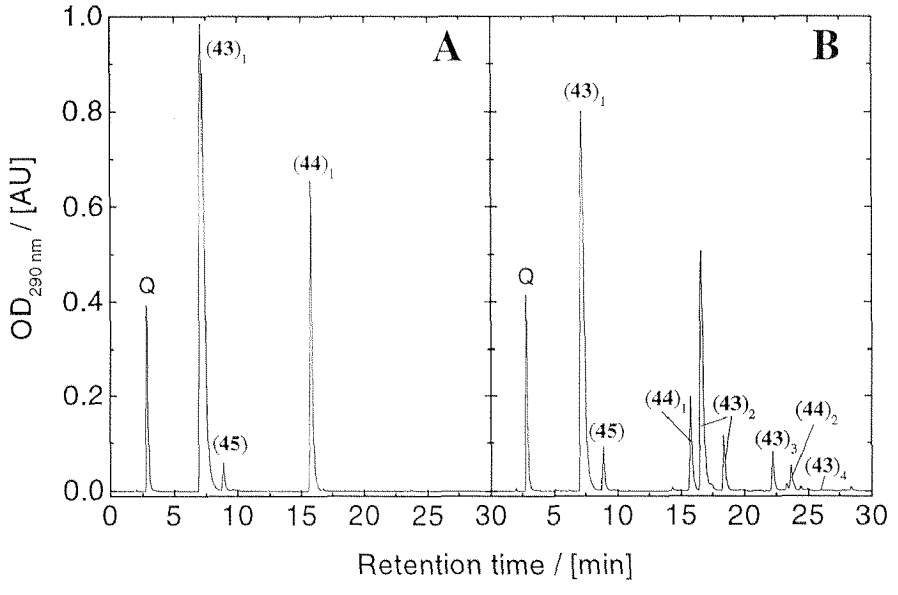


Figure 48: 1 ml of a 5 mM H-TrpTrp-OH, 50 mM phosphate solution at pH 5.90 (A) or a 5 mM H-TrpTrp-OH, 100 nm extruded 25 mM POPC liposome suspension in 50 mM phosphate buffer at pH 5.90 (B) was incubated for 26 hours at room temperature with 12 µl of a 0.169 M EEDQ stock solution in ACN. The resulting reaction sample was 2 mM in EEDQ. Q stands for quinoline and the numbers in parentheses correspond to the molecular structures in Figure 46, whereas the subscript indicates the degree of oligomerization.

Similar experiments with H-PhePhe-OH or H-TrpPhe-OH failed because of the low solubility of these peptides at pH 5.90. One experiment was carried out with H-LeuTrp-OH under the conditions described above. The results obtained were less pronounced than in the case of H-TrpTrp-OH. The POPC (reference) system yielded: 17.7% (30.1%) cyclo(-LeuTrp-), 21.8% (4.4%) H-LeuTrpLeuTrp-OH, and 15.3% (61.2%) EtO-CO-LeuTrp-OH of the reacted dipeptide. The dialysis experiment yielded an association constant K_a of 6.14±0.36 M^{-1} for POPC liposomes in accord with expectations based on the ΔG_{wif}-value of Leu given in Table 10: H-LeuTrp-OH binds less efficiently to the POPC bilayer interface at pH 5.90 than does H-TrpTrp-OH. Of the 21% of the initial H-LeuTrp-OH that reacted in the liposome system, only 63% was bound to the membrane at the beginning. This weak binding is probably responsible for the moderate effect of

the liposomes in this case. The same was observed for the tripeptide H-PheHisLeu-OH under the same conditions, where the low membrane affinity (K_a: 0.62±0.36 M^{-1}) led only to the formation of EtO-CO-PheHisLeu-OH in both the reference system and the POPC system.

5.1.2.3. Liposome-Assisted Selective Peptide Condensation

If the binding of a peptide is the critical factor for enhanced liposome-assisted oligomerization, then selective peptide condensation of hydrophobic peptides should be possible. To test this selection principle with dipeptides as the building blocks, an experiment was carried out starting from a mixture of four different dipeptides, namely the hydrophobic H-TrpTrp-OH and the more hydrophilic dipeptides H-TrpGly-OH, H-TrpAsp-OH, and H-TrpGlu-OH. In this experiment, selectivity towards oligo-Trp sequences was observed. The results are given in Table 11 and compared with the POPC system for each of the dipeptides.

DLS measurements confirmed that the hydrodynamic radii of the liposomes did not change with the reaction for any of the peptides used. Dialysis experiments performed with each dipeptide and the peptide mixture in the presence of 100 nm extruded POPC liposomes gave the following association constants ($K_a/[M^{-1}]$): 1.63±0.20 for H-TrpGly-OH, 0.11±0.11 for H-TrpAsp-OH, 0.076±0.032 for H-TrpGlu-OH, 58.28±0.96 for H-TrpTrp-OH, and 60.63±0.50 for H-TrpTrp-OH in the mixture of all four peptides. H-TrpTrp-OH is the only dipeptide out of the peptide pool that exhibits high affinity to the liposome membrane and is therefore selected out of the dipeptide pool and enriched 76 times on the bilayer surface of the mixture POPC system. This is the reason why the hydrophilic dipeptides hardly underwent oligomerization in the POPC system when used alone or in the mixture of all four dipeptides. On the other hand H-TrpTrp-OH was activated on the membrane by EEDQ, which was also present, and readily formed oligomers as was shown in Figure 48. For each of the hydrophilic dipeptides, the main product formed was its carbamate in yields of about 80% of the dipeptide reacted. About 12% went into the tetrapeptide or its corresponding carbamate. It was not clear to what extent the Asp- and Glu-containing dipeptides coupled at the γ or δ carbonyl instead of the *C*-terminal carboxylic acid. It seems likely, however, that such branching does occur. When all four dipeptides are mixed with each other, their products are suppressed in favor of H-TrpTrp-OH oligomerization. The theoretically possible co-oligomers among

all of the peptides used here make up only about 15% of the total peptides reacted, i.e. out of the 16 possible tetrapeptides, H-Trp_4-OH makes up about 70% of all the tetrapeptides formed.

Table 11: This table emphasizes the liposome-assisted selectivity for the co-oligomerization of dipeptides. All solutions were incubated for 26 hours on a vortex at room temperature and the products analyzed by LC-MS. (H-TrpX-OH) are the POPC systems for each of the dipeptides, which were later used in the mixture with POPC liposomes (Lip) and the reference system with no liposomes (Ref).

Products:		**One peptide only:**				**Mixture:**	
		H-TrpX-OH:[a]				Lip:[b]	Ref:[c]
in % of dipeptide(s) reacted	X:	*Gly*	*Asp*	*Glu*	*Trp*	*Trp*	*Trp*
	Y,Z:					*Gly, Asp, or Glu*	
Peptides:							
cyclo(-TrpX-)		-	-	-	0.8	0.9	1.6
H-$(TrpX)_2$-OH		8.2	8.6	7.3	62	43	0.6
H-$(TrpX)_3$-OH		0.7	-	-	5.9	2.1	0.03
H-$(TrpX)_4$-OH		-	-	-	0.3	0.03	-
cyclo(-TrpY-)/H-TrpYTrpZ-OH						8.0	13
H-Trp_3Y-OH/H-TrpYTrp_2-OH						7.0	6.4
Derivatives:							
EtO-CO-TrpX-OH		81	80	78	15	11	33
EtO-CO-$(TrpX)_2$-OH		4.0	4.5	4.9	4.3	1.6	0.1
EtO-CO-$(TrpX)_3$-OH		0.5	-	-	0.7	0.1	-
EtO-CO-TrpY-OH						22	43
rest		5.7	6.6	9.9	11	4.2	2.5

a. 5 mM H-TrpX-OH, 100 nm extruded 25 mM POPC liposomes, 50 mM phosphate, pH 5.90, 2 mM EEDQ, 1.2% v/v ACN.
b. 5 mM H-TrpTrp-OH, 5 mM H-TrpGly-OH, 5 mM H-TrpAsp-OH, 5 mM H-TrpGlu-OH, 100 nm extruded 25 mM POPC liposomes, 50 mM phosphate, pH 5.90, 2 mM EEDQ, 1.2% v/v ACN.
c. 5 mM H-TrpTrp-OH, 5 mM H-TrpGly-OH, 5 mM H-TrpAsp-OH, 5 mM H-TrpGlu-OH, 50 mM phosphate, pH 5.90, 2 mM EEDQ, 1.2% v/v ACN.

5.1.2.4. Multiple EEDQ Feeding

Based on the knowledge that multiple addition of monomer can influence product length and distribution in NCA-Trp condensations in the presence of liposomes, multiple-feeding in EEDQ-induced condensation was tested. Since the increase in H-TrpTrp-OH through multiple-feeding would have affected the size and shape of the liposomes, as was demonstrated earlier, the dipeptide was maintained at a 5 mM concentration. The EEDQ was added in three aliquots at time intervals of 3 hours so that the total added EEDQ corresponded to a 2 mM concentration. The experiments were performed at pH 5.00 in a 50 mM phosphate buffer in the presence and absence of 100 nm extruded 25 mM POPC liposomes and were compared with the equivalent single-feeding experiments (see Table 12).

Table 12: Multiple-feeding with ($POPC_{multi}$) and without (Ref_{multi}) liposomes is compared with the single-feeding experiments (Ref and POPC). Either 12 µl 0.168 M EEDQ in ACN (POPC) or three times 10 µl 0.056 M EEDQ in ACN at time intervals of 3 hours ($POPC_{multi}$) were added to 1 ml of a 5 mM H-TrpTrp-OH, 100 nm extruded 25 mM POPC liposomes, 50 mM phosphate, pH 5.00 suspension and incubated for 26 hours at room temperature. In both types of the experiments, the total EEDQ added corresponded to a final concentration of 2 mM. Products are given in % of reacted H-TrpTrp-OH.

Products:	**Ref:**	**POPC:**	**Ref_{multi}:**	**$POPC_{multi}$:**
Peptides:				
cyclo(-TrpTrp-)	2.9	3.3	3.3	18
H-Trp_4-OH	3.5	66	-	60
H-Trp_6-OH	-	6.7	-	4.9
H-Trp_8-OH	-	0.4	-	-
Derivatives:				
EtO-CO-Trp_2-OH	83	9.6	83	9.6
EtO-CO-Trp_4-OH	1.7	3.0	-	3.1
rest	8.9	11	13.7	4.4

The single-feeding experiment in the presence of lipid bilayers at pH 5.00 revealed that carbamate formation was even more suppressed than at pH 5.90 (see Table 11). However, in spite of this, the yields of oligopeptides did not change significantly. This might be attributed to the practically unchanged association constant ($K_a/([M^{-1}])$: 59.1±1.4) at pH

5.00. Comparison of the single-feeding with the multiple-feeding experiment at pH 5.00 showed that in the latter case the degree of oligomerization and the number of by-products, aside from carbamate, was reduced, but the amount of diketopiperazine had drastically increased. Multiple-feeding of EEDQ obviously favors cyclization over chain elongation. Given these unspectacular results, multiple-additions of EEDQ was not further investigated.

5.1.3. Concluding Remarks to the POPC Liposome-Assisted Condensation

In conclusion, the results presented above for CDI- and EEDQ-induced condensation show that the POPC bilayer membrane of liposomes can promote the polycondensation of hydrophobic amino acids and peptides that exhibit affinity to the bilayer membrane. In repeated feeding experiments with hydrophobic aromatic NCA-amino acids, the presence of liposomes leads to oligomers with polymerization degrees of over 20. The elongation to higher water-insoluble oligomers takes place only on the membrane bilayer. Furthermore, sequence-selective condensation of dipeptides can be achieved on the liposomes by using EEDQ as a lipophilic condensing agent. In contrast to hydrophilic dipeptides, hydrophobic dipeptides bind to the bilayer membrane, where they are locally concentrated and preferentially oligomerize.

5.2. Peptide Condensation on Charged Liposomes

The results presented so far on the liposome-assisted polycondensation made use of hydrophobic interactions. In principle, charged membranes should also be able to bind amino acids or peptides on the basis of electrostatic interactions, as has been shown for Asp, Glu and *O*-phospho-serine in the case of CTAB micelles. [453] The possibility of carrying out polycondensation based on both hydrophobic and electrostatic interactions appears to be a more challenging undertaking. If successful, it would allow the formation of chains consisting of different types of amino acids, which is a prerequisite for obtaining polypeptides with functionality in a prebiotic scenario.

5.2.1. CDI-Induced Glu/Trp Block Oligomer Formation

In the section 5.1.1. it was shown that POPC membranes assist the condensation of hydrophobic NCA-amino acid, especially when NCA-Trp is employed. This characteristic of the liposomes was attributed to the affinity of the reactants for the lipid

bilayer interfaces. We also investigated NCA-Trp condensations in the presence of positively charged mixed liposomes, as well as the co-oligomerization of NCA-Trp and NCA-Glu.

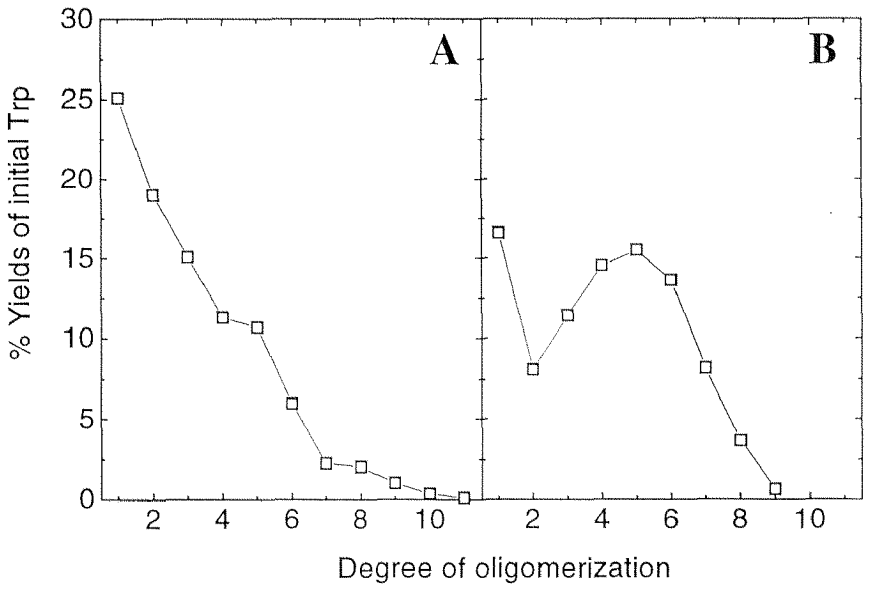


Figure 49: The condensation of NCA-Trp in the presence of positively charged liposomes (B) leads to a different product distribution than in the case of neutral lipid bilayers (A). 20 mM NCA-Trp, 0.4 M imidazole/HCl, pH 7.50, was mixed 1:1 (v/v) with either a 100 nm extruded 25 mM POPC liposome suspension in 0.4 M imidazole/HCl buffer of pH 7.50 (A) or 100 nm extruded 10 mM POPC/10 mM DDAB liposome suspension in 0.4 M imidazole/HCl buffer of pH 7.50 (B). The resulting mixture was incubated for 12 hours at room temperature.

The cationic surfactant didodecyldimethylammonium bromide (DDAB) (**35**) is reported to form liposomes[3] spontaneously, with no tendency for liposome fusion at concentrations of 1-10 mM. [510]-[512] DDAB is further known to form stable mixed liposomes together with POPC or other PCs. [513] If 10 mM of NCA-Trp was incubated in the presence of DDAB containing mixed POPC liposomes at pH 7.50, the polycondensation yielded higher amounts of Trp-oligomers than in the POPC system (see Figure 49). The nonamer of Trp was the highest oligomer observed in the mixed liposome system, which was lower than for the pure POPC system where the highest oligomer of Trp was the 11mer. A possible explanation for this observation might be the possibility of π-cation interaction between the Trp residues and the DDAB ammonium group, which leads to better binding of the oligo-Trps to the mixed DDAB/POPC liposomes than to the POPC system. [472] Longer oligomers now have a higher affinity

[3] Please note that DDAB was the first synthetic amphiphile known to form liposomes. [510]

for the positively charged mixed liposomes when compared to the neutral POPC liposomes. They bind more tightly, especially the di-, tri-, tetra-, and pentamer bind to a greater extent, which is favorable for their elongation at the beginning of the polycondensation process. The liposomes might be viewed at as macromolecules with a finite number of saturable sites for solute binding. Therefore, as the reaction proceeds, an increased affinity of Trp-oligomers to the lipid bilayer decreases the number of free binding sites left for NCA-Trp to bind to. After the first short oligomers have formed and bound to the bilayer membrane, this reduces the NCA-Trp concentration in the lipid interface region of the DDAB/POPC liposomes when compared to the POPC system. A reduced NCA-Trp concentration in the lipid membrane however is not favorable for the chain elongation and results in lower degrees of oligomerization compared to the POPC system.

In order to perform block co-oligomerization experiments in the presence of POPC and positively charged DDAB/POPC liposomes, sequential feeding of NCA-Trp and NCA-Glu was executed at pH 7.50 and the resulting product distributions were compared to each other. In these experiments, NCA-Trp was added first to either the POPC or the mixed DDAB/POPC liposomes and incubated for 24 hours. After incubation, the same amount of freshly prepared NCA-Glu was added to both liposome systems and incubated for another 24 hours. Samples were then withdrawn and analyzed by LC-MS. The UV-traces at 280 nm are shown for the two systems in Figure 50. At this wavelength, only the products containing Trp are seen. However, mass spectrometry also indicated the presence of some Glu-oligomers in both systems (but longer ones in the case of DDAB/POPC liposomes). Detailed analysis of the NCA-Glu condensation reaction in the presence of DDAB/POPC liposomes involves some analytical problems, as the reversed-phase separation must be replaced with ion-exchange chromatography which is not compatible with on-line LC-MS coupling. It is apparent that the positively charged, mixed DDAB/POPC liposomes assisted in the formation of higher block oligomers. For example, in block oligomers containing five Trps, the decapeptide H-Glu_5Trp_5-OH was formed at yields of 0.2% based on initial Trp in the DDAB/POPC system, whereas in the POPC system peptides no longer than H-Glu_2Trp_5-OH were detected (0.2%).

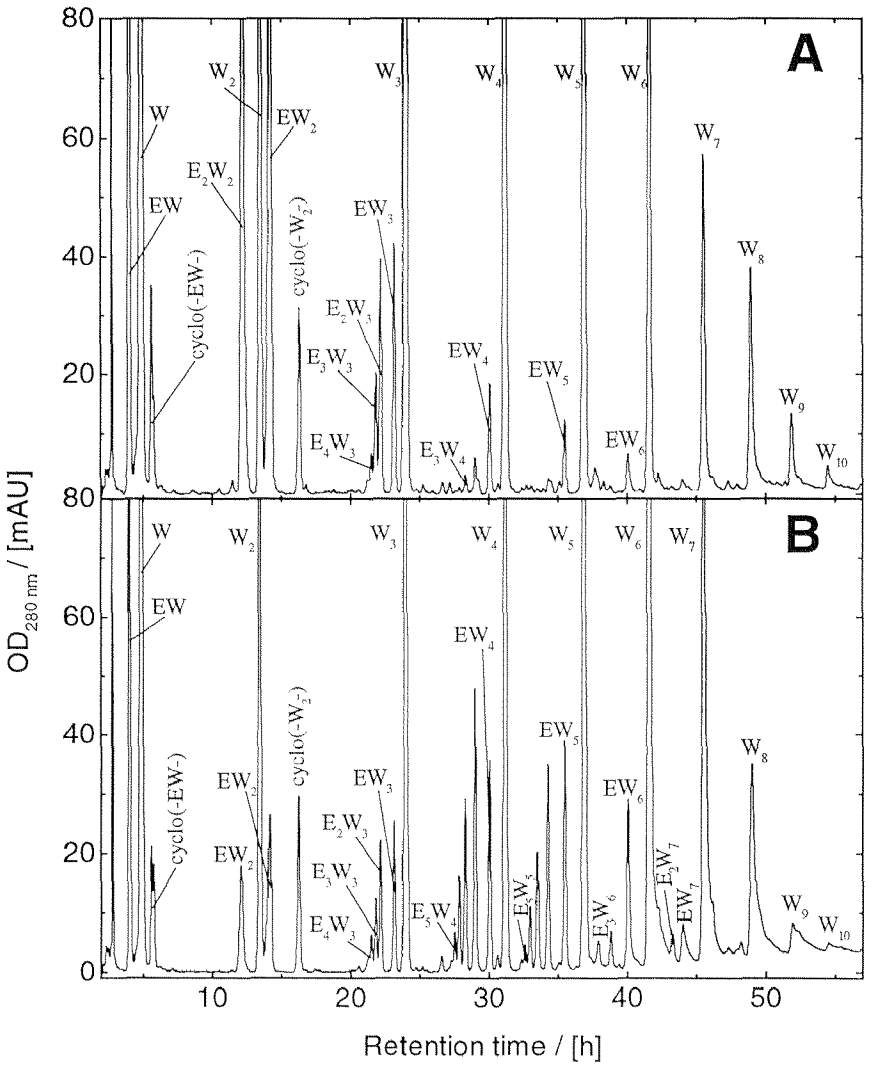


Figure 50: 40 mM NCA-Trp in 0.4 M imidazole/HCl, pH 7.50, was mixed first 1:2 (v/v) with either 100 nm extruded 20 mM POPC liposome suspension in 0.4 M imidazole/HCl buffer of pH 7.50 (A) or 100 nm extruded mixed 10 mM DDAB/10 mM POPC liposome suspension in 0.4 M imidazole/HCl buffer of pH 7.50 (B) for 24 hours at room temperature. After that period, the same volume (as for NCA-Trp) of a 40 mM NCA-Glu solution in 0.4 M imidazole/HCl, pH 7.50, was added to both systems and incubated for another 24 hours. E and W are one letter codes for Glu and Trp, respectively.

Hydrophobic and π-cation interactions most likely increased both the NCA-Trp concentration and the concentration of its oligomer products in the charged lipid bilayer membrane interface during the first feeding reaction. As seen in Figure 49, the higher affinity of the Trp residues for the positively charged lipid membrane leads to higher yields of the tetra- to octamer of Trp. After an incubation of 24 hours, all the initially added NCA-Trp had either oligomerized to oligo-Trps or hydrolyzed to Trp. Now the negatively charged NCA-Glu (pK_a ~ 4.5) [475] was added, which was expected to interact electrostatically with the positively charged membrane, but insignificantly with

the overall neutral membrane. The use of a quarternary amine (DDAB) makes it unlikely that the NCA-Glus are specifically oriented on the surfaces of the liposomes and also makes it unlikely that acid-base catalysis is important. It is more likely that negative-charged NCA-Glu monomers are concentrated close to the cationic surface, and the polyanionic oligomers and co-oligomers, once formed, attach or remain attached to the surface with an affinity that increases with oligomer length. Both the hydrophobic and π-cation interactions for Trp residues and the electrostatic interaction for Glu residues contribute to an increased affinity in the case of DDAB/POPC liposomes. Thus, the *N*-terminus of the oligo-Trps formed in the first feeding, as well as that of the oligo-Glus and Glu/Trp co-oligomers produced in the second feeding, are in an environment where the concentration of the activated amino acid exceeds that in free solution. Consequently, the probability that an oligomer will be extended at its *N*-terminus increases with its length, at least until it is long enough to attach to the liposome interface in a stable fashion.

The CDI-induced formation of Trp and Glu block oligomers shows that liposome-assisted condensation of aromatic hydrophobic NCA-amino acids can be extended to charged amino acids by making use of electrostatic interactions. In the previously described experiments, this was done by introducing a positive charge on the lipid membrane surface using the positively charged, double-chain ammonium surfactant DDAB. Negatively charged POPC liposomes can be analogously exploited in EEDQ-induced polycondensations.

5.2.2. EEDQ-Induced Condensation of H-ArgTrp-OH

In section 5.1.2., the EEDQ-induced condensation of dipeptides in the presence of POPC liposomes was discussed. The results indicated that selective oligomerization of hydrophobic dipeptides occurs in the presence of neutral liposomes, whereas hardly any polycondensation at all took place in their absence. The following two sections extend the liposome-assisted condensation to the more hydrophilic dipeptides H-ArgTrp-OH and H-HisTrp-OH by introducing negative charge on POPC liposomes.

1,2-Dioleoyl-*sn*-glycero-3-phosphate (DOPA) (**36**) forms stable liposomes in aqueous solutions in the absence of calcium ions and at neutral pH values. [514] It can also be used together with POPC or other phosphatidylcholines to form mixed, negatively charged liposomes. [515] The pK values reported for DOPA are greatly dispersed as in

the case for other phospholipids with ionizable groups. [516], [517] This is due to the fact that a rather large variety of techniques have been used to measure the pK values. Most of the values reported concern apparent pK values which were determined for different ionic conditions in the bulk water phase. This also accounts for the difficulty which exists in determining an intrinsic pK at water/lipid interfaces. For the present research, only the apparent pK values ($pK_{1,app}$: 3-4 and $pK_{2,app}$: 8-9) [516], [517] for phosphatidic acid are of immediate interest.

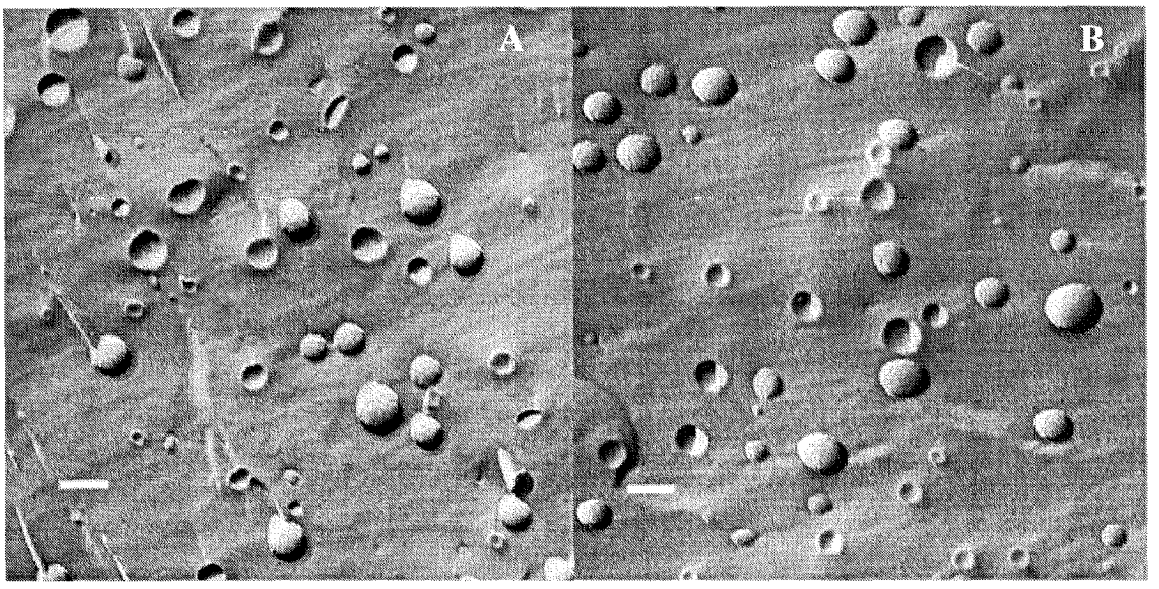


Figure 51: The freeze-fracture electron microscopy pictures are shown for 5 mM H-ArgTrp-OH, 100 nm extruded 12.5 mM DOPA/12.5 mM POPC liposomes, 50 mM phosphate, 1.2% (v/v) ACN, pH 6.40, before (A) and after incubation of 26 hours with 2 mM EEDQ (B). The length of the white bars corresponds to 100 nm.

First, H-ArgTrp-OH was chosen as the positively charged dipeptide (the pK_a for the Arg side chain is about 12, [475] so it is always protonated under the conditions used) to be oligomerized in the presence of negatively charged DOPA/POPC liposomes. 5 mM H-ArgTrp-OH, 12.5 mM DOPA, and 12.5 mM POPC were suspended in 50 mM phosphate buffer of pH 5.90, resulting in a suspension of pH 6.40 which was frozen ten times in liquid nitrogen and thawed in a water bath, prior to being extruded. The resulting 100 nm extruded DOPA/POPC liposome suspension was analyzed before and after condensation with 2 mM EEDQ by DLS and ff-EM. The DLS data (60°: 51.89±0.84 nm (51.82±0.46), 90°: 46.52±0.51 nm (46.74±0.69), and 120°: 45.13±0.30 nm (45.22±0.46)) and the ff-EM pictures (see Figure 51) revealed that the 1:1 composition of DOPA and POPC formed stable liposomes as expected and showed no transformation during the

condensation reaction. Table 13 compares the product distributions of the H-ArgTrp-OH condensation at two different pH values, 5.90 and 6.40, for either the aqueous reference system without liposomes, the POPC liposome system, or the negatively charged DOPA/ POPC liposome system. There is a clear preference for oligopeptide formation in the presence of negatively charged DOPA/POPC liposomes.

Table 13: A 5 mM H-ArgTrp-OH, 50 mM phosphate, 1.2% (v/v) ACN solution was incubated with 2 mM EEDQ (Ref), and either 100 nm extruded 25 mM POPC (POPC) or 12.5 mM DOPA/ 12.5 mM POPC liposomes (DOPA/POPC) for 26 hours at room temperature.

Products:	pH:	5.90			6.40	
% of dipeptide reacted		**Ref:**	**POPC:**	**DOPA/POPC:**	**Ref:**	**DOPA/POPC:**
Peptides:						
cyclo(-ArgTrp-)		8.4	8.0	18	-	-
H-$(ArgTrp)_2$-OH		5.1	6.4	43	-	56
H-$(ArgTrp)_3$-OH		-	1.7	6.0	-	11
Derivatives:						
EtO-CO-(ArgTrp)-OH		76	44	7.0	92	16
EtO-CO-$(ArgTrp)_2$-OH		3.3	2.9	6.0	-	5.0
rest		7.2	37	19	8.0	12

As we have seen for H-TrpGly-OH at pH 5.90, a weak association to the POPC liposome membrane (K_a: 1.63±0.20 M^{-1}) is sufficient to produce slightly enhanced oligomerization (see Table 11). The same holds true for H-ArgTrp-OH, which has an association constant K_a of 2.29±0.27 M^{-1} at pH 5.90 for POPC bilayer membranes. However, the effect was not as pronounced as for the negatively charged DOPA containing liposomes, where the K_a is 71.70±0.66 M^{-1} at pH 5.90 and 49.7±2.0 M^{-1} at pH 6.40, respectively. The percent yields of dipeptide reacted listed in Table 13 are misleading if two DOPA/POPC systems at different pHs are compared. The amount of dipeptide that reacted during the 26 hour incubation period was different in both cases. At pH 5.90, it was 26% for the reference, 22% for the POPC, and 54% for the DOPA/ POPC system, whereas at pH 6.40, it was only 9.2 and 22%, respectively. Taking this into account, the DOPA/POPC system at pH 5.90 (6.40) led to 580 µM (320 µM) tetrapeptide and 50 µM (40 µM) hexapeptide. In addition, although Arg was chosen

instead of Lys with the aim of preventing branching at the side chain, the peaks of the oligopeptides were broad and often consisted of several overlapping peaks. It has to be concluded from these observations that branching probably occurred to a certain extent during the polycondensation, but detailed analysis and quantification was difficult. For the same reason, the values in Table 13 are strongly dependent on the specific integral parameters used to evaluate the chromatograms. Therefore, the results should be regarded as qualitative rather than quantitative.

Despite the analytical difficulties, it is clear that the introduction of negative charge at the liposome interface favors oligomerization of the dipeptide H-ArgTrp-OH. As mentioned previously, the guanidinium function of Arg has a pK of about 12 and is therefore completely protonated under the conditions employed here. On the other hand, the $pK_{1,app}$ of DOPA is between 3-4 and the $pK_{2,app}$ between 8-9, so that most of the phophatidic acid head groups have one negative charge around neutral pH values. The possibility for electrostatic interactions, in particular the formation of ionic H-bonds of the guanidinium ion with the phosphate group (as known for phosphodiesters inhibitors in staphylococcal nuclease (see Figure 52), [518], [519]) probably increase the affinity of Arg-containing peptides for the negatively charged lipid membrane surface. In addition, the interactions of the Trp residues with the lipid bilayers must still be taken into account, so that both hydrophobic and electrostatic interactions contribute to the increased affinity and therefore the local accumulation of H-ArgTrp-OH at the DOPA/ POPC liposome membranes. As in the case of H-TrpTrp-OH, the lipophilic condensing agent EEDQ activates and oligomerizes the peptides at the membrane interfaces.

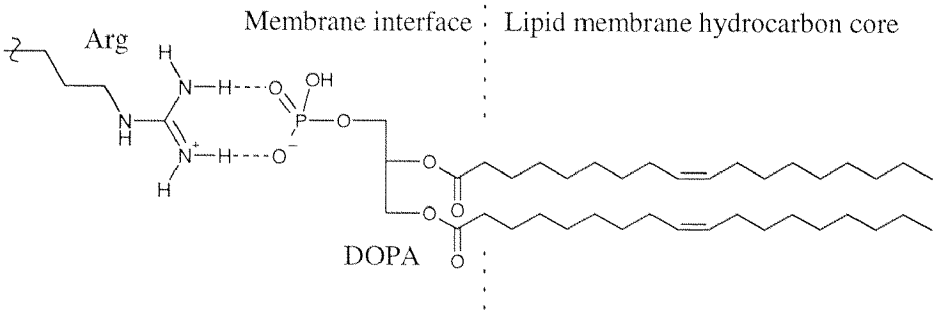


Figure 52: Guanidinium ions may form strong ionic H-bonds with phosphates, as schematically shown here for the probable interaction of the Arg-residues in H-ArgTrp-OH and its oligomers.

Since both peptides, H-ArgTrp-OH and H-TrpTrp-OH, can be oligomerized by liposome-assisted polycondensation, the question was posed as to whether the co-

condensation of these two dipeptides is possible. Since H-TrpTrp-OH is expected not only to bind to zwitterionic membranes but also to negatively charged lipid bilayers, co-oligomerization should occur when H-ArgTrp-OH and H-TrpTrp-OH are incubated in the presence of EEDQ and DOPA/POPC liposomes.

Table 14: A 5 mM H-TrpTrp-OH, 5 mM H-ArgTrp-OH solution in 50 mM phosphate buffer, pH 6.60, was incubated for 26 hours at room temperature with 2 mM EEDQ (in 1.2% (v/v) ACN) and either 100 nm extruded 12.5 mM DOPA/12.5 mM POPC liposomes (POPC/DOPA) or in the absence of liposomes (Reference).

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

The results for the DOPA/POPC liposome system and the aqueous reference system are provided in Table 14. In the presence of negatively charged lipid bilayer membranes, 28% of the dipeptides had reacted, for the aqueous reference system 16%. Analysis by DLS displayed no significant changes of the liposomal shapes and sizes during the

reaction. The hydrodynamic radii before (after) the polycondensation reaction were 47.06±0.59 nm (46.94±0.65) at 60°, 45.72±0.40 nm (46.46±0.58), and 44.95±0.57 nm (45.84±0.38) at 120°. Looking at each dipeptide individually reveals that in the liposome (reference) system about 13% (2%) of H-ArgTrp-OH and 15% (14%) of H-TrpTrp-OH had reacted. Of the total dipeptides reacted, 63% formed tetrapeptides and 11% hexapeptides. Of all the oligopeptides formed, 64% were co-oligomers made up of both dipeptide monomers and the co-oligomer products were overrepresented compared to the statistical value, when assuming the same reactivity and local concentration for both monomers. Therefore, the oligomers consisting of only one type of monomer were clearly underrepresented. Co-oligomers having -TrpTrp- residues were obtained in higher yields than these containing -ArgTrp-. This is consistent with the observation made above and leads to the conclusion that H-TrpTrp-OH is probably more reactive on its *N*-terminal amine than H-ArgTrp-OH. This would also explain the excess of H-ArgTrp$_3$-OH, as well as the higher yield in oligo-Trp carbamates. However, more detailed studies will be needed to confirm this assumption.

In conclusion, the liposome-assisted co-oligomerization of hydrophobic and positively charged dipeptides in the presence of negatively charged lipid bilayers is possible and yielded peptides in reasonable amounts. The corresponding reference system failed to yield co-oligomers.

5.2.3. EEDQ-Induced Condensation of H-HisTrp-OH

In enzymes, the most common nucleophilic groups that are functional in catalysis are the serine, cysteine and tyrosine hydroxyl and the imidazole of histidine. The latter usually functions as an acid-base catalyst and enhances the nucleophilicity of hydroxyl and thiol groups, although it sometimes acts as a nucleophile in phosphoryl transfer. [475] Furthermore, imidazole [520] and histidine derivatives [521] have been shown to have weak catalytic activity in the hydrolysis of activated esters. Some histidine containing peptides have been reported to associate with micelles or lipid bilayers and have shown enhanced catalytic activity. [522]-[535] To emphasize the importance of the introduction of His into oligopeptides exhibiting affinity to liposomes, the following experiment was carried out with POPC liposomes, using the hydrophobic substrate *p*-nitrophenyl palmitate (C16-ONp) [536]-[538] and the hydrophobic *N*-protected, histidine containing tripeptide Z-PheHisLeu-OH. This experiment will demonstrate that binding of

catalyticly active peptides to liposome membranes can lead to enhanced overall reaction rates for hydrophobic substrates. This characteristic is believed to be important with respect to the origin of life.

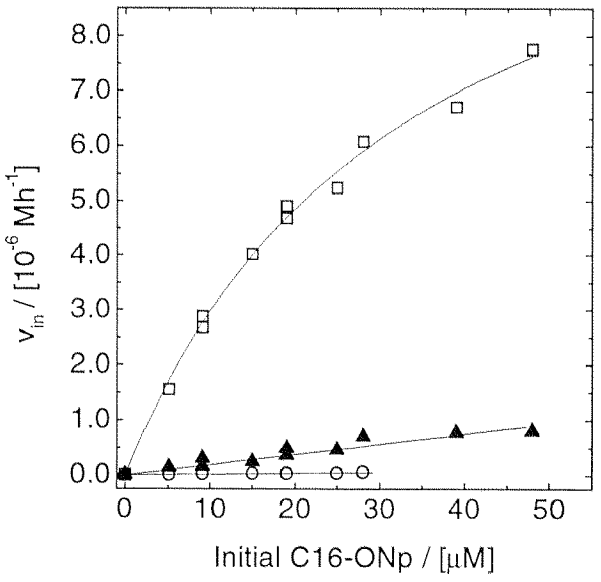


Figure 53: The dependency of initial hydrolysis rates (v_{in}) for the hydrolysis of C16-ONp on different substrate concentrations is shown. Hydrolysis at 25 °C was either performed in a suspension of 50 nm extruded 10 mM POPC liposomes, 50 mM borate buffer, pH 8.50, 1.2% (v/v) ACN in the absence (○) or presence (□) of 1 mM Z-PheHisLeu-OH, or 1 mM Z-PheHisLeu-OH in 50 mM borate buffer of pH 8.50, 1.2% (v/v) ACN with no liposomes (▲).

The initial hydrolysis rates for different conditions and concentrations of C16-ONp are shown in Figure 53. When this compound was suspended in water at pH 8.5, practically no hydrolysis was observed (data not shown). However, in the control experiment performed with C16-ONp in the presence of POPC liposomes only (no peptide), slight hydrolysis was observed. At fixed lipid concentration, the initial velocity for the hydrolysis reaction increased linearly with increasing C16-ONp concentration (see Figure 53). Likewise, it has been shown for oleic acid/oleate liposomes that at fixed substrate concentrations, the velocity increases with increasing fatty acid concentration. [535] As a further control, the catalytic efficiency of the Z-PheHisLeu-OH peptide towards the hydrolysis of C16-ONp was studied in aqueous buffer and the absence of liposomes. It is clear from the representative data in Figure 53 that the peptide itself has only a small effect on the initial hydrolysis rate of C16-ONp in the absence of liposomes. A large increase in the initial hydrolysis rate was observed, however, when C16-ONp was incubated in the presence of POPC liposomes and peptide. Interestingly, the

dependence of the initial velocity on the substrate concentration at fixed lipid and peptide concentrations follows a saturation profile which is reminiscent of Michaelis-Menten kinetics[4], with an apparent K_M value of 35±3 μM and v_{max} being 13±1 μMh^{-1}.

The normalized catalytic activity of peptide containing POPC liposomes towards the hydrolysis of C16-ONp is two orders of magnitude larger than without the peptide. Considering that under the conditions used only 17% of Z-PheHisLeu-OH was bound to the POPC liposomes, and by normalizing this to bound peptide, one concludes that the membrane-peptide complex has a catalytic power which is about 2.5 orders of magnitude greater than that of the lipid membrane itself (or 1.5 orders of magnitude greater than the tripeptide alone). Similar results were obtained with Z-HisTrp-OH (data not shown), where the initial hydrolysis rate at pH 8.50 and room temperature in the presence of 1 mM dipeptide and 50 nm extruded 10 mM POPC liposomes was 50 times higher compared to the peptide alone, using a substrate concentration of 25 μM C16-ONp. Taking into account the association constant of Z-HisTrp-OH to POPC liposomes (17.4±1.7 M^{-1}, corresponds to about 15% dipeptide bound), the catalytic power is about 2.5 orders of magnitude greater than that of the dipeptide alone.[5]

With this rate enhancement in mind - an enhancement was induced by a tertiary complex of peptide, substrate and liposome - the liposome-assisted oligomerization of a His-containing dipeptide, namely H-HisTrp-OH was attempted. From the experience gained in the experiments described above, the binding of a dipeptide to the lipid bilayer is a prerequisite for efficient EEDQ-induced oligomerization. For this reason, the initial binding studies of H-HisTrp-OH were performed under different conditions.

The pK of the π-imidazole nitrogen of His is around 6.5, [539] but depending on its microenvironment it can range from 5 to 8. [475] At pH 5.90, the association constant K_a for 5 mM H-HisTrp-OH to 100 nm extruded 25 mM POPC liposomes was measured 0.54±0.09 M^{-1}. At this pH, the imidazole residue of His is expected to be partially protonated and hence increases the hydrophilicity of the dipeptide compared to the

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[4] It should be kept in mind that for unilamellar 50 nm POPC liposomes, the POPC aggregation number is about 27'000 so that the liposome concentration at 10 mM POPC lipid corresponds to 0.37 μM, which is far lower than the substrate concentrations used (5-50 μM). However, there are probably many independent binding sites on the surface of each liposome (on the basis of the binding data for Z-PheHisLeu-OH, K_a is 22±1 M^{-1}, each POPC liposome contains about 450 tripeptide bound molecules) and it is therefore difficult to establish whether the liposome system shows turnover.

[5] Please be aware of the warning given at the end of this section.

deprotonated residue. In order to determine whether better affinity can be obtained at higher pH values for the deprotonated His, K_a was determined at pH 8.00 for POPC liposomes. The value was 0.70±0.17 M^{-1} and thus did not provide a great improvement. This led to the conclusion that electrostatic interactions had to be introduced. With the help of both charged lipids and lower pH values, it should be possible to get reasonable peptide binding and eventually oligomerization. To test this assumption, 100 nm extruded 8.3 mM DOPA/16.7 mM POPC liposomes were used and the K_a values determined for 5 mM H-HisTrp-OH at different pH values. At pH 4.00, K_a was 48.84±0.64 M^{-1}; at pH 5.00, 42.65±0.80 M^{-1}; at pH 5.70, 38.8±1.0 M^{-1}; and at pH 6.24, 24.29±0.68 M^{-1}. The decreasing trend of the association constants with higher pH values displays the effect of the π-imidazole nitrogen pK_a of H-HisTrp-OH and the apparent pK_as of DOPA on the dipeptide affinity to the DOPA/POPC lipid membrane. Between pH 4 and 7, DOPA is expected to have one negative charge, whereas around pH 6.5 about 50% of the His residues are protonated. Beyond that value the amount of protonated species increases with decreasing pH and vice versa. Therefore, higher affinity is expected closer to pH 4 and that is exactly what was observed.

Table 15: A 5 mM H-HisTrp-OH, 50 mM phosphate, 1.2% (v/v) ACN solution of either pH 4.00, 5.00, 5.70, or 6.24, was incubated with 2 mM EEDQ in the absence of liposomes (Ref), or in the presence of 100 nm extruded 8.33 mM DOPA/16.67 mM POPC liposomes (Lip) for 26 hours at room temperature.

Products:	pH:	4.00		5.00		5.70		6.24	
in % of dipeptide reacted		**Ref:**	**Lip:**	**Ref:**	**Lip:**	**Ref:**	**Lip:**	**Ref:**	**Lip:**
Peptides:									
cyclo(-HisTrp-)		3.5	21	0.5	21	-	17	-	9.7
H-$(HisTrp)_2$-OH		2.4	34	3.5	34	2.3	34	0.6	23
H-$(HisTrp)_3$-OH		-	1.3	-	2.1	-	1.6	-	-
Derivatives:									
EtO-CO-HisTrp-OH		71	20	77	21	78	27	83	35
EtO-CO-$(HisTrp)_2$-OH		1.1	4.7	2.0	6.9	0.7	5.4	0.4	5.3
rest		22	19	17	15	19	15	16	27

The EEDQ-induced condensation at all four pH values was performed for H-HisTrp-OH in the presence and absence of DOPA/POPC liposomes (see Table 15). The amount of

oligomers produced was higher in the presence of bilayer membranes at all pH values. In the reference systems, the carbamate of the di- and tetrapeptide was the major product. Diketopiperazine formation seemed to be more favored at lower pH values, but the opposite holds true for the side reaction involving carbamate formation. The same trends had already been observed for H-TrpTrp-OH and H-ArgTrp-OH (see Table 10, Table 12 and Table 13). Maximum oligopeptide formation was reached in the liposome system at pH 5.00, where about 40% of H-HisTrp-OH had reacted. At this pH, the hexapeptide yield was 14 µM, whereas at pH 5.70 it was 7.1 µM, at pH 4.00 only 5.8 µM and at pH 6.24 no hexapeptide was formed at all.

As we have seen above, His-containing peptides that exhibit affinity to lipid bilayers may promote the hydrolysis of the hydrophobic C16-ONp. This observation was one of the reasons for performing liposome-assisted condensations with His-containing peptides. In order to test the H-HisTrp-OH oligomers for their catalytic behavior towards C16-ONp hydrolysis, H-HisTrpHisTrp-OH was synthesized. This tetrapeptide is assumed to exhibit more affinity to the liposomal membrane than the dipeptide at alkaline pH values, where the His residues are no longer protonated. If the pH is chosen below the pK_a of the *N*-terminal amine (ca. 8), [475] one would expect this amine to be protected by protonation and therefore it should not interfere as a nucleophile in the C16-ONp hydrolysis. However, the pH value cannot be set too low, as the His residue has to be deprotonated for the imidazole catalyzed hydrolysis of esters (see Figure 54).

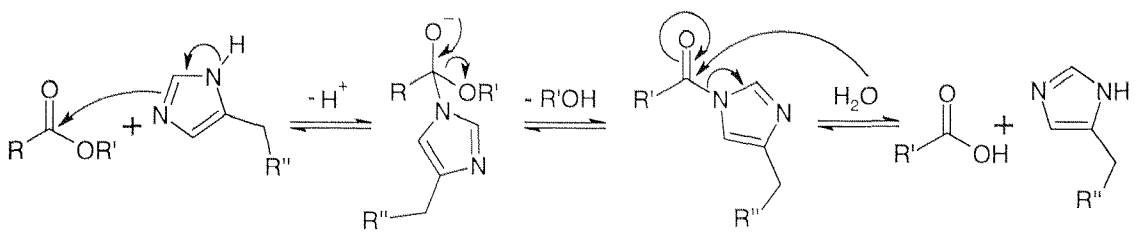

Figure 54: Reaction scheme for the hydrolysis of carboxylic acid esters catalyzed by the imidazole of a histidine residue. [540], [541]

For this reason, dialysis experiments were first performed for 1 mM H-HisTrpHisTrp-OH and either 50 nm extruded 10 mM POPC or 50 nm extruded 3.33 mM DOPA/6.67 mM POPC liposomes at pH 7.70. For the latter system, pH 8.50 was also tested. These dialysis experiments were done in order to get information on the tetrapeptide binding properties. At pH 7.70, the K_a obtained for the POPC system was 5.15±0.80 M^{-1}

whereas for the DOPA/POPC system it was much higher, namely 134±12 M^{-1}. This significant difference suggests that at the higher pH value some of the His residues are still protonated and the electrostatic forces that contribute to peptide binding are still dominant. However, at pH 8.50, the K_a for the DOPA/POPC system drastically decreased to 23.3±2.0 M^{-1}, probably because the pH was now above the pK_a of both His residues in the tetrapeptide and close to the $pK_{2,app}$ of DOPA.

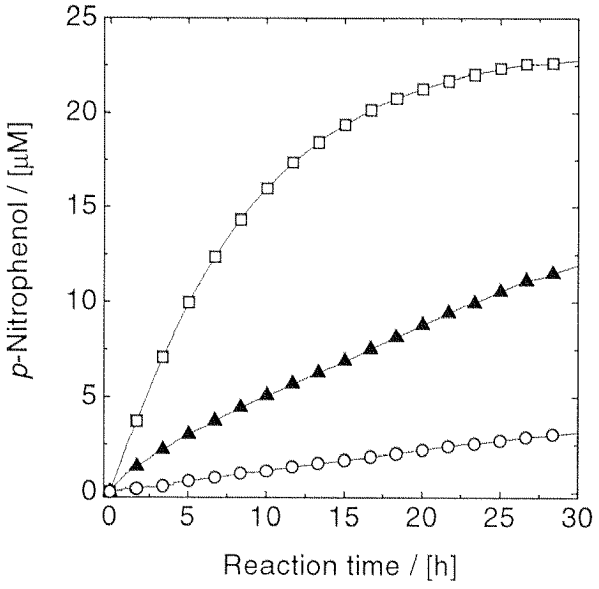

Figure 55: The release of the *p*-nitrophenol is shown for the incubation of 25 μM C16-ONp at 25 °C in a suspension of 50 nm extruded 3.33 mM DOPA/6.67 mM POPC liposomes, 50 mM borate buffer, pH 8.50, 1.2% (v/v) ACN in the absence (○) or presence (□) of 1 mM H-HisTrpHisTrp-OH, or 1 mM H-HisTrpHisTrp-OH in 50 mM borate buffer, 1.2% (v/v) ACN of pH 8.50, 1.2% (v/v) ACN with no liposomes (▲).

C16-ONp hydrolysis experiments were performed for the POPC, as well as the DOPA/ POPC liposomes alone, the liposomes in the presence of tetrapeptide, and the tetrapeptide alone, for a concentration of 25 μM C16-ONp at the above mentioned pH values. As expected from the K_a values and the scheme in Figure 54, the rates of *p*-nitrophenol release in the hydrolysis of C16-ONp were greatest in the liposome/peptide system (2.5-fold faster) compared to the peptide alone at pH 8.50 (see Figure 55). In order to check whether the *N*-terminal amine formed an amide with the plamitate by transamidation of the ester, LC-MS analysis was conducted on all samples after incubation. The chromatograms for the peptide only samples monitored at a wavelength of 290 nm displayed a H-HisTrpHisTrp-OH peak (ESI^+ m/z: 665.3) at low retention time (16.3 min), and very low amounts of impurities which were too low to be identified by

the MS. In the liposome samples with no peptide present, no peaks were observed at 290 nm, since no Trp was present in any of the compounds in these samples. If however, the tetrapeptide containing liposome samples were analyzed, two peaks appeared in the chromatograms at 290 nm: the H-HisTrpHisTrp-OH peak (ESI^+ m/z: 665.3, retention time: 16.3 min) and a second peak at a higher retention time (35.5 min). The later peak was also present in the peptide only samples, but at much lower intensity. The UV-Vis spectra of this peak clearly revealed that the compound in question contained Trp (see Figure 56A) and its MS spectrum displayed a monoisotopic mass in the ESI^+ mode of 903.5, which corresponds to the *N*-palmitoylated tetrapeptide (**50**) (see Figure 56B).

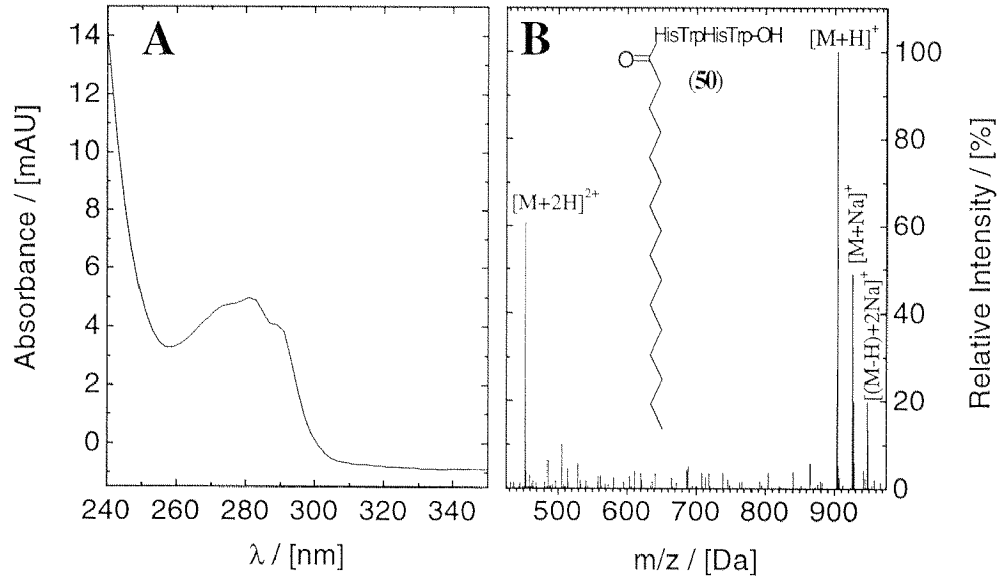

Figure 56: (A) UV-Vis spectrum of the peak at 35.5 min retention time in the liposome/peptide sample after incubation with 25 μM C16-ONp. (B) Mass spectrum of the peak at 35.5 min retention time. The peaks from left to right correspond to the masses: 452.3, 903.5, 925.5, and 947.5 Da, whereas the first is the doubly charged species and the last two are sodium adducts.

Integration of the two peaks at 16.3 and 35.5 min retention time (based on the assumption that the extinction coefficient of (**50**) was the same as for H-HisTrpHisTrp-OH) led to the following results and conclusions: 1.9 to 2.1% of the initial H-HisTrpHisTrp-OH, which corresponds to 19 and 21 μM peptide, had reacted with C16-ONp to the amide (**50**). Therefore, at least 75 to 85% of C16-ONp did not hydrolyze, but underwent transamidation. The crux of the whole story is that the substrate C16-ONp in the 'hydrolysis' experiments was only 2.5% of the amount of peptide catalyst present. Therefore, small amounts of highly reactive nucleophiles (i.e. deprotonated *N*-terminal amines of the peptide or surfactant impurities) might have reacted with the ester to form

an amide or other by-products and have released the *p*-nitrophenol, leading to the assumption that hydrolysis had occurred.

A re-evaluation of the C16-ONp 'hydrolysis' by the suspension of the liposome/Z-HisTrp-OH, revealed by-products with spectra like the one in Figure 56 (A) and the masses 539, 584, 734, and 756 Da in the ESI^+ mass spectra. This was observed despite the fact that the *N*-terminal amine was Z-protected. Impurities present in the commercial Z-HisTrp-OH might have reacted with the substrate and caused the release of *p*-nitrophenol, thus falsifying the hydrolysis rates measured. The same might hold true for Z-PheHisLeu-OH and many other published imidazole catalysts for *p*-nitrophenyl ester hydrolysis in the presence of surfactant aggregates. In most of these experiments, the imidazole catalysts were used in equimolar amounts or higher, often in the presence of surfactants in great excess. [522]-[534], [542]-[548] Extensive analysis of the reaction mixtures at the end of the reaction was seldom performed. In addition, the hydrolysis of *p*-nitrophenyl acetate in the presence of mixed micelles of *N*,*N*-dimethyl-*N*-2-hydroxyethylstearyl ammonium bromide and long-chain 4-imidazolyl derivatives is known to occur by fast acylation of the imidazole, followed by fast quantitative transfer of the acyl group to the hydroxy group. [549]

Based on these observations, I suggest that all published data on *p*-nitrophenyl ester hydrolysis experiments catalyzed by imidazole derivatives in the presence of surfactant aggregates should be treated with great care. In order to address the problem of misleading secondary reactions in imidazole catalyzed ester hydrolysis, some of the experiments in the literature will have to be repeated and their products analyzed more extensively with methods such as LC-MS.

With respect to the observations made on the use of His-containing peptides as catalysts in the ester hydrolysis reactions, the by-products formed as the carbamates of the oligomers in EEDQ-induced condensation become more interesting, since they are protected at the *N*-terminal amine. This protection should in principle prevent them from undergoing amidation at the *N*-terminus. Another promising direction for future research would be investigations of acyl transfer reactions catalyzed by multiple His-containing peptides in the presence of liposomes, as such experiments in the absence of surfactant aggregates have been recently published, showing that this type of catalysis occurs. [550]

5.2.4. Concluding Remarks on the Condensation on Charged Liposomes

The results presented above show that the liposome-assisted condensation of either NCA-amino acids or dipeptides with the hydrophobic condensing agent EEDQ can be extended to charged, hydrophilic amino acids and peptides. The introduction of either positively or negatively charged lipids into the POPC membranes increased the bilayer affinity of oppositely charged NCA-amino acids and peptides: the NCA-amino acids and peptides accumulate at the membrane interfaces by electrostatic (combined with hydrophobic) interactions. In the case of CDI-induced condensation, the increased affinity of oppositely charged NCA-amino acids and peptides leads to preferential condensation in the presence of charged liposomes because the longer oligomers bind more tightly to the liposome interface and are elongated by the accumulated NCA-amino acid. In the case of EEDQ-induced condensation, the accumulated, oppositely charged peptides are activated by the lipophilic EEDQ and oligomerized.

In summary, accumulation, due to electrostatic and/or hydrophobic interactions, leads to higher local concentrations of the activated amino acids, peptides, and oligopeptides on the lipid bilayer. This favors the oligomerization of charged hydrophilic as well as hydrophobic amino acids and peptides. In this way, oligopeptides consisting of hydrophobic and hydrophilic amino acids such as Trp, Glu, Arg, His were obtained. Such His-containing peptides in the presence of liposomes seem to at least have an effect on the selectivity of the C16-ONp 'hydrolysis' reaction. Side reactions complicate the interpretation of the experimental results as to whether or not catalysis occurs.

5.3. Liposome-Assisted Condensation of Racemic NCA-Trp Mixtures

Enantiomers have identical physico-chemical properties in a symmetrical environment,[6] but their properties are different in an asymmetrical environment (i.e enantiomers may react at different rates in the presence of a chiral surface, they may have different solubilities in an optically active solvent, etc. [553]). In most cases however, these differences are too small to be measured. Equimolar mixtures of enantiomers are called racemates (or racemic mixtures[7]). Their properties are not always the same as those of

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[6] Interactions between electrons, nucleons, and certain components of nucleons (e.g. bosons), called weak interactions, violate parity (mirror image interactions do not have the same energy). It has been claimed that such interactions cause one of a pair of enantiomers to be (slightly) more stable than the other. [551], [552]

the individual enantiomers. [554] In the gaseous state, liquid state, or in solution, racemates usually have the same properties (within the experimental error) as each individual enantiomer, since such mixtures are nearly ideal (no significant intermolecular interaction between the enantiomers). However, properties involving the solid state (i.e. melting point, solubilities (see experimental section), and heats of fusion) are often different. [555]

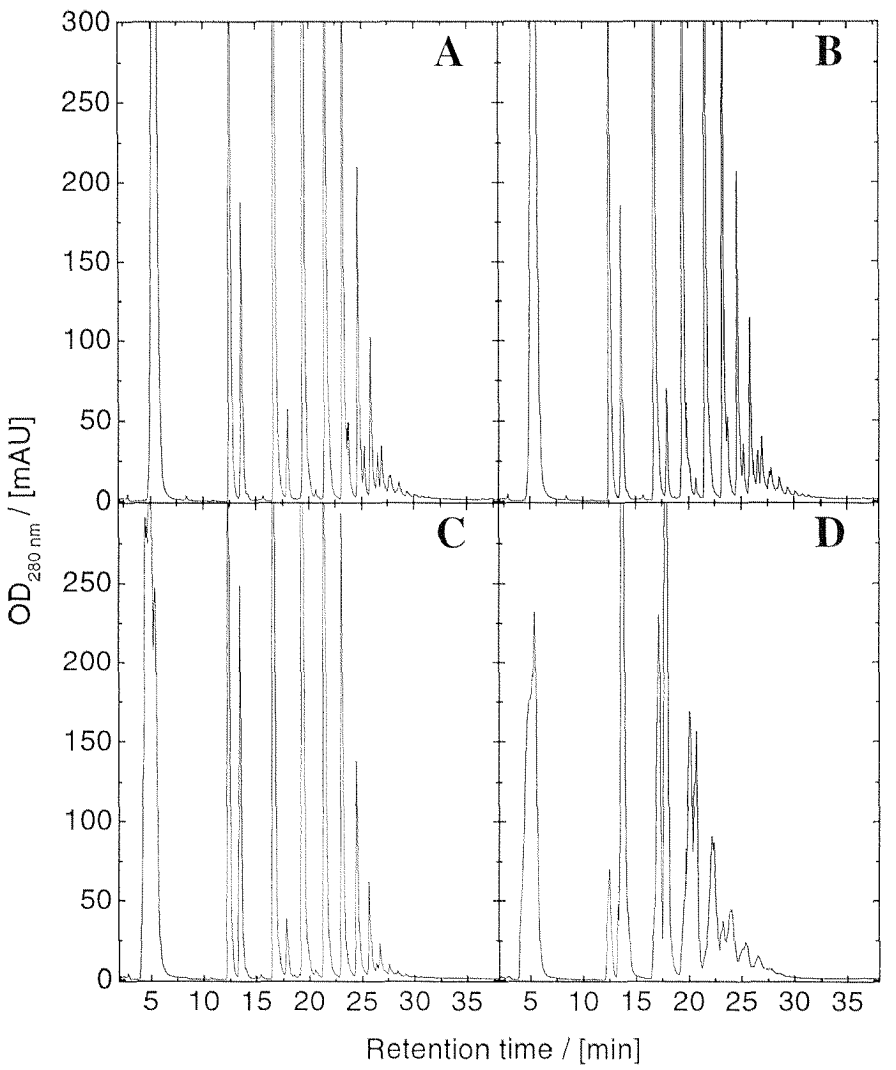

Figure 57: HPLC chromatograms (recorded at 280 nm) of the CDI-induced condensation in the presence of POPC liposomes and either NCA-L-Trp (A), NCA-D-Trp (B), NCA-L-Trp(d_5) (C), or NCA-D-Trp/NCA-L-Trp(d_5) racemate (D) are shown here. In all four single-feeding experiments, a freshly prepared solution of 60 mM NCA-amino acid(s), 0.4 M imidazole/HCl, pH 6.80, was mixed 1:3 (v/v) with a 100 nm extruded 100 mM POPC liposome suspension in 0.4 M imidazole/HCl at pH 6.80 and incubated for 12 hours at room temperature.

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[7] The term racemic mixture is sometimes restricted only to the solid phase. In the present study this expression will, however, also be used for the solution.

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (**34** in Figure 34), which was used in the preparation of the POPC liposomes described above, is optically active and we have seen that L-Trp-oligomers exhibit affinity for the POPC bilayer membranes. As described in paragraph 5.1.1., the effect of the POPC liposomes on the NCA-L-Trp condensation causes the formation of longer Trp-oligomers. The results indicated that the elongation of longer oligomers occurs only in the presence of the liposomes, because the oligomers are solubilized at the lipid membrane interfaces. This characteristic of the liposome-assisted condensation of NCA-L-Trp suggests that a difference might be observed for the oligomerization of its enantiomer NCA-D-Trp, since the environment, being a chiral POPC liposome suspension, is asymmetric. Therefore, a set of experiments was carried out using the CDI-induced condensation of either L- or D-Trp, or the racemate of NCA-Trp in the presence of POPC liposomes (see Figure 57). In the experiments with the racemate, deuterated L-Trp(d_5) was used instead of L-Trp. In this way, the CDI-induced condensation of NCA-L-Trp(d_5) could be compared with that of undeuterated NCA-L-Trp (see Figure 57 A and C).

Evaluation of four independent single-feeding experiments with NCA-D-Trp in the presence of liposomes revealed average experimental errors of 3 to 20% (standard deviations of the chromatogram peak areas at 280 nm for each oligo-Trp produced). The experimental error was normally greater for longer oligomers where yields were smaller. The mean experimental error for all Trp-oligomers was about 8%. These experimental errors restrict the observation of small effects induced by the presence of the asymmetric lipid membrane. Only differences of greater than 20% deviation from the chromatogram of the NCA-L-Trp condensation in the presence of the POPC liposomes can be considered to be significant in the condensation of NCA-D-Trp or NCA-L-Trp(d_5). Extensive evaluation of the chromatograms (A) to (C) shown in Figure 57 and their corresponding UV-Vis and MS spectra did not reveal any significant differences. Apparently, the influence of the asymmetric lipid membrane on the CDI-induced condensation of either the L-Trp (L-Trp(d_5)) or D-Trp is not large enough to be detected by the analytical methods used. This might be due to the fact that the condensation was performed above the transition temperature of POPC (T_m = -3°C), [556] where it is in the liquid (liquid-crystalline) analog phase. More stereochemical influence might be expected for a lipid with a higher T_m than room temperature, since the solid analog (gel) phase generally has a higher packing asymmetry. [557], [558] The use of L-Trp(d_5), with

a deuterated indole ring, did not seem to have any significant influence on the CDI-induced condensation in the presences of liposomes when compared to the undeuterated system. The same was observed in the absence of liposomes (data not shown).

If a racemic mixture of NCA-L-Trp(d_5) and NCA-D-Trp was oligomerized in the presence of POPC liposomes, the chromatogram of the resulting suspension after reaction (see Figure 57D) was significantly different from the chromatograms just described (A-C). This difference was expected, as any of the diastereomeric Trp-oligomers of the same length (consisting now of L- and/or D-Trp) produced in the racemic condensation experiment would have slightly different retention times. The enantiomeric pairs of each oligo-Trp diastereomer, however, have the same retention times, since the reversed-phase column (C_{18}) used was achiral. Analysis of the product length revealed that the use of racemic NCA-Trp instead of NCA-L-Trp or NCA-D-Trp generally led to (i) a reduction in the yield of oligomers obtained and (ii) a decrease in the length of the longest detectable oligomer. The same was observed in the absence of liposomes (data not shown). If NCA-L-Trp was used instead of the deuterated species in racemic condensation experiments, congruent chromatograms were obtained (data not shown). This underscores the statement made above that deuteration of the indole ring has no significant influence on the chemical behavior of L-Trp in CDI-induced condensations.

Similar results to those just described for the racemic NCA-Trp condensation have been previously reported for experiments with NCA-Glu in aqueous systems. [412]

If the different types of NCA-L/D-Trp condensation in the presence and absence of liposomes were extended to multiple-feeding experiments (two feedings), no significant qualitative difference to the results obtained in single-feeding experiments was observed. The chromatograms of the multiple-feeding experiments in the presence of liposomes are shown in Figure 58. As in the single-feeding experiments described earlier, the experimental errors of the different oligomer peaks were determined for multiple addition of NCA-D-Trp to the POPC system. These values were between 4 and 20% (standard deviation in % of mean value) with a mean experimental error of 9%. In some experiments a slight trend towards higher yields of the longer oligomers was observed for the case of NCA-D-Trp, when compared to the NCA-L-Trp(d_5) condensation. However, with respect to the experimental error, the differences in peak areas for the highest oligomers detected seldom exceeded 20% and were never higher than 55%.

Therefore, the slight trend towards higher yields in NCA-D-Trp condensation was not regarded to be significant. The condensation of the racemic NCA-Trp (Figure 58 D) clearly showed a reduction in the yield of higher oligomers and a decrease in the length of the longest detectable oligomer obtained when compared to NCA-L-Trp (Figure 58 A and C) or NCA-D-Trp condensations (Figure 58B).

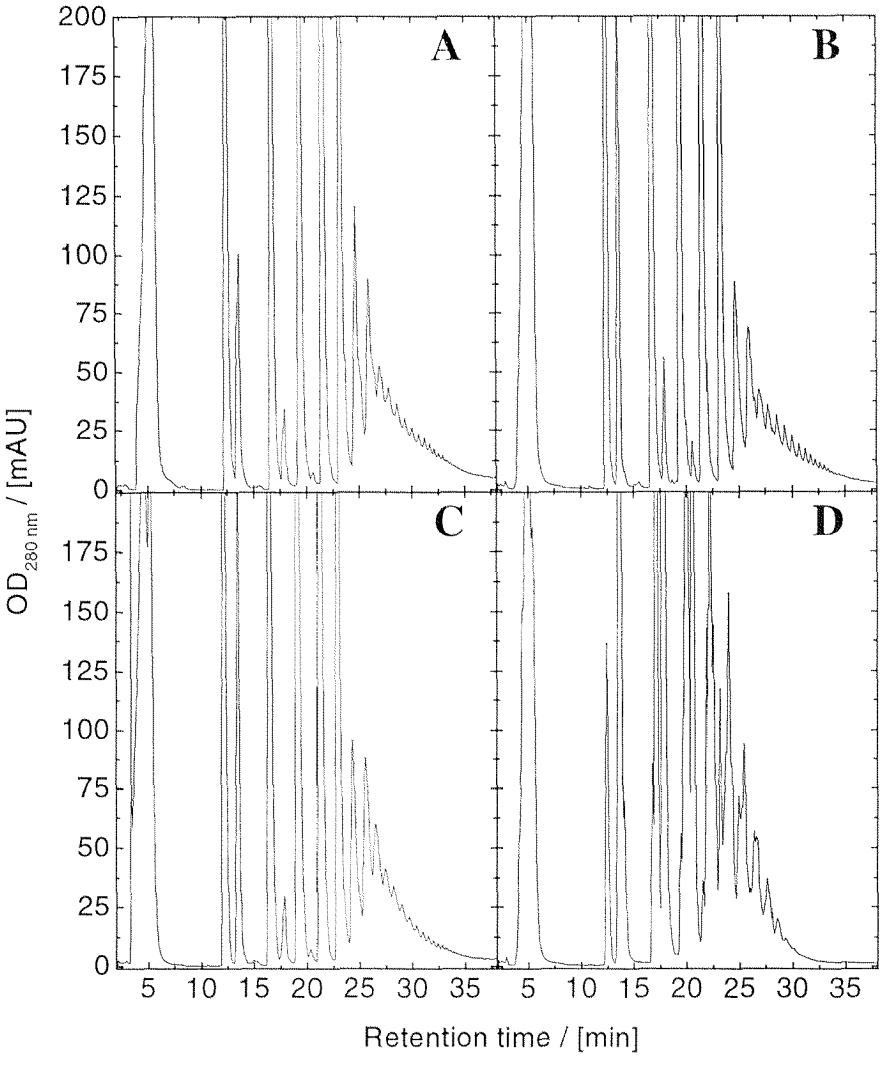


Figure 58: HPLC chromatograms recorded (at 280 nm) of the CDI-induced condensation in the presence of POPC liposomes are shown, with two-feedings of either NCA-L-Trp (A), NCA-D-Trp (B), NCA-L-Trp(d_5) (C), or NCA-D-Trp/NCA-L-Trp(d_5) racemate (D). In the first feeding a freshly prepared solution of 60 mM NCA-amino acid(s), 0.4 M imidazole/HCl, pH 6.80, was mixed 1:3 (v/v) with a 100 nm extruded 100 mM POPC liposome suspension in 0.4 M imidazole/HCl at pH 6.80 and incubated for 12 hours at room temperature. The same volume of NCA-amino acid was added in the second feeding and incubated for another 12 hours.

Obviously, the fluid, chiral lipid surface in the POPC liposome-assisted condensation of one or the other enantiomer of NCA-Trp does not induce a large difference for either of the two enantiomers. The racemic NCA-Trp condensation, however, produces shorter

oligomers in the liposome system as well as in the aqueous reference system (data not shown), when compared to the homochiral oligomerization of only one enantiomer. Let us now take a closer look at the different stereoisomers produced in the racemic NCA-Trp condensation in the presence and absence of POPC liposomes.

5.3.1. Stereoisomer Distribution in Racemic NCA-Trp Condensation

The racemic co-condensation of NCA-L-Trp and NCA-D-Trp leads in principle to a distribution of stereoisomers within each oligo-Trp group of a fixed length n. The number of the 2^n ($2^n/2$ enantiomer pairs of diastereomers) theoretically possible stereoisomers increases with the oligomer length n. The experimental separation and determination of all the 262'143 theoretically possible stereoisomers of all the different Trp-oligomers formed (up to the 18mer) in Figure 58 D seems to be an insurmountable task. In the LC separation chromatogram shown in Figure 58, an average of 64 different types of diastereomeric oligo-Trps eluted per second. If we restricted the analysis only to the determination of the stereoisomers consisting of the same number of D-Trp (p) and L-Trp (q) subunits, there would still be 189 different stereoisomeric oligo-Trp subgroups to be separated, which corresponds on average to about 6 overlapping subgroups eluting per minute. Conventional analytical methods, however, demand non-overlapping peaks for good detection and quantification. The peaks of the subgroups of interest strongly overlap under the conventional separation used and the chance of finding a single acceptable run analysis that unravels the peaks according to their subgroups (D_pL_q) of each oligomer of length n (= p+q) appears minimal. To address this problem, a different approach was chosen.

Quantitative analysis in pharmacokinetic studies often faces similar problems. [559] The large number of test samples in high-throughput-screening analyses today allows only short elution times in LC. This restricts separation to an absolute minimum which causes heavy coelution, especially when dealing with metabolites in animal blood plasma. [560] The qualitative and quantitative analysis is then performed by LC-MS, as this method can handle the analysis of coeluting samples by using internal standards. If quantitative LC-MS analysis has to be performed for such samples, an internal standard must always be used. [559] Otherwise coeluting substances can quench the desired MS signal or precipitates may plug-up parts of the capillary inlet to the MS detector and influence the signal intensities over time. Ideally, a stable isotopically labelled analog of the substance

to be quantified is chosen as internal standard, so that both have similar elution behavior and similar response in the mass spectrometer. The stable isotope analogs are usually labelled with D, ^{13}C, ^{15}N, or ^{18}O. Even though the difference in masses may be distinguishable by mass spectrometry, great care must be taken to ensure that the mass peaks monitored are not common to both compounds. For this reason, molecular weights of the analyte and its isotope analog should be at least three units apart from each other (no interference with the isotope pattern of the non-labelled compound). Finally, the quotient of the areas of elution peaks in either the SIM-chromatogram of the analyte or the internal standard should correspond to their relative amount in the sample analyzed by LC-MS.

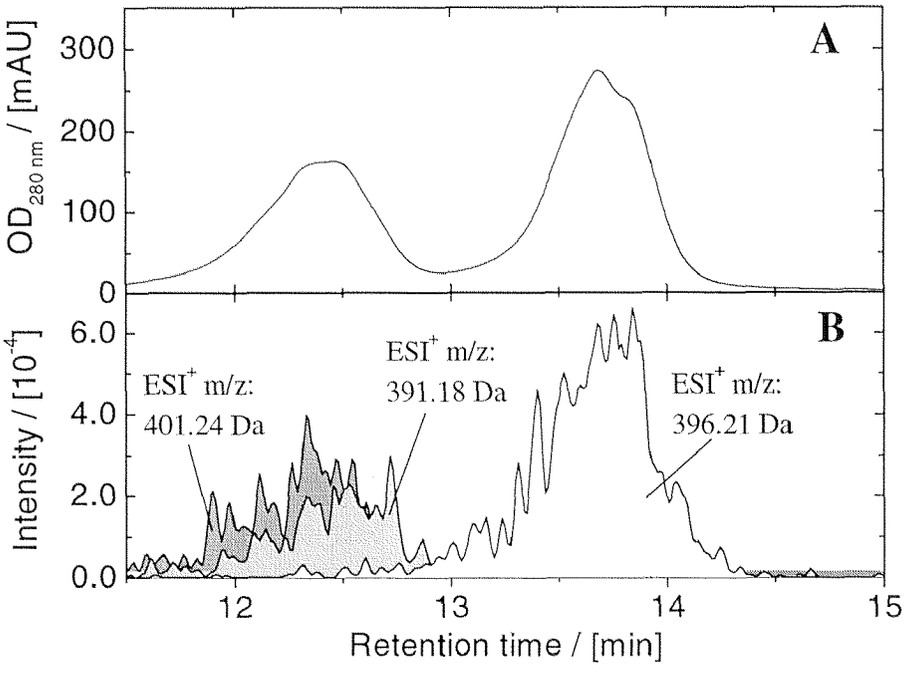


Figure 59: The UV-chromatogram (A) and the MS SIM-chromatograms (B) are shown for H-TrpTrp-OH stereoisomeric subgroups. Only the chromatogram interval for the times of dimer elution is shown. Dimers consisting of only L-Trp(d_5) (L_2: dark gray), only D-Trp (D_2: light gray), or both (DL or LD: white) can be traced according to their masses (B).

Let us now return to the racemic NCA-Trp condensation. If the racemic condensation is performed using the penta-deuterated NCA-L-Trp(d_5) and nondeuterated NCA-D-Trp, the stereoisomeric subgroups (D_pL_q) of the Trp-oligomer products with length n will have different masses ($m_{all\ D}$ + 5q), where $m_{all\ D}$ is the mass of all D homochiral oligo-Trp. The masses of different D_pL_q-subgroups are separated by at least 5 and maximally 5n Da. If the peaks in the SIM-chromatograms of the corresponding masses are

integrated and compared to each other (shown in Figure 59 for n = 2), the stereoisomer distribution of D_pL_q-subgroups of each oligomer of length n can be determined, provided that the ionization behavior of the stereoisomers is similar under the conditions used.

To check whether oligo-Trp stereoisomers have similar sensitivities in ES-ionization, non-overlapping peak areas in the UV-chromatogram of diastereomeric and enantiomeric subgroups were compared, according to equation 1, with the corresponding areas in MS SIM-chromatograms.

$$\frac{UVarea_{280nm}}{n \cdot MSarea_{SIM}} \approx Const \qquad \text{(Eq. 1)}$$

If there is no significant difference in the ionization sensitivity for oligo-Trp stereoisomeric subgroups of the same length, then one expects the ratios of the UV-peak areas at 280 nm and the SIM-peak areas of the corresponding masses to be constant. The values calculated for the NCA-Trp condensation in the absence of liposomes using equation 1 are listed in Table 16 for different oligomer lengths. 60 mM of either NCA-D-Trp, NCA-L-Trp, NCA-L-Trp(d_5), or racemic NCA-D-Trp/NCA-L-Trp(d_5) were mixed 1:3 (v/v) with buffer solution and incubated for 12 hours on a shaker at room temperature. The samples were then injected into the HPLC and analyzed by LC-MS.

Table 16 displays mean values with standard deviations for seven injections of 10 to 50 µl volume. The MS-signals were proportional to the amount of sample injected (data not shown), which verifies the linearity of the MS-signal. The same was true for the UV-signal at 280 nm. For the di-, tri-, and tetramer of Trp, two subgroups of diastereomers could be discerned, having UV-signals sufficiently separated to be integrated independently (see Figure 59 (A) for the case of the dimer). In the case of oligomers longer than the tetramer, all UV-signals of the stereoisomers of the same length were strongly overlapping and could not be related to different groups of diastereomers. For oligomers higher than the pentamer, stereoisomers of different length started to overlap in the UV-chromatogram, so that not even all ((all) in Table 16) stereoisomers of the same length could be integrated independently from the stereoisomers of the neighboring chain lengths. The values given in Table 16 for equation 1 are similar for different stereoisomers of the same length.

Table 16: The UV-signal/MS-signal ratios divided by the oligomer length n were calculated for different oligo-Trp stereoisomeric subgroups. D: represents D-Trp, L: L-Trp, and $L(d_5)$: L-Trp(d_5). $D_pL(d_5)_q$: represents all oligomers of length n (= p+q) consisting of p D-Trps and q L-Trp(d_5). All: corresponds to of all the stereoisomers formed with length n.

Oligomer:	**Composition:**	**UV-area$_{280nm}$/(n•MS-area$_{SIM}$)•10^4:**	**Average:**
Dimer:	D_2[a]	157.4±3.8	
	L_2[a]	122.5±4.8	
	$L(d_5)_2$[a]	162±27	
	all[b]	114.2±6.1	139±28
	D_2 and $L(d_5)_2$[b]	114.8±1.7	
	$DL(d_5)$[b]	109.7±8.0	112.3±8.2
Trimer:	D_3[a]	54.1±3.7	
	L_3[a]	38.7±1.4	
	$L(d_5)_3$[a]	50.9±6.6	
	all[b]	38.5±1.9	45.6±7.9
	D_3 and $L(d_5)_3$[b]	29.3±1.8	
	$D_2L(d_5)$ and $DL(d_5)_2$[b]	43.7±2.1	36.5±2.8
Tetramer:	D_4[a]	31.3±2.2	
	L_4[a]	22.6±1.6	
	$L(d_5)_4$[a]	31.0±4.7	
	all[b]	24.9±0.6	27.5±5.5
	D_4 and $L(d_5)_4$[b]	19.5±0.6	
	$D_3L(d_5)$ and $DL(d_5)_3$[b]	27.2±0.7	23.4±0.9
Pentamer:	D_5[a]	29.5±2.9	
	L_5[a]	21.4±0.6	
	$L(d_5)_5$[a]	24.9±0.6	
	all[b]	15.3±0.7	22.8±3.1

a. Single-feeding single enantiomer condensation of either NCA-D-Trp, NCA-L-Trp, or NCA-L-Trp(d_5).

b. Single-feeding racemic condensation of NCA-L-Trp(d_5) and NCA-D-Trp.

For each Trp-oligomer length, all the ratios obtained for stereoisomer subgroups are within a range of 25% around the mean value of single enantiomer and racemic condensation (average). The mean values in Table 16 decrease with increasing length and approach a constant value of about 20 for oligomers higher than the tetramer. Standard deviations typically were lower for longer oligomers.

These observations led to the following conclusion: the integral areas of stereoisomeric subgroup peaks in the MS SIM-chromatogram representation are proportional to the total relative abundance of the diastereomers in that subgroup. This means that the relative distribution of the oligo-Trp subgroups of D_pL_q-stereoisomers of length n, formed in the racemic NCA-Trp condensation, can be determined by LC-MS analysis and isotopic labelling of one of the two enantiomeric monomers.

The stereoisomeric oligo-Trp distributions for multiple-feeding experiments of racemic NCA-Trp condensation (as in Figure 58D) are shown in Figure 60 for the absence (A) and presence (B) of POPC liposomes (light gray columns with error bars). In the absence of liposomes, only traces of the 8mer were detected and the determination of the stereoisomer distribution was possible up to the 7mer. In the case of POPC liposomes, oligo-Trps up to the 18mer were formed and the experimental determination of the stereoisomer distribution was possible up to the decamer.

The experimentally determined distributions (light gray columns with error bars) were compared with the statistically distributions (white columns), assuming that L-Trp(d_5) and D-Trp had the same probability of being present in each of the theoretically possible stereoisomers. In both systems (Figure 60 A and B), the homochiral oligo-Trps (all L or all D) were found first to be underrepresented compared to the statistically expected distribution (i.e. 1.1 times for the 2mer in A1 and 1.8 times for the 2mer in B1). With increasing oligomer length, however, the homochiral oligomers became increasingly overrepresented (i.e. 8.3 times for the 7mer (A6) and 40 times for the 10mer (B9)).

Interestingly, the change from under- to overrepresentation occurs at shorter oligomer length if the maximal degree of oligomerization is smaller. In the absence of liposomes, the change occurs at the 3mer for both the single- (data not shown) and multiple-feeding experiment (A2). In the presence of POPC liposomes however, where multiple-feeding induces higher maximal degrees of oligomerization, the change occurs at the 5mer for the single-feeding experiment (data not shown) and the 7mer for the multiple-feeding experiment (B6). Being aware of the fact that with the exception of the homochiral

sequences (far left and right columns in the histograms) all the stereoisomer groups consist of more than one diastereomer, it is clear that the two enantiomer homochiral oligo-Trps are the most abundant of all stereoisomers when they are longer than a critical length.

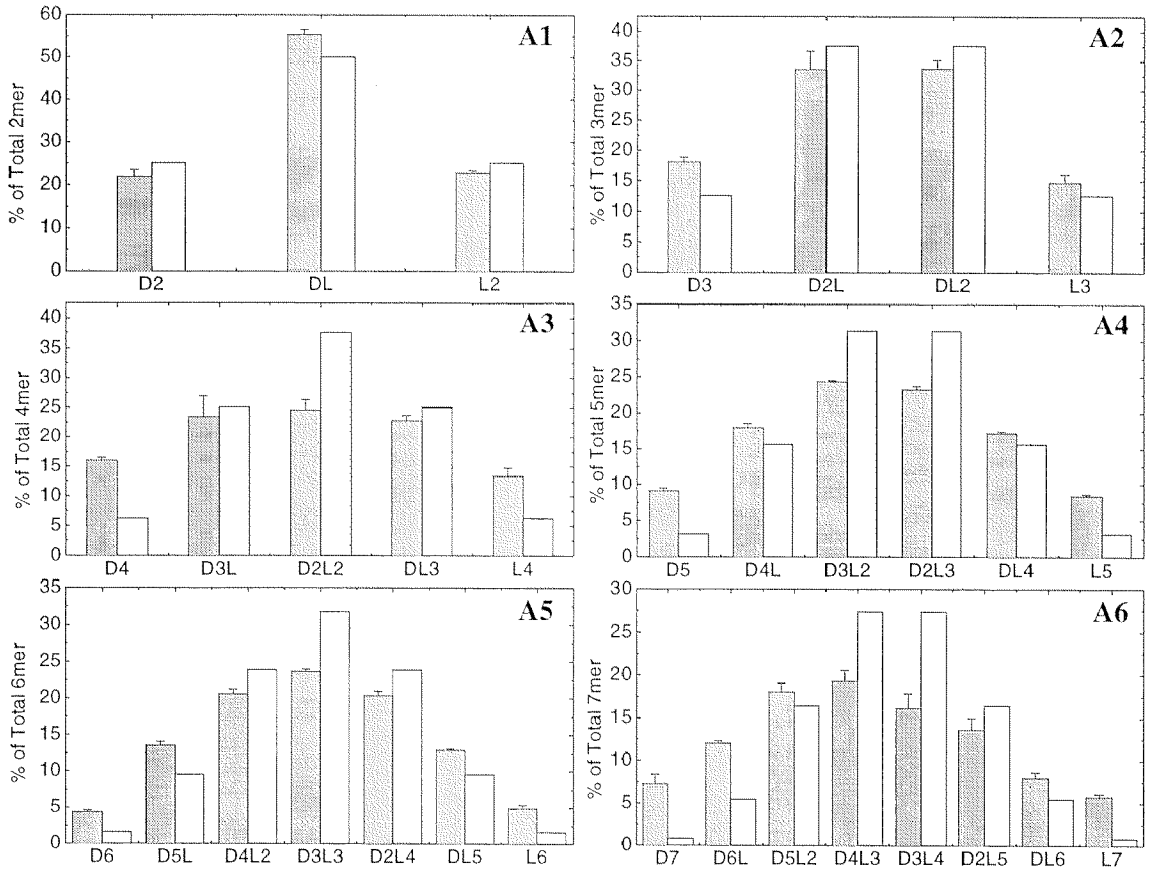
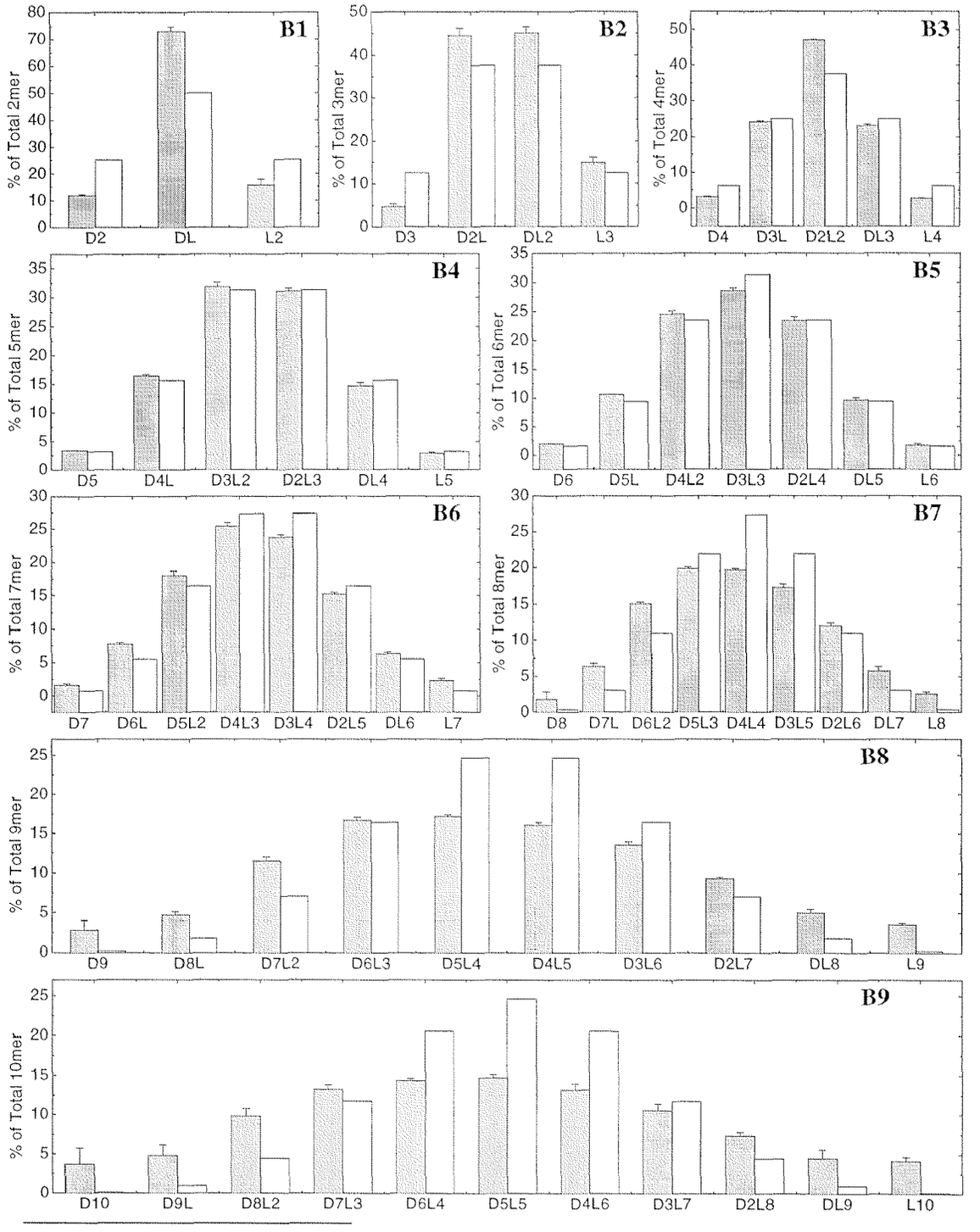


Figure 60: Relative abundances for D_pL_q-stereoisomer groups of the oligo-Trp n-mers are shown for two racemic NCA-Trp feedings in either the absence (A) or presence of POPC liposomes (B). In the first feeding, a freshly prepared solution of 60 mM racemic NCA-D-Trp/NCA-L-Trp(d_5) in 0.4 M imidazole/HCl, pH 6.80, was mixed 1:3 (v/v) with either buffer (A) or a 100 nm extruded 100 mM POPC liposome suspension in 0.4 M imidazole/HCl at pH 6.80 (B) and incubated for at least 12 hours at room temperature. The same volume of racemic NCA-D-Trp/NCA-L-Trp(d_5) was added again in the second feeding and incubated for another 12 hours. For each oligomer length, the SIM-chromatograms for all the D_pL_q stereoisomer subgroup masses were integrated. The total of all SIM-peak areas of a particular n-mer corresponds to 100%. The relative abundances of the D_pL_q stereoisomer subgroups (light gray columns) are mean values of three measurements. Standard deviations are given as error bars. The white columns correspond to the theoretical distribution, assuming a statistical oligomerization (0. order Markov process[8]).

[8] In a zero order Markov process (see Figure 62) the probability for the addition of either a L-Trp(d_5) or a D-Trp monomer in the next elongation step does not depend on the growing oligomer end.

So far, it can be concluded from the stereoisomer distributions in Figure 60 A and B, that the racemic NCA-Trp condensation seems to be stereoselective[9]. In the following section a brief overview will be provided on the work published to date on the stereoselective copolymerization of racemic NCA-amino acids and will used to discuss the results presented above.



[9] If out of two or more possible stereoisomeric products one is formed preferentially, the reaction is called stereoselective. [561]

5.3.2. Stereoselective Copolymerization of Racemic NCA-Amino Acids

Many reviews have been published on the NCA-amino acid polymerization in organic solvents, but only few address the topic of racemic copolymerization. [562]-[564] In all of the studies devoted to the stereoselectivity of NCA copolymerization, only the copolymerization of L- and D-amino acids and some of their derivatives was considered. As a rule, L- and D-isomers of the same amino acid were studied. In some cases mixtures of enantiomeric pairs of more than one amino acid were copolymerized. [565], [566] Polymerization was generally initiated by either a primary, secondary, or tertiary amine. After 1970, several papers appeared dealing with the stereospecificity[10] of the NCA-amino acid polymerization based on the observation that mixtures of enantiomeric NCAs are polymerized more slowly than optically pure NCAs. [567]-[578] Most experiments were carried out with NCA-γ-benzyl glutamate, NCA-alanine, and NCA-leucine monomers. The experiments were analyzed either by enzymatic digestion, by kinetic methods or by a study of the optical rotation of the resulting polypeptides. At that time, the results obtained by these rather indirect analytical methods led to the conclusion that high stereoselectivity and stereospecificity is only expected if the growing peptide chain possesses a helical secondary structure. The slower kinetics observed with racemates were attributed to the monomer sorption of opposite optical configuration on the growing polypeptide chains. [576], [578] Later, a series of ^{13}C- and ^{15}N-NMR measurements of the products produced in racemic NCA-amino acid polymerization argued against stereospecificity in the reaction and made the importance of the secondary structure of the growing chain for stereoselectivity uncertain. [565], [566], [579]-[585] Despite the insight provided by these studies, the stereoselectivity of NCA polymerization is still not entirely understood. The following sections summarize the main features accepted to date.

5.3.2.1. Two Polymerization Mechanisms

Polymerization of NCA can be initiated by amines, alkoxy compounds, hydroxyl anions, various salts, and also by heating. [563], [586], [587] Despite the apparent simplicity of

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[10] Reactions in which only a single stereoisomer can be detected are called stereospecific. [561] The term stereospecificity in racemic NCA-amino acid polymerization stands for the theoretical formation of exclusively homochiral (only all L or all D but no other stereoisomers) polyamino acids. Some of the authors cited misleadingly used the term 'stereospecificity' instead of 'stereoselectivity'.

the NCA condensation reaction, the general mechanism of polymerization for all NCAs or all types of initiators has not yet been described. [563] The polymerization of NCA is very sensitive to a number of chemical and physical factors, including polymer solubility and the conformation of the growing polymeric chain. In addition, the tendency of polypeptides to intermolecular aggregation complicates the elucidation of the polymerization mechanisms.

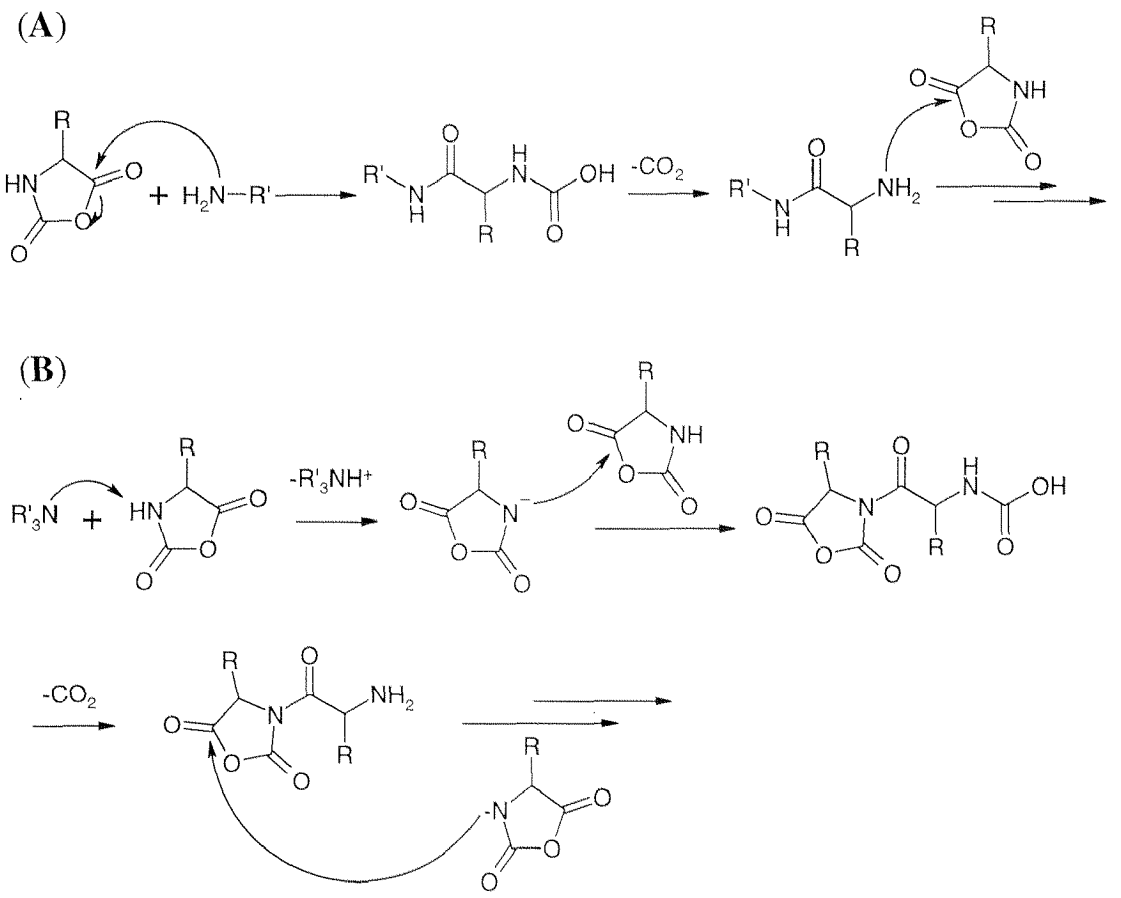

Figure 61: The amine mechanism (A) and the activated monomer mechanism (B) for the polymerization of NCAs (adapted from [564], [588]).

The most likely modes of NCA polymerization are the so-called amine (or normal) mechanism and the activated monomer mechanism, as depicted in Figure 61. The amine mechanism (A) is initiated by protic nucleophiles, such as water, alcohols, or primary amines. The amine mechanism is the one which is most likely to occur in water (see Figure 41). Nucleophilic secondary amines with small substituents behave like primary amines. However, secondary amines with bulky substituents can initiate the activated monomer mechanism (B). The activated monomer mechanism (B) is always initiated by

tertiary amines. A change of temperature, concentration, or solvent can affect the rate of polymerization, but does not change the reaction mechanism. The activated monomer mechanism is more sensitive to changes in the reaction conditions. [580]

5.3.2.2. Low Stereoselectivity Depends on the Mechanism

In the literature, ^{13}C- and ^{15}N-NMR analysis of products obtained by racemic NCA-amino acid polymerization in organic solvents of either Ala, Leu, γ-methyl glutamic acid, Phe, or Val led to the following observations. [580]-[582]

For Leu, γ-methyl glutamic acid, and Phe, the formation of isotactic[11] blocks was slightly favored under conditions where the amine mechanism is expected to occur. Isotactic blocks also predominanted under the activated monomer mechanism polymerization. If pyridine was used as initiator, syndiotactic[11] sequences were favored. Ala and Val behaved somewhat differently. Under the amine mechanism, Ala preferentially formed atactic[11] and Val isotactic sequences. If the conditions were set for an activated monomer mechanism, Ala formed isotactic and Val syndiotactic and heterotactic[11] sequences. However, in all cases the average lengths of the stereoblocks never exceeded 4 monomer units. Low stereoselectivity of most racemic NCA-amino acids was also confirmed by solubility tests of the resulting poly-D,L-amino acids in aprotic solvents. [582] Penultimate effects in the growing chain on the type of monomer added next were reported to be either weak or absent. The authors therefore concluded that most NCA polymerizations follow a first order Markov process (see Figure 62), where the last unit in the growing chain influences the type of the monomer added next. [582] This is in contradiction to the data reported for NCA-amino acid addition to stereoisomeric preoligomers of either γ-methyl aspartic acid, γ-methyl glutamic acid, Phe, or Val. In these studies, the addition rate of a D (L) monomer to a R-DD* (R-LL*) end was greater than to a R-DL* or R-LD* end ($k_{DDD} = k_{LLL} > k_{DLD}$, $k_{LDD} = k_{LDL}$, k_{LDL}). The latter rate was further reported to be greater than the addition to a R-LL* (R-DD*) end (k_{DLD}, $k_{LDD} = k_{LDL}$, $k_{LDL} > k_{DDL} = k_{LLD}$). [562] These data appear to suggest a second order Markov process (see Figure 62), where the last two units of the growing chain influence the type of monomer added next.

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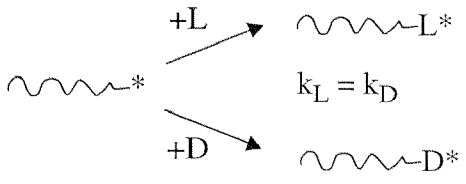
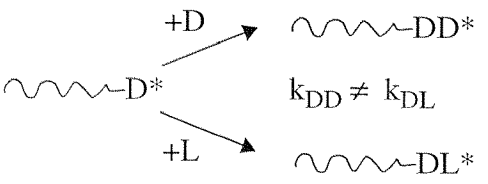
[11] isotactic: $[D]_n$ or $[L]_n$; syndiotactic: $[DL]_n$ or $[LD]_n$; heterotactic: $[LLDD]_n$ or $[DDLL]_n$; atactic: non specific (random) order of D and L units.

It can be concluded that the type of reaction mechanism is mainly responsible for the degree of stereoselectivity, whereas the direction of the stereoselectivity seems to depend primarily on the nature of the amino acid. In no case was the NCA polymerization observed to be stereospecific. The degree of stereoselectivity is low in all polymerizations, so that the average length of isotactic blocks never exceeded four monomer units. [580]-[582] The reactions are first or second order Markov polymerization processes.

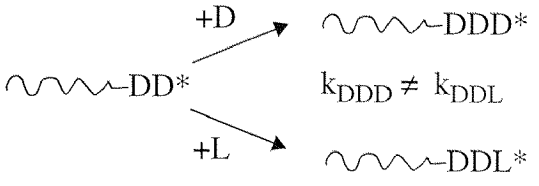
0. Order Markov process:
(Bernoulli process)



1. Order Markov process:



2. Order Markov process:



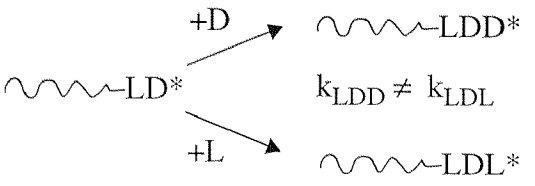


Figure 62: Schematic representation of the zero, first, and second order Markov processes. The zero order Markov process is also called a Bernoulli process. In the first and second order Markov processes, the enantiomeric reactions were not explicitly drawn, as their rates are the same as for their mirror reactions in a symmetrical environment. k_i is the kinetic constants for the reactions and the asterix (*) symbolizes the growing chain end. Be aware that some of the authors cited above [582] misleadingly used the term Bernoulli polymerization for the first order Markov and the term first order Markov for the second order Markov process.

5.3.2.3. Secondary Structures and Slower Kinetics

Polymerization of NCA-D,L-amino acids is complex. The main kinetic feature is the decreasing rate of conversion, as the mole ratio of NCA-D- and NCA-L-amino acid approximates unity (racemic mixture). It has been argued that this depression of the rate may be, at least partially, the result of different secondary structures in the growing chain. [588] While poly-L-Ala, poly-L-Phe, poly-L-Leu, and poly-L-γ-methyl glutamate certainly take on a helical secondary structure, [589], [590] the corresponding poly-L,D-amino acids partially adopt a β-sheet structure. [591] Since the end groups of helical polypeptides are sterically more accessible than those of pleated-sheet structures, faster

polymerizations of pure enantiomer NCAs are likely to occur. However, the slower polymerization may also be the result of a stronger association between enantiomeric amino acids. There is some spectroscopic evidence that NCAs dimerize via hydrogen bonding in less polar solvents. [592]

5.3.3. Concluding Remarks on the Racemic NCA-Trp Condensation

The racemic NCA-Trp condensation in the absence and presence of POPC liposomes led to lower yields in longer oligo-Trps and the maximal degree of oligomerization was reduced, when compared to the condensation using one of the two enantiomers. This observation might be attributed to the slower oligomerization kinetics often observed for racemic NCA condensations. Slower oligomerization favors hydrolysis of the activated monomers, consequently reducing the yields and the length of the oligomer products. However, the presence of liposomes assists in the formation of longer oligomers (hydrophobic interactions).

The use of LC-MS for stereoisomer quantification in racemic oligomerization reactions by isotopic labelling of one of the optical pure enantiomers is novel. To the best of my knowledge, it has never been reported before. The application of this analytical method to the racemic NCA-L-Trp(d_5)/NCA-D-Trp condensation revealed non-Bernoullian oligomerization in the absence, as well as in the presence of POPC liposomes. The reaction was stereoselective but not stereospecific. The homochiral oligomers were underrepresented in short oligo-Trp and overrepresented in long oligo-Trp products, if compared to the Bernoulli distribution (see Figure 60). The point of change from under- to overrepresentation occurred at longer oligomer lengths if the maximal degrees of oligomerization were higher. The change and its increase towards longer oligomers with higher degrees of oligomerization can only be explained by a second order Markov process. First order Markov oligomerization would not have a point of change at all. The formation of isotactic sequences is obviously favored (k_{DDD} (k_{LLL}) > $k_{DLD} \approx k_{LDD}$ ($k_{DLL} \approx k_{LDL}$) > k_{LLD} (k_{DDL})) and homochiral sequences tend to react further during condensation and therefore become overrepresented in the longer oligo-Trps. This observation is consistent with data reported earlier on the kinetics of racemic NCA-amino acid condensation. [562], [582] The stereoisomer distributions are symmetrical, which agrees with the earlier observation that the optically pure POPC membrane in its fluid state (above T_m) has no significant influence on the condensation. The POPC

membrane assists solely in the formation of longer oligomers, as it did in the case of enantiomeric NCA-Trp condensation experiments. However, longer homochiral sequences were obtained in higher yields in the presence of liposomes.

Preliminary results for racemic NCA-L-Leu(d_{10})/NCA-D-Leu and NCA-L-Ile(d_{10})/NCA-D-Ile condensation exhibited similar stereoisomer distributions to racemic NCA-Trp condensation in the absence of liposomes (data not shown). It will be of great interest to see whether in future work mixtures of racemic NCA-amino acids lead to preferential formation of homochiral oligopeptides consisting of more than one type of amino acid.

6. Final Conclusions and Outlook

The deterministic hypothesis that life arose from the operation of natural laws on a certain physico-chemical system which then evolved over time governed by physical principles allows us to undertake a scientific study on the origin of life. Even though the details need not be totally deterministic in every aspect, the overall behavior must have followed a predictable path.

It must be the aim of every scientist investigating the origin of life to experimentally demonstrate short sequences from the accumulation, pre-organization or the maturation period (see Figure 12), in order to clarify the overall picture by increasing the resolution of the patchwork made up by such experiments. The present study focused on the pre-organization period, namely on the transition from chemical to molecular evolution. Despite the many unanswered questions about primitive lipids (i.e. prebiotic synthetic pathways that lead to reasonable yields of lipids), their existence at an early stage of the pre-organization period seems likely. [194], [257] In the present study, POPC, DOPC, DDAB, and DOPA lipids were used as model systems. Some of these lipids are chiral (POPC, DOPC, DOPA). It is clear that these lipids can not be considered to be prebiotic molecules. They were chosen for the sake of their commercial availability and well characterized behavior. However, one can consider these lipids as models for prebiotically more relevant surfactants. There is no solid argument why the rather nonspecific interactions of amino acids and peptides with lipid membranes, of hydrophobic or electrostatic nature, should not be present for other liposome-forming and presumably prebiotic amphiphiles (i.e. mono-*n*-alkyl phosphates, polyprenyl phosphates, dipolyprenyl phosphates, or fatty acids). Interactions between peptides and membranes have been suggested to have played a central role in prebiotic chemistry and precellular, molecular evolution. [74], [593]-[595] However, little experimental work has been done on such interactions with respect to the origin of life. [213]-[217], [426] In addition, the formation of polypeptides (and other macromolecules) is still one of the unresolved problems in the origin of life field. [257], [409] Understanding how functional polypeptides formed is believed to be one of the key points for understanding the transition to life. For these reasons, the experimental part of this thesis focused on the condensation of amino acids and peptides in aqueous solutions in the presence of liposomes. On one hand, prebiotically relevant *N*-carboxyanhydride (NCA)-activated

amino acids [390]-[394] and, on the other, a model compound for a lipophilic condensation agent, 2-ethoxy-1-ethoxycarbonyl-1,2-dihydrochinoline (EEDQ), were used for the oligomerization of amino acids and dipeptides. It is not clear whether EEDQ itself is of prebiotic nature, [596] but comparable lipophilic condensing agents based on carbodiimides were probably present on the primitive earth. [154], [285] The amino acids used in this study, namely Asp, Arg, Glu, Gly, His, Ile, Leu, Met, Phe, Trp, Tyr, and Val, can all be considered to be prebiotically relevant. [90], [291]-[298], [597] Starting from dimers of these amino acids needs no further justification, since dipeptide formation is easily achieved under various prebiotic conditions, as was summarized in paragraph 4.2. The present thesis clearly demonstrates that liposomes can assist in the formation of oligopeptides.

The hydrophobic nature of the POPC liposome membrane is usful for the polycondensation of hydrophobic amino acids and peptides in two ways.

First, we have seen that the presence of liposomes yields oligomers in multiple-feeding experiments with hydrophobic aromatic NCA-amino acids (i.e. 70-80% of initial Trp in the NCA-Trp condensation), in some cases with degrees of polymerization of over 20. The elongation to higher water-insoluble oligomers takes place only in the presence of the POPC membrane bilayer. The efficiency, observed in the liposome-assisted condensation, of forming long oligopeptides is comparable to the results obtained in experiments in which negatively charged NCA-amino acids were added to clay or other minerals. [154], [362], [413], [414] Unfortunately, the ability of POPC liposomes to enhance the formation of long oligopeptides from hydrophobic NCA-amino acids seems to be restricted to aromatic amino acids such as Trp, Phe, and probably also Tyr. All CDI-induced condensations in the presence of POPC liposomes with other hydrophobic but aliphatic amino acids, such as Ile, Leu, and Val, did not significantly increase the chain length of the oligomers formed when compared to the aqueous reference system. The same holds true for EEDQ-induced dipeptide condensations. If a Trp residue in H-TrpTrp-OH is replaced by another non-aromatic hydrophobic amino acid residue, oligomers are almost completely absent and the major by-product, the carbamate of the dipeptide, dominates not only in a liposome-free aqueous system but also in the presence of liposomes. Equilibrium dialysis experiments performed for peptides and POPC liposomes revealed that the preferential elongation of aromatic amino acids and peptides

compared to the aliphatic ones is mainly due to the higher membrane affinity of the aromatic residues.

Secondly, sequence-selective condensation of dipeptides can be achieved on the POPC liposomes by using EEDQ as a lipophilic condensing agent. In contrast to the hydrophilic dipeptides, hydrophobic dipeptides bind to the bilayer membrane, where they accumulate at the liposome interfaces (local peptide concentration up to a 80 times higher than in the aqueous buffer). This is the reason why hydrophobic peptides are preferentially incorporated into of the oligomers formed in such experiments. In an EEDQ-induced condensation experiment with a mixture of H-TrpTrp-OH and three more hydrophilic dipeptides in the presence of POPC liposomes, H-Trp_4-OH made up about 70% of the 16 theoretically possible tetrapeptides. Consequently, the POPC liposome membrane not only assists in the formation of longer oligopeptides when compared to the aqueous reference system, but also leads to selective formation of hydrophobic oligomers starting from a pool of different dipeptides.

We have further seen that the liposome-assisted CDI-induced condensation of aromatic amino acids and the EEDQ-induced oligomerization of hydrophobic dipeptides in the presence of liposomes can be extended to charged, hydrophilic amino acids and peptides by introducing either positively or negatively charged lipids into the POPC membranes. The use of charged lipids increases the bilayer affinity of oppositely charged NCA-amino acids and peptides. This allows either the enhanced formation of block oligomers, as demonstrated for the sequential feeding of NCA-Trp and NCA-Glu to positively charged DDAB/POPC liposomes, or the formation of oligopeptides made up of hydrophobic and positively charged amino acids such Trp, Arg, and His. The latter was confirmed in the case of EEDQ-induced condensation of both H-TrpTrp-OH and H-ArgTrp-OH, or either H-ArgTrp-OH or H-HisTrp-OH alone, in the presence of negatively charged DOPA/ POPC liposomes.

In summary, accumulation, due to electrostatic and/or hydrophobic interactions, leads to higher local concentrations of activated amino acids, peptides, and oligopeptides on the lipid bilayer. This favors the oligomerization of charged hydrophilic, as well as of hydrophobic amino acids and peptides. In this way, oligopeptides consisting of hydrophobic and hydrophilic amino acids such as Trp, Glu, Arg, or His were obtained. His-containing peptides in the presence of liposomes seem to have a catalytic effect on

the hydrolysis of the *p*-nitrophenyl ester C16-ONp. However, side reactions complicate the interpretation of the experimental results and must be further investigated.

We have seen that the racemic NCA-amino acid condensation in the absence and presence of the POPC liposome membranes leads to lower yields of longer oligomers and that the maximal degree of oligomerization is reduced when compared to the condensation of one of the two enantiomers. This observation can be attributed to the slower oligomerization kinetics often observed in racemic NCA condensations. [567]-[578] Slower oligomerization favors hydrolysis of the activated monomers and consequently reduces the length and yield of the longer oligomer products formed. The chiral (fluid) POPC bilayer had no significantly different influence on the CDI-induced condensation of either L- or D-Trp. This observation illustrates once again that rather unspecific hydrophobic interactions cause the NCA-amino acid and peptide accumulation at the lipid membrane interfaces, and that this effect is very likely to occur in the presence of liposomes other than the POPC type. It remains to be demonstrated whether the principles of liposome-assisted polycondensation of amino acids and peptides can be extended to liposomes of prebiotically relevant lipids.

To the best of my knowledge, LC-MS was demonstrated to be useful in stereoisomer quantification for the first time in the present thesis. The isotopic labelling of one of the two enantiomers in racemic oligomerization reactions allows the relative quantification of stereoisomer subgroups for a fixed oligomer length. The application of this analytical method on the racemic NCA-amino acid condensation revealed non-Bernoullian oligomerization in the absence as well as in the presence of POPC liposomes. The racemic condensation reaction is stereoselective in both cases, but not stereospecific. In both systems, the homochiral oligomers (all L or all D) are overrepresented for longer oligomers compared to the statistical values (assuming a Bernoullian elongation process). For example, in multiple-feeding experiments of NCA-Trp the homochiral sequence was 8.3 times higher for the 7mer in the absence and 40 times higher for the 10mer in the presence of POPC liposomes than would be expected for a statistical distribution. The two enantiomeric homochiral oligomers are the most abundant stereoisomers formed for oligomers longer than a critical length. These results can best be explained by a second order Markov process. Preliminary results suggest similar

behavior for racemic NCA-L-Leu(d_{10})/NCA-D-Leu and NCA-L-Ile(d_{10})/NCA-D-Ile condensation in the absence of liposomes.

It will be of great interest to see whether mixtures of different racemic NCA-amino acids lead to a preferential formation of mixed homochiral oligopeptides and whether sequence selectivity can be induced by choosing the right lipid composition of the liposome membranes.

Liposomes can assist selective amino acid and peptide condensations and lead to longer oligopeptides and specific sequences. Peptides that exhibit high affinity for the lipid membrane are the preferred products of such reactions. NCA-amino acid condensation preferentially forms homochiral stereoisomers above a certain oligomer length. Liposomes have been shown to enhance reaction rates of various reactions [598] and we have seen that the presence of liposomes can also enhance the catalytic activity of certain peptides. Such peptides, bound to lipid bilayer microcompartments, can in principle lead to enhanced reaction rates on the inside as well as on the outside of the liposomes. The permeability property of the lipid membrane, which might also be affected by the products of the liposome-assisted polycondensation, leads to concentration gradients of the substrates and products throughout the liposomal microreactor. Therefore, the internal microenvironment will be different from the outside bulk solution and this leads to new interesting properties that might have been important to the origin of life. It will therefore be of great interest to focus on specific and selective liposome-assisted condensation of amino acids and peptides containing functional groups, some of them being of homochiral nature. The aim of such reactions should be to form catalytically active peptides which affect liposomal permeability at the bilayer membrane, in order to start a primitive, peptide-catalyzed metabolism in the inner aqueous pool of the liposomes.

7. Experimental Section

7.1. Chemicals

7.1.1. General Chemicals

Angiotensin I (8-10), human peptide (H-PheHisLeu-OH) (>99%) was purchased from Advanced Chemtech (Louisville, KE, USA). 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was a gift from the Pharma Research Department of Novartis International Ltd. (Basel, Switzerland) for all EEDQ-induced condensation and related experiments. For the CDI-induced condensation experiments, POPC as well as 1,2-dioleoyl-*sn*-glycero-3-phosphate monosodium salt (DOPA) and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). H-ArgTrp-OH·1.5HCl·0.5H_2O (peptide content: 85%), L-Glu (>99%), H-HisTrp-OH (98%), D- and L-Ile (>99%), D- and L-Leu (>99%), H-LeuTrp-OH·H_2O (peptide content: 94%) , L-Met (>99%), L-Phe (99%), H-PhePhe-OH (>99%), H-TrpAsp-OH·H_2O (peptide content: 93%), H-TrpGlu-OH·0.5H_2O (peptide content: 97.4%), H-TrpGly-OH·H_2O (peptide content: 93%), H-TrpPhe-OH (>99%), H-TrpTrp-OH·2H_2O (peptide content: 91%), L-Tyr (>99%), L-Val (>99%), Z-HisTrp-OH (>98%), and Z-PheHisLeu-OH (>98%) were purchased from Bachem (Bubendorf, Switzerland). Fmoc-His(Trt), Fmoc-Trp(Boc) and Fmoc-Trp(Boc)-Wang resin (0.4 mmol/g) were purchased from Calbiochem-Novabiochem (Läuflingen, Switzerland). L-Isoleucine (D10) (98%), L-leucine (D10) (98%), and the L-tryptophan (indole-D5) (>98%) were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Acetic acid (AcOH) (>99.5%), boric acid (>99.5%), Celite Filter Cel, ceric sulfate tetrahydrate (≥90%), chloroform (CHCl3) (≥99.8%), didodecyldimethylammonium bromide (DDAB) (≥98%), ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) (≥99%), 2-ethoxy-1-ethoxycarbonyl-1,2-dihydrochinoline (EEDQ) (>99%), L-His (>99%), methanol (MeOH) (>99.8%), potassium dihydrogen phosphate (KH_2PO_4) (>99.5%), phosphomolybdic acid hydrate (puriss. p.a.), 10% Pd-C, quinoline (≥97%), sodium dihydrogen phosphate dihydrate ($NaH_2PO_4·2H_2O$) (≥99%), sulphuric acid (95-97%), trifluoroacetic acid (TFA) (≥99% HPLC grade), triisopropylsilane ($^iPr_3SiH$) (>99%), D- and L-Trp (>99.5%), and imidazole (≥99.5%) were purchased from Fluka (Buchs, Switzerland). Acetonitrile (ACN) and methanol (MeOH) (HPLC grade) were purchased

from Macherey-Nagel (Oensingen, Switzerland). Sodium hydrogen carbonate ($NaHCO_3$) (≥99.5%), standard 1 M solutions of HCl (Titrisol) and NaOH (Titrisol) were purchased from Merck (Darmstadt, Germany). The deionized water was deionized a second time by a Milli-Q RG from Millipore (Volketswil, Switzerland). Argon (4.5), helium (5.6), hydrogen, nitrogen gas (4.5) were purchased from PanGas (Zürich, Switzerland). *N,N'*-Carbonyldiimidazole (CDI) and *p*-nitrophenyl palmitate (C16-ONp) were purchased from Sigma (St. Louis, MO, USA).

7.1.2. Synthesized Chemicals

The tetrapeptide H-HisTrpHisTrp-OH was synthesized using the Fmoc-Trp(Boc)-Wang resin (0.615 g, 4-fold excess), and for each coupling step either Fmoc-His(Trt) (0.620 g, 2-fold excess) or Fmoc-Trp(Boc) (0.527 g, 2-fold excess) using a FastMoc protocol (Fmoc-strategy) on an Applied Biosystems 433A peptide synthesizer from Perkin-Elmer in the group of Prof. Hilvert (Laboratory of Organic Chemistry, ETH). According to protocol, the resin was dried at high vacuum overnight (0.4015 g). The protecting groups were removed by incubation for 2 h with a cleavage cocktail of TFA (9.5 ml), water (0.25 ml), and $^{i}Pr_3SiH$ (0.25 ml) as a scavenger. Rotatory evaporation led to precipitation. This suspension was washed three times with ice cold ether and the precipitate was separated each time by centrifugation at 3500 rpm for 5 min. The remaining precipitate was dried under high vacuum resulting in H-HisTrpHisTrp-OH (0.146 g, >95% purity (HPLC, $OD_{220\,nm}$)), a white powder, which was stored at -20°C until further use.

H-PheHisLeu-OH was synthesized by dissolving Z-PheHisLeu-OH (215 mg) in EtOH (250 ml) and evacuating (water pump) and flushing with argon several times before 10% Pd-C (39 mg) was added under argon, evacuated again and refilled with H_2. The dispersion was stirred rigorously under H_2 (balloon, >1 atm) for 3 days until there was no educt left. The reaction was followed by TLC ($CHCl_3$:MeOH:AcOH, 15:4:1, $R_{f,\,Z\text{-}FHL\text{-}OH}$: 0.24, molybdenum-bath: 12.5 g phosphomolybdic acid hydrate, 5 g ceric sulphate tetrahydrate, 30 ml conc. H_2SO_4, 940 ml H_2O). At the end of the reaction the solution was filtered through a Celite filled (2 cm) glass filter and dried in the rotatory evaporator. The resulting peptide was dissolved in water and lyophilized (yield: 76%, >90% purity (HPLC, $OD_{220\,nm}$)).

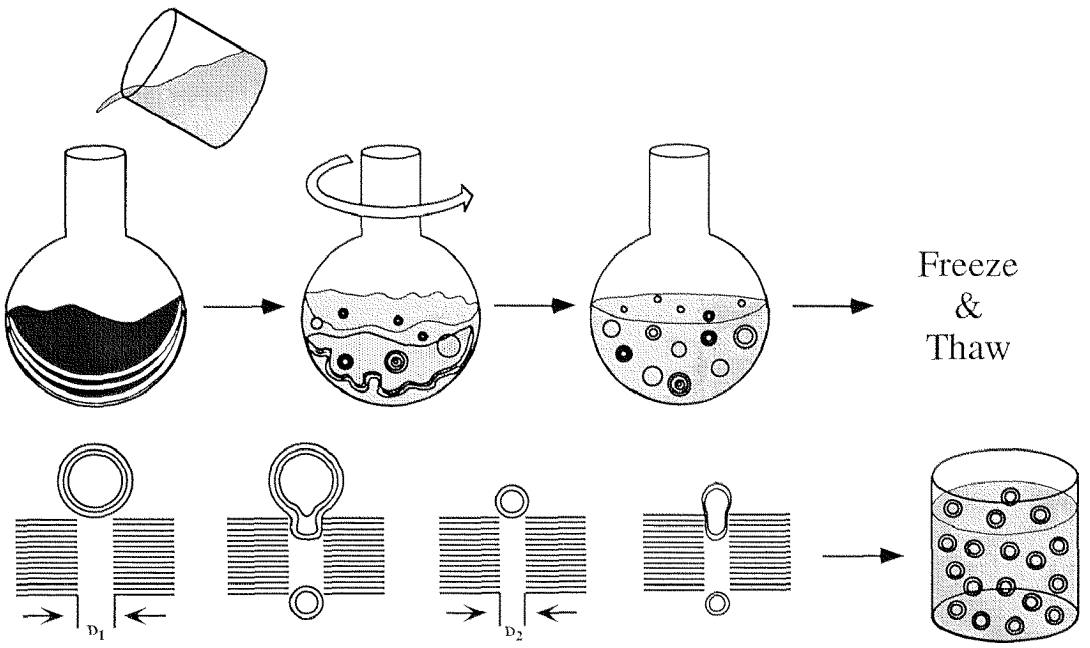


Figure 63: Schematic description of the preparation of liposomes: The buffer solution was added to the high vacuum dried lipid film and the film dispersed by shaking. The number of small unilamellar liposomes was decreased by freezing in liquid nitrogen and thawing in a water bath. The resulting suspension was then homogenized by subsequent extrusion through polycarbonate filters of different sizes (D_i).

7.2. Methods

7.2.1. Liposome Preparation

Liposomes were prepared from POPC or DOPC only, or in the case of mixed liposomes a mixture of POPC and the according amount of DOPA in $CHCl_3$ or DDAB, respectively. The lipids were first dissolved in $CHCl_3$ in a round-bottom flask. After evaporation of the solvent by rotatory evaporation under reduced pressure at 40°C, the thin film was dried overnight at high vacuum. The dried lipid film was dispersed in buffer, or buffered peptide solution, and put on a shaker for about 15 min to help speed up the dispersion process, which led to the formation of mainly multilamellar liposomes of a considerable heterogeneity in size. [434], [599] For the case of DOPA containing liposomes, concentrated solutions were used for dispersion and the pH (using a Radiometer (Copenhagen, Denmark) PHM82 pH-meter and an InLab 423 electrode from Mettler Toledo, Nänikon-Uster, Switzerland) and volume were adjusted thereafter. The liposome suspension was then frozen ten times in liquid nitrogen (-195°C) and thawed in a water

bath at 40°C (freeze-thaw cycles) in order to reduce the number of small unilamellar liposomes and increase the content of multivesicular liposomes. [444], [600] A significant decrease in size and lamellarity was achieved by successively passing the liposome suspension through two Nucleopore polycarbonate membranes (d: 25 mm) from Sterico AG (Dietlikon, Switzerland) with mean pore diameters of 400 nm (10 times), 200 nm (10 times), 100 nm (10 times), and in the hydrolysis experiments also 50 nm (10 times), using an extruder (The Extruder supplied by Lipex Biomembranes Inc., Vancouver, Canada) (see Figure 63). [444] Liposomes were characterized by ff-EM and DLS. The 100 nm extruded liposome suspensions were normally freshly prepared, especially when containing peptides, and were never stored for longer than two weeks.

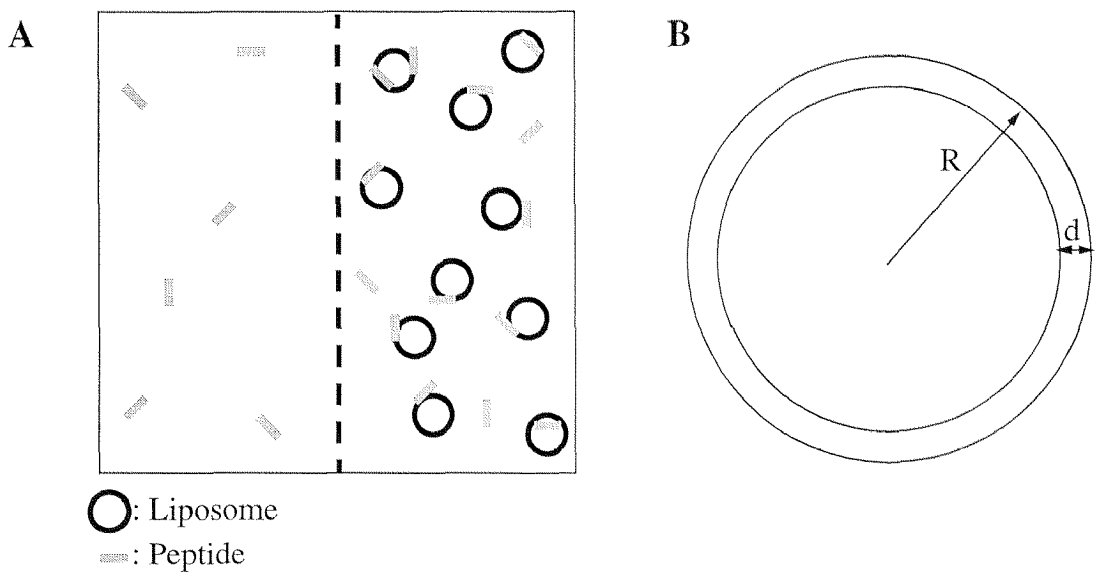


Figure 64: Equilibrium dialysis experiments: A) A schematic representation of the two dialysis chambers that are separated by a dialysis membrane with a molecular weight cut-off of 5'000 Da. Only the peptide can permeate through the membrane, but not the liposomes in the right chamber. B) The schematic drawing of a liposome shows the outer radius R and the bilayer thickness d.

7.2.2. Determination of the Association Constant (K_a)

The association properties of peptides to liposomes were studied quantitatively by using the equilibrium dialysis method (see Figure 64). [601], [602]

Dialysis experiments were performed by using a Dianorm equilibrium dialyzer (Dianorm, München, Germany) and a highly permeable dialysis membrane (made from neutral cellulose) with a molecular weight cut-off of 5'000 Da. 1.0 ml of a typically buffered, 5 mM in peptide and 100 nm extruded 25 mM lipid liposome-containing suspension and 1.0 ml of a 5 mM peptide buffered solution were put into the two

chambers of each of the five teflon cells separated by the dialysis membrane (d: 63 mm, A: 4.5 cm^2), as shown in figure 64. The dialysis membranes were incubated before use for 10 to 15 min in boiling, deionized water (ca. 1 l), containing one spatula of sodium hydrogen carbonate ($NaHCO_3$) and two spatulas of ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA), washed several times with deionized water and later equilibrated overnight in the type of buffer used in the dialysis experiments that followed. The dialysis experiments were performed by rotating the cells at a constant speed of 8 rpm for 24 h at room temperature. It was shown that after this time equilibrium distribution is reached between the two chambers and DLS measurement confirmed that the size of the liposomes did not change during dialysis. The solutions were then removed from the cells, and each chamber was analyzed for peptide content using LC-MS.

The distribution of solute molecules in the bilayer and in the aqueous phase has been theoretically discussed in the literature either using the partition model or the binding model. [603] The partition model assumes that the bilayers form a second phase and play the role of a solvent. It defines the partition coefficient P of a solute as the ratio of equilibrium concentrations in the bilayers and in the aqueous phase. On the other hand, the binding model emphasizes the limited capacity of the bilayers to incorporate the solute molecules. A finite number of saturable sites are assumed to be available for the solute binding, just as in the case of multiple binding sites of proteins. Therefore, the equilibrium distribution of a solute between water and bilayer is expressed as an association (or binding) constant K_a. The partition model is suitable for low solute concentrations, while the binding model is often more reasonable for higher solute concentrations, as were used in the present research.

The overall association constant K_a is defined as described by equation 2, assuming the overall concentration of bilayer-associated peptide $[Pep]_b$ being proportional to the product of the overall concentration of free aqueous peptide $[Pep]_w$ and lipid $[Lip]_0$ in terms of total volume ([M]).

$$K_a = \frac{[Pep]_b}{[Pep]_w[Lip]_0} \qquad \text{(Eq. 2)}$$

The peptides were quantified by HPLC. The area of the absorption peak in the HPLC chromatogram at the characteristic wavelength of the peptide under investigation (i.e.

wavelength of 280 nm for Trp containing peptides), is proportional to the concentration of the peptide in the sample. Therefore, the concentration $[Pep]_w$ in equation 2 can be replaced by the absorption peak area $OD_\lambda area_{Aq}$ in the chromatogram of the dialysis chamber sample containing only the aqueous buffer. Since the concentration of bilayer-associated peptide $[Pep]_b$ corresponds to the difference between the total peptide concentration in the chamber containing the lipid bilayer $[Pep]_{Lip}$ and the peptide concentration in the buffer chamber $[Pep]_{Aq}$ (=$[Pep]_w$), it can be replaced by the difference between the corresponding peak areas of the chromatograms (see Eq. 3).

$$K_a = \frac{OD_\lambda area_{Lip} - OD_\lambda area_{Aq}}{OD_\lambda area_{Aq}[Lip]_0} \qquad \text{(Eq. 3)}$$

The partitioning coefficient P for a peptide can be defined as follows. The volumes of the bilayer V_b and the aqueous phase V_w are used to calculate the local peptide concentration in each phase ($[Pep]_{b,local}$ and $[Pep]_{w,local}$) from their overall concentrations ($[Pep]_b$ and $[Pep]_w$). Assuming that the volume of the aqueous phase V_w is approximately equal to the total volume of the chamber V_0, provides:

$$P = \frac{[Pep]_{b,\,local}}{[Pep]_{w,\,local}} = \frac{[Pep]_b V_w}{[Pep]_w V_b} \approx \frac{[Pep]_b V_0}{[Pep]_w V_b} \qquad \text{(Eq. 4)}$$

The volume of the bilayer phase V_b is a function of the outer radius R (as determined by DLS) of the liposome, the bilayer thickness d (3.7 nm for POPC liposomes), and the mean head group area of the lipid used a_0 (0.72 nm^2 for POPC liposomes). [604], [605] Using the geometrical formulas for the spherical volume and the surface area and taking the lipid concentration $[Lip]_0$ in the total sample volume V_0, the volume of the bilayer phase V_b is calculated as follows for the liposome containing chamber:

$$V_b = \frac{a_0 N_A [Lip]_0 V_0 (R^3 - (R-d)^3)}{3(R^2 + (R-d)^2)} \qquad \text{(Eq. 5)}$$

Replacing V_b in equation 4 and using equation 2, provides the final equation for an approximate determination of the partitioning coefficient of peptides into liposomal membranes:

$$P \approx K_a \frac{3(R^2 + (R-d)^2)}{a_0 N_A (R^3 - (R-d)^3)} \qquad \text{(Eq. 6)}$$

7.2.3. Freeze-Fracture Electron Microscopy (ff-EM)

In order to determine the size and shape of liposomes before and after the condensation experiments, electron microscopy analysis was carried out using the freeze-fracture method.

The sample was frozen at -180°C using a liquid propane jet (6 bar). The frozen sample was then transferred into the freeze-fracture apparatus (Blazer BAF 300), and fractured with a knife at low temperatures (-150°C) and under high vacuum ($2*10^{-7}$ mbar). This creates shear forces which result in a shear plane passing through the sample. Experience has shown that when the shear plane passes through a liposomal membrane, it preferentially crosses the equator of the spherical shape, thus dividing the membrane in two half-spheres which exposes their interior. To avoid any sublimation of water, a replica of the surface was immediately created. It is this replica that was actually looked examined in the electron microscope. The replica was produced by shadowing the sample with a 2 nm platinum-carbon layer at approximately a 45° angle to highlight topological features. It is the carbon which gives the replica mechanical strength. The sample was then thawed and the replica floated off and picked up on an appropriate grid for examination, which was performed in a Philips EM 301 electron microscope at 80 kV. [606]

The ff-EM of the samples was carried out by Michaela Wessicken (Laboratory for Electron Microscopy I, ETH-Zentrum).

7.2.4. Dynamic Light Scattering (DLS)

30 µl of sample containing a typical concentration of 25 mM in lipid was diluted with 1470 µl buffer before and after the condensation experiment and transferred into a round-bottom light-scattering cuvette. The DLS was performed on a fiber-optics-based spectrometer consisting of an argon-ion-laser (Innova 200-10, λ_0 = 488 nm, Coherent), a digital autocorrelator (ALV 5000) and computer-controlled rotational stage (Model 496, Newport and Controller PMC 400). A photomultiplier (ALV/PM-15) was used as the detector. [607] The intensity of the scattered laser-beam, reduced by a 9.5%-transmission filter, was measured 10 times over a period of 90 s at each angle (60°, 90°, and 120°). The data was evaluated using a second-order cumulant fit (Angle 1.3, ALV DLSm software) which provided mean radius size of the liposomes for each angle.

7.2.5. CDI-Induced Amino Acid Condensation

In a typical single-feeding experiment, a concentrated amino acid solution (i.e. 60 mM in H-Trp-OH) in a 0.4 M imidazole/HCl buffer of pH 6.80 was incubated with a 2.5-fold excess of *N,N'*-carbonyldiimidazole (CDI) at 0°C for 2 min. Yields of up to 95% of NCA-amino acid and *N*-[imidazolyl-(1)-carbonyl]amino acid were obtained. [154], [361], [362], [390], [411]-[413], [453] The resulting NCA-amino acid solution was mixed 1:1 (v/v) with either an aqueous 40 mM lipid suspension of 100 nm extruded liposomes in 0.4 M imidazole/HCl buffer, pH 6.80, or 0.4 M imidazole/HCl buffer, pH 6.80, with no liposomes in an 1.5 ml eppendorf tube. The resulting solutions were incubated on a Vortex-Genie 2 shaker (Scientific Industries, USA) for 12 h at room temperature. After this time, neither NCA-amino acid nor N-[imidazolyl-(1)-carbonyl]amino acid was detected, indicating that the reaction was complete. 25 µl of the reaction mixtures with liposomes were injected into the HPLC immediately after removal from the shaker. This was done to ensure a homogeneous distribution of the oligomers formed. It was observed that vesicular samples left to stand for few hours with no shaking, tended to precipitate especially at high initial NCA-amino acid concentration and in multiple feeding experiments of hydrophobic amino acids. In some cases the liposome-containing reaction mixtures were diluted 1:1 (v/v) with a 100 mM "100 nm" extruded POPC, 0.4 M imidazole/HCl, pH 6.80, suspension before injection (50 µl) to prevent precipitation. Mixtures containing no liposomes were diluted 1:1 (v/v) with acetonitrile after incubation in order to solubilize the precipitated products and 50 µl were injected into the HPLC.

In a typical multiple-feeding experiment, 50 µl of a NCA-amino acid solution was either mixed with 150 µl of a 100 nm extruded 100 mM lipid suspension or buffer alone and incubated for 12 h. Another 50 µl of a freshly prepared NCA-amino acid solution were added to the reaction mixture and incubated again for 12 h and so forth. If a racemate of an amino acid was oligomerized, the NCAs of the D- and L-enantiomer were prepared separately and then mixed 1:1 (v/v) at 0°C just before adding the resulting racemic mixture to the liposomal or buffer solution. The reason for this procedure was that the solubility of the amino acid racemates was generally lower than for their corresponding racemic NCA-solutions or the enantiomers of the amino acids used in these studies.

7.2.6. EEDQ-Induced Peptide Condensation

In a typical experiment, 12 µl of a 0.169 M 2-ethoxy-1-ethoxycarbonyl-1,2-dihydrochinoline (EEDQ) stock solution in acetonitrile was added either to 1 ml of a 5 mM H-TrpTrp-OH, 50 mM phosphate, 25 mM lipid 100 nm extruded liposome suspension of pH 5.90 (liposome system) or 1ml of a 5 mM H-TrpTrp-OH, 50 mM phosphate, pH 5.90, (reference system) in a 1.5 ml eppendorf tube and rapidly mixed on a vortex. The resulting mixture, at 2 mM EEDQ, was incubated on a Vortex-Genie 2 shaker (Scientific Industries, USA) for 26 h at room temperature. Then 25 µl of the resulting solution were injected into the HPLC connected to a mass spectrometry detector for product analysis.

In the case of the liposome system, the solutions were characterized before and after incubation with EEDQ by ff-EM and DLS.

7.2.7. Liquid Chromatography Mass Spectrometry (LC-MS)

The condensation products of the non-diluted (EEDQ-induced condensation) and the diluted reaction samples (CDI-induced condensation), were analyzed either by HPLC (HP1050 LC, Hewlett Packard) connected to a diode array detector (HP1050 DAD, Hewlett Packard), by HPLC (HP1100 LC, Hewlett Packard) connected to a diode array detector (HP1100 DAD, Hewlett Packard) and a single quadrupole mass spectrometry detector (HP1100 MSD, Hewlett Packard), or by HPLC (SpectraSystem, Thermo Separation Products) connected to a diode array detector (UV6000LP, Thermo Separation Products) and an ion-trap mass spectrometry detector (LCQdeca, Finnigan).

A C_{18} column (ET 250/4 Nucleosil 100-5, Macherey-Nagel) was used to separate the condensation products according to their hydrophobicity (reverse-phase chromatography). Typical parameter settings for the HPLC were a 1 ml/min flow rate at room temperature, using two buffer solvents A and B (A: 0.1% TFA; B: 99.9% ACN, 0.1% TFA), starting with a 2 min isocratic flow of 10% B and then driving a typical gradient of 2.0% B/min up to 90% B. At high ACN levels, lipids such as POPC precipitate on the column and therefore the column had to be washed with about 100 ml MeOH after every 10 lipid-containing injections in order to guarantee good, reproducible separation on the column.

For the qualitative detection of peptides containing the amino acids used, UV-Vis spectra from the diode array detector were compared with the absorption known from amino

acid spectra. In addition, atmospheric pressure electrospray ionization in the positive mode (ESI^+) was used to verify the expected molecular mass of the peptide oligomers. Typical settings for the single quadrupole instrument HP1100 were: 350°C gas temperature, 10 l/min drying gas flow, 60 psig nebulizing pressure, 3500 V capillary voltage, and 95 V fragmentor voltage. Typical settings for the ion-trap instrument LCQdeca were: 350°C capillary temperature, 80 units sheath flow rate, 20 units aux flow rate, 4.5 kV I-spray-voltage, 39.0 V capillary-voltage and -60.0 V tube lens offset. To determine the exact structure of the peptides and some by-products and to verify the presence of certain amino acids in the peptides, MS/MS was applied by either in-source fragmentation (HP1100 MSD) or the collision-induced dissociation technique (LCQdeca).

Quantification of Trp-containing peptides was performed by measuring the optical density at 280 or 290 nm and integrating the chromatogram peaks over time, assuming that the extinction coefficient for each tryptophan-indole chromophore of the monomer did not change when incorporated into a peptide that is an oligomer of itself. [15]

In the case of co-condensation of D- and deuterated L-amino acids, quantification of stereoisomeric subgroups (consisting of p D-amino acids and q L-amino acids within (p+q)-oligomer group) was made by MS, using the areas of the SIM-chromatogram peaks integrated over time. Since the deuterated enantiomer has a higher molecular mass of Δm, the number of deuterated L-enantiomers q in an oligomer of the mass m_{p+q} is given by the mass difference of the oligomer looked at and the homochiral, undeuterated oligomer of the same length n (= p+q), namely $q = (m_{p+q}-m_n)/\Delta m$.

7.2.8. Kinetics of Z-PheHisLeu-OH Catalyzed C16-ONp Hydrolysis

A peptide stock solution in 50 mM boric acid/borate of pH 8.50 was mixed 1:1 (v/v) with a 50 nm extruded liposome suspension in 50 mM boric acid/borate buffer of pH 8.50 and incubated for at least 10 hours. 3 ml of the peptide-liposome suspension (or the corresponding reference reaction samples with either buffer only, peptide but no liposomes, or liposomes only) were added into a UV quartz cuvette (3 ml / 1 cm). For each system, the baseline was taken against the reference cell containing the same composition as the sample itself prior to addition of the substrate. 36 µl of a C16-ONp stock solution in ACN were then added to each reaction sample and 36 µl ACN to the reference cells, respectively. The cells were mixed through shaking and the time-

dependent absorbance was measured at 400 and 480 nm. The concentration change of *p*-nitrophenolate/*p*-nitrophenol was calculated from the difference in the optical densities at 400 and 480 nm using a molar extinction coefficient $\varepsilon_{400\ nm}$ of 14'700 $cm^{-1}M^{-1}$ for the POPC suspensions, 16'300 $cm^{-1}M^{-1}$ for the DOPA/POPC liposome suspensions and 18'400 $cm^{-1}M^{-1}$ for samples containing no liposomes.

8. References

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Curriculum Vitae

Name:		Markus Christoph Blocher
Date of Birth:		April 16, 1971
Place of Birth:		Männedorf ZH, Switzerland
Education:	1978 - 1984	Primary School, Feldmeilen ZH
	1984 - 1987	Secondary School, Meilen ZH
	1987 - 1988	Mathematisch- Naturwissenschaftliches Gymnasium, Rämibühl Zürich
	1988 - 1989	Senior at Brunswick High School, Ohio, USA,
	1989	Graduation Diploma
	1989 - 1991	Mathematisch- Naturwissenschaftliches Gymnasium, Rämibühl Zürich
	1991	Matura, Typus C
	1991 - 1997	Undergraduate Student at the ETH Zürich, Department of Chemistry
	1997	Diploma in Chemistry
	1996 - 2000	Ph.D. Student at the ETH Zürich
	March 2000	Promotion Exam

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