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Archaenal lipids in oral delivery of therapeutic peptides

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Abstract

Archaenae contain membrane lipids that differ from those found in the other domains of life (Eukarya and Bacteria). These lipids consist of isoprenoid chains attached via ether bonds to the glycerol carbons at the sn-2,3-positions. Two types of ether lipids are known, polar diether lipids and bipolar tetraether lipids. The inherent chemical stability and unique membrane-spanning characteristics of tetraether lipids render them interesting for oral drug delivery purposes. Archael lipids form liposomes spontaneously (archaeosomes) and may be incorporated in conventional liposomes (mixed vesicles). Both types of liposomes are promising to protect their drug cargo, such as therapeutic peptides, against the acidic environment of the stomach and proteolytic degradation in the intestine. They appear to withstand lipolytic enzymes and bile salts and may thus deliver orally administered therapeutic peptides to distant sections of the intestine or to the colon, where they may be absorbed, eventually by the help of absorption enhancers. Archael lipids and their semisynthetic derivatives may thus serve as biological source for the next generation oral drug delivery systems.

The aim of this review is to present a systematic overview over existing literature on archaenae carrying diether and tetraether lipids, lipid diversity, means of lipid extraction and purification, preparation and in vitro stability studies of archaenal lipid-based liposomal drug carriers and in vivo proof-of-concepts studies.

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1. Introduction & Background

Despite tremendous research efforts undertaken in recent years, oral delivery of labile drug compounds such as therapeutic proteins and peptides still is challenging primarily for two reasons: 1) due to the harsh environment of the lumen of the gastrointestinal (GI) tract, including acidic pH and proteolytic enzymes, which the drug needs to withstand and 2) the intestinal barrier, which the drug needs to come across on its way to the site of action. The GI tract’s physiological role is the digestion of nutrients, including proteins and peptides, and subsequent absorption of digested fragments, including amino acids and short peptides. To this end, the GI tract plays an important role in preventing “foreign” compounds, including proteins and larger peptides, from entering the body as these may be pathogenic or allergenic. These essential features of the GI tract account for the challenges encountered with the oral administration of peptide drugs.

On the other hand, therapeutic peptides represent a rapidly growing class within the pharmaceutical market with currently 60 US Food and Drug Administration (FDA) approved peptide drugs and 140 and over 500 peptide drugs in clinical and preclinical trials, respectively (Fosgerau and Hoffmann, 2015). The oral route in general is the preferred way of administration as it combines ease and convenience of self-application with low production cost. However, routine oral application of therapeutic peptides has been accomplished in only a few cases (e.g. Desmopressin, Cyclosporin A) with a few more in various phases of clinical trials. Despite major research efforts in recent years, oral administration of peptide and protein drugs has not been accomplished for the vast majority of compounds to date (Renukuntla et al., 2013). Clearly, novel and more efficient oral formulation strategies suitable for this class of compounds would yield a tremendous step forward in treatment options for a number of diseases where peptide based drugs are essential.

Different formulation strategies, for example the use of absorption enhancers, enzyme inhibitors, bioadhesive systems, site specific delivery systems and particulate carrier systems, such as liposomes or solid lipid nanoparticles, have been investigated to enable the oral drug
Nanoparticulate carriers are regarded as promising for several reasons: 1) they may protect their drug load against the harsh environment of the upper intestine 2) they may release their drug load in a sustained or site specific manner (e.g. where the pH value is close to neutral and/or the activity of proteolytic enzymes reduced) 3) they may slow down the drug’s passage along the GI tract and/or convey intimate contact with the mucosa (e.g. through muco-adhesive surface characteristics). We shall focus here on a specific type of nanoparticulate carrier, liposomes.

Liposomes are (sub-)microscopic vesicular structures composed of one or several phospholipid (PL) bilayer(s) enclosing an aqueous core. Both, the aqueous core and the bilayer(s) can accommodate drug compounds. Hydrophilic compounds like most peptides may be encapsulated and retained within the aqueous core for extended periods of time if they are sufficiently large and charged, such that leakage across the bilayer(s) is slow. Hydrophobic compounds may be incorporated within the bilayer(s) and their apparent solubility thus improved (Brandl, 2001). The general structure of liposomes is shown in Fig. 1.

Encapsulation of therapeutic peptides within liposomes may offer some protection against the harsh environment in the GI tract. Thereby, the premature degradation of the therapeutic compound can be prevented. Recent studies support this by indicating that oral bioavailability of poorly soluble and low bioavailable drugs could be enhanced by liposome formulations (Fricker et al., 2010). Even though these results are promising, conventional liposomes only exhibit a limited stability at low pH in the presence of bile salts and lipases (Lasic, 1998), thus demonstrating the clear need for an improvement of liposomal formulations for oral use. Different strategies to stabilize liposomes in the GI tract environment have been investigated. For example, the linking of polymers such as polyethylene glycol (PEG), the sugar chain portion of mucin or polyvinyl alcohol to the liposome surface (surface coating), has a stabilizing effect on liposomal formulations (Silva et al., 2012). Another promising technique to increase liposomal stability, which will be the focus of this review, is the use of archaeal membrane lipids in the formulation of liposomes (Jacquemet et al., 2009).

Archaea are a diverse group of prokaryotic microorganisms. Even though several archaeal species were already identified at the beginning of the 20th century, (for a recent review see (Petitjean et al., 2015), archaea were not classified as a separate group of prokaryotes and were initially called archaebacteria. With the introduction of a novel technique to reconstruct evolutionary relationships that is based on the comparison of 16S ribosomal RNA (rRNA) sequences (Woese and Fox, 1977), archaea were found to be different from bacteria. Subsequently, Woese and coworkers proposed the three domain model based on the 16S rRNA phylony, which divides the tree of life into three main domains, Bacteria, Eukaryota and Archaea (Woese et al., 1990). With the separation of Archaea into a distinct domain, the epithet “bacteria” became redundant and hence literature uses the term Archaea instead of archaebacteria today.

Originally, two major kingdoms (same as phyla in Bacteria) were recognized for the domain Archaea, Euryarchaeota and Crenarchaeota. The kingdom Euryarchaeota consists of cultivated species that are methanogens, halophiles or thermophiles and is a phenotypically heterogeneous group. The kingdom Crenarchaeota is a more homogenous group, mainly consisting of thermoacidophiles and thermophiles (Woese et al., 1990). With the use of environmental molecular techniques ‘non-extreme’ archaea (mainly mesophiles) were found in many environments, including the ocean, freshwater and soils. Research concerning archaea is rapidly progressing and, besides mesophile Eury-
and Crenarchaeota, recently the additional kingdoms of Korarchaeota, Nanoarchaeota, Thaumarchaeota and Aigarchaeota were discovered and defined (Villanueva et al., 2014). An overview over phylogenetic relationships is given in Fig. 2.

Many archaeal species can withstand adverse conditions found in extreme environments including high temperatures (thermophiles and hyperthermophiles), high pressures (barophiles), high salt concentrations (halophiles) and low or high pH (acidophiles and alkaliophiles, respectively). Among other factors, their unique membrane lipids that are not generally seen in bacteria and eukaryotes are regarded as one key adaptation making archaea able to survive in these extreme environments (Konings et al., 2002). Two major groups of archaeal membrane lipids are known diphytanylglycerol diether lipids (DELs) and its derivatives, which are also called “archaeol”, as well as the dimeric dibiphytanylglycerol tetraether lipids (TELs) and its derivatives, which are also called “caldarchaeol” and “nonitolcaldarchaeol” respectively (Kates et al., 1993) (see Fig. 3).

Archaeal membrane lipids are attracting considerable attention due to their specific characteristics. Their stability may be utilized in biotechnological or pharmaceutical applications. As stated earlier, the stabilization of liposomes against the harsh environment of the GI tract is one possible pharmaceutical application. However, archaea produce various membrane lipids and still it is not fully understood which lipid characteristics are most important when archaeal membrane lipids are used in this context.

In this review structural features of archaeal membrane lipids and their relation to different archaeal species, different lipid extraction techniques and liposome preparation methods will be discussed. Finally, results from both in vitro and in vivo studies investigating the stability and performance of liposomes containing archaeal membrane lipids will be summarized and discussed.

It is important to mention that herein there will be a differentiation between liposomes consisting entirely of archaeal membrane lipids from natural as well as synthetic origin, which will be termed archaeosomes, conventional liposomes consisting entirely of PLs and cholesterol (Chol) and liposomes partly consisting of PLs and archaeal membrane lipids, which will be termed mixed vesicles (see Fig. 1.). Furthermore, it has to be mentioned that archaeosomes and mixed vesicles have been studied extensively in the context of vaccination and gene delivery. As these potential applications focus on different features of archaeal lipids, these will not be further discussed here.

2. Method

A computer-based literature search was conducted using the search engines/databases Scopus® and SciFinder®. Different search strategies were tested to cover the topic in a comprehensive manner. Search strategies and corresponding results are presented below.

Regardless of the database, in which a search was conducted, a combination of the following keywords was used.

2.1. Liposome, archaeosome, archaea, oral drug-delivery, pharmaceutical formulation, tetraether lipids, stability

A search in Scopus® was based on searching for separate keywords, as Scopus® has a function, which lets you search for additional keywords within the results. Using this function, results were narrowed down to more specific results matching the topic of this review.

As vaccination and gene-delivery will not be discussed here, results dealing with vaccination and gene-delivery were excluded using an additional feature of Scopus®, which lets you exclude certain keywords from the results. Excluded keywords included...
“adjuvants, immunologic”, “adjuvant”, “vaccine”, “humoral immunity”, “immune response”, “cellular immunity”, “gene-delivery”, “gene therapy” and “gene transfer”. Furthermore, keywords related to dermal and transdermal drug-delivery were excluded as well. Excluded keywords included “skin”, “skin absorption”, “skin permeability”, “transcutaneous” and “keratinocytes”.

From the articles obtained by this procedure, the most relevant hits were selected for further evaluation. Articles were selected from the first 25 results sorted by relevance. Furthermore, the article had to be cited at least 10 times and/or had to be from 2015 to be selected for further evaluation.

In contrast, a search in SciFinder® was initiated with searching for a whole sentence. Results for the initial search were grouped according to concepts. For a given combination of concepts, potentially two groups of results were created one where concepts are “closely associated with one another” and one where concepts “were present anywhere in the reference”. To obtain results matching the topic of this review, only groups containing three or more concepts from the original search were chosen. Additionally, the groups’ concepts had to be “closely associated with one another” and contain the keyword “archaea”, “archaeosome” or “tetraether lipid” to exclude studies only investigating conventional liposomes. SciFinder® has a function, which lets you analyze results by “index terms”. Obtained results were analyzed by the index terms “Drug-delivery systems” and “Pharmaceutical liposomes”. Furthermore, results dealing with vaccination, gene-delivery, dermal and transdermal drug-delivery were excluded manually. Finally, articles with available full text cited 10 or more times and/or all articles from 2015 and later were selected.

In total four searches were performed in the database SciFinder®. The four searches were based on the sentences: “Liposomes containing lipids from archaea in pharmaceutical formulations for oral administration”, “Archaeosomes for oral drug-delivery”. “Liposomes containing tetraether lipids for oral drug-delivery” and “Stability of liposomes containing lipids from archaea”.

For a higher degree of comprehensibility, a search based on author names identified from the searches in the databases Scopus® and SciFinder® was conducted. For this purpose, the database Scopus® was used.

An overview and summary of all identified articles, including original research articles and reviews, sorted by date of publication is given in Table in supplementary material.

3. Results & discussion

This section is divided into four subsections dealing with archaeal membrane lipids in general, methods for extraction of archaeal membrane lipids, methods for the preparation of liposomes and results of in vitro and in vivo studies.

3.1. Archaeal membrane lipids: lipid structure and variability of lipids

Here we present a general description of archaeal membrane lipids, especially with emphasis on structural features. Additionally, we will discuss how different growth conditions can influence structural features in various archaea.

Archaeal membrane lipids generally are composed of an C_{20–40} isoprenoid core (phytanyl) linked to either glycerol and/or nonitol, which can be substituted or unsubstituted with polar or non-polar head groups (Hanford and Peeples, 2002). In contrast to membrane lipids found in bacteria and eukaryotes, which have a sn-1,2 stereochemistry, archaeal membrane lipids possess a sn-2,3 stereochemistry (Hanford and Peeples, 2002). As stated before, two major groups of archaeal
membrane lipids are known, the monomeric, monopolar DELs and the dimeric, bipolar TELs (De Rosa and Gambacorta, 1988; Hanford and Peeples, 2002). DELs can vary in chain length, stereochemistry, head group and/or the nature of the polyol. Furthermore, macrocyclic DELs (see Fig. 2) have been discovered (De Rosa and Gambacorta, 1988). Similar to conventional PL membranes, DELs membranes are arranged in bilayers (Hanford and Peeples, 2002). Bipolar TELs are macrocyclic in nature and generally divided into two classes, glycerol-dialkyl-glycerol-tetraethers (CDGT) and glycerol-dialkyl-nonitol-tetraethers (GDNT) (Jacquet et al., 2009). Furthermore, the lipid core of TELs can contain up to four cyclopentane rings per isoprene chain (Hanford and Peeples, 2002). TELs can vary in chain length, stereochemistry, head groups and/or the number of cyclopentane rings in the lipid core. In contrast to PLs and DELs, TELs tend to self-arrange in monolayers (Hanford and Peeples, 2002) but may also be incorporated within PL and/or DEL bilayers. Fig. 2 shows examples of archaeal lipid structures.

The membrane lipid composition and lipid characteristics vary both, within and among archaeal species and seem to be related to their habitat. Lipid characteristics that often are regarded to vary with the habitat are the TEL-to-DEL ratio and the degree of cyclization of the TEL lipid core.

The membrane of halophiles mainly consists of DELs while those of methanogens consist of 50–100% DELs and 0–50% TELs (Chong, 2010). TELs make up the majority (90–95%) of the membrane lipids of thermoacidophiles and hyperthermophilic neutrophiles (Chong, 2010). Studies by D. Lai et al. (2008) and G. D. Sprott et al. (1991) have shown that the TEL-to-DEL ratio of different archaea is related to the culturing temperature. For example, the membrane of *Methanococcus jannaschii* grown at 50 °C consisted of 60% DELs, 22% TELs and 18% macrocyclic DELs. At 75 °C its membrane consisted of 47% TELs, 36% macrocyclic DELs and 17% DELs (Sprott et al., 1991). Furthermore, the TEL-to-DEL ratio of *Archaeoglobus fulgidus* increased from 0.3 ± 0.1 at 70 °C to 0.9 ± 0.1 at 89 °C (Lai et al., 2008). In the same study, it could be shown that the number of cyclopentane rings in the lipid core also seems to be related to the culturing temperature, as the number of TELs from *A. fulgidus* grown at 70 °C showed less cyclization than TELs from *A. fulgidus* grown at higher temperatures (Lai et al., 2008). Identical conclusions could be drawn from other studies (Chong, 2010). For example, H. Shimada and Co-workers were able to show that the average number of cyclopentane rings in the polar lipid fraction from *Thermoplasma acidophilum* increased from 3.6 at 45 °C to 4.5 at 60 °C, thereby supporting results from an earlier study by I. Uda et al. (Shimada et al., 2008; Uda et al., 2001). A more recent study by S. M. Jensen et al., which investigated the lipid composition of *Sulfolobus islandicus* and *Sulfolobus tokodaii* membranes at different temperatures and growth phases, further supported the correlation between growth temperature and number of cyclopentane rings in the lipid core. The results showed that for both species in all biological replicates the number of cyclopentane rings increased with increasing growth temperature. Furthermore, S.M. Jensen et al. could show that as an additional factor growth phase seemed to have an influence on the number of cyclopentane rings in the lipid core. For bulk lipids of both *S. islandicus* and *S. tokodaii* the number of cyclopentane rings decreased from lag to exponential phase and increased from exponential to stationary phase again (Jensen et al., 2015b).

Additional environmental factors as for example salinity, pressure, nutrients and pH, may have an influence on lipid characteristics (Oger and Cario, 2013). However, these are not as extensively studied as temperature. Considering the effect of pH, it was shown that the number of cyclopentane rings in the total polar lipid fraction from *Pyrococcus horikoshii*, which optimally grows at 95 °C and pH 6–8, is lower than that from *Sulfolobus acidocaldarius*, which optimally grows at 70–75 °C and pH 2 (Chong, 2010), indicating that pH indeed may have an influence on the degree of cyclization. However contradicting results were achieved when studying the effect of pH on cyclization (Jensen et al., 2015b). For example, the number of cyclopentane rings in *T. acidophilum* surprisingly decreased when pH was decreased (Shimada et al., 2008).

### 3.2. Extraction of lipids from archaea and preparation of liposomes

#### 3.2.1. Extraction of archaeal lipids

A key requirement for using archaeal lipids in liposome formulations is the extraction of the lipids from the archaeal cell (biomass). Not only the efficiency at which the lipids can be extracted (yield) but also the lipid composition, i.e. whether certain lipids are preferentially extracted, may be of importance for the usefulness of the gained extracts.

Extraction separates the desired compound(s) from a matrix e.g. other compounds present in the mixture. Most lipid extraction procedures take advantage of the fact that lipids are highly soluble in non-polar solvents, when compared with other biomolecules (e.g. proteins, nucleic acids and sugars), inorganic salts and other contaminating compounds. Thus, lipids are often extracted by various forms of organic solvent extractions. Another factor of variation is whether and how the archaeal cells are disrupted prior to extraction.

#### 3.2.2. Cell disruption

Prior to the actual lipid extraction, the cell samples are commonly disrupted to facilitate the transfer of lipids from cells to organic solvents. Common methods for cell disruption are sonication (Sturt et al., 2004) or homogenization (Harrison, 1991), exposure to solvents or heat (Huguet et al., 2010). Cells which have a sturdy protein-based cell wall require a more effective cell disruption/homogenization process compared to soft tissues (Folch et al., 1957). Archaeal cell samples are composed of a single cell membrane often coated with an S-layer. In the field of biotechnology, a common cell disruption method is bead-beating/milling, where the mechanism is based on mechanical disruption with glass or metal beads (also called ball milling). Apart from conventional ball mills, this can be obtained by using Dual asymmetric centrifugation (DAC). Hereby, the combination of two centrifugal forces gives vigorous agitation of a mixture of cells and beads (Massing et al., 2008). DAC has been introduced by Jensen et al. for the disruption of archaeal cells (Jensen et al., 2015a).

#### 3.2.3. Lipid extraction process

Several extraction protocols exist and the choice depends on the objectives. The requirements for an optimal archaeal lipid extraction are:

1. The lipids should preferentially be extracted in intact form, i.e. no loss of polar head groups should occur.
2. The extraction yield should be high, i.e., extraction should be efficient.
3. The resulting lipid extract should be free of contaminating compounds.
4. The extraction protocol should secure extraction of the most relevant type of lipid (typically TELs) at good efficiency.

If the aim is to obtain a highly purified fraction of lipid with minimal contamination, the tradeoff is typically reduced recovery, i.e. some of the material will probably be lost during the extraction process. On the other hand, a highly efficient extraction method will likely extract other compounds soluble in organic solvents, such as residues from the media, which will affect the subsequent use of the lipid extract. Therefore, it is necessary to find a balance between these two requirements. With the amount of lipid being around 8–10% of dry archaeal cell weight it is important to have an efficient extraction method (De Rosa et al., 1980; Lo et al., 1989; Nishihara and Koga, 1987).

Lipid extraction protocols can be divided into two groups 1) solvent extraction (e.g. liquid-liquid extraction or Soxhlet extraction) or 2) solid phase extraction. Selected extraction methods will be described below with a particular emphasis on methods applied for the extraction of archaeal lipids. Since solid phase extraction has not been applied to archaeal lipids, hence it will not be discussed further here.
3.2.4. Liquid-liquid extraction

The principle of liquid-liquid extraction is to transfer the desired compound(s) from a matrix to typically an organic solvent in which the target compounds are soluble. Often liquid-liquid extractions comprise two steps, first a monophasic system, where a specific ratio of solvents is used to obtain a miscible system. This is followed by a second step where the ratio of the solvents is changed to induce phase separation.

The “classical” method for the extraction of lipids according Bligh and Dyer (B&D) in 1959 (Bligh and Dyer, 1959) is by the use of a blend of chloroform (CHCl₃):Methanol (CH₃OH):water (H₂O) as solvent blend, which matches the solubility of a variety of lipid classes. In this method the lipids are first extracted from the biomaterial by organic solvents and then separated from contaminating compounds (e.g. protein, sugars, nucleic acids and salt) by partition. This is carried out by mixing the homogenized cells with specific CHCl₃:CH₃OH-ratios creating a miscible (monophasic) system.

B&D use a ratio of 1:2:0.8 (CHCl₃:CH₃OH:H₂O, v/v) during extraction and then a ratio of 2:2:1.8 (CHCl₃:CH₃OH:H₂O, v/v) to induce partitioning (Bligh and Dyer, 1959). Since the 1960’s many modifications have been suggested for the B&D protocol to optimize the method. The H₂O content has been replaced with 5% trichloroacetic acid for the extraction of archaeal lipids, described as the modified Bligh and Dyer (M-B&D), increasing the yield from 0.98% to 5.38% lipid content of dry cells weight (Nishihara and Koga, 1987). Another variation was applied to the M-B&D protocol by replacing the 5% trichloroacetic (TCA) with phosphate buffered saline (PBS) and sonication of the extract (Sturt et al., 2004). The reason for using PBS instead of TCA-buffer was not further discussed in the paper. However, for extracting TELs the author recommended that TCA should be used in the last two steps of the extraction.

3.2.5. Soxhlet extraction

Another example of a solvent extraction procedure for lipids is a Soxhlet extraction. The principle of Soxhlet is lixiviation, i.e. separation of soluble material from a solid sample with solvents by continuous refluxing (Luque de Castro and García-Ayuso, 1998). The sample is placed in a sleeve within a reservoir. During operation the solvent is heated and it evaporates to rise up to the condenser to be condensed and drop down into the reservoir with the sample. The reservoir is gradually filled with solvent and when the liquid reaches the overflow level it will flow through the siphon back to the distillation flask. Since there is dripping freshly distilled solvent into the reservoir, analytes that are soluble in the solvent are transferred from the solid matrix to the liquid and the operation may be continued until complete extraction is achieved (Luque de Castro and García-Ayuso, 1998).

The Soxhlet method has been applied as a preliminary step before M-B&D extraction for the extraction of lipids from S. acidocaldarius (Lo et al., 1989) and used as a separate method for the extraction of lipids (Huguet et al., 2010). The yield of lipids was similar to the M-B&D (9.5% compared to 9.1% of dry cell weight (Lo et al., 1989)), indicating that the Soxhlet method here does not provide an improvement in yield for the extraction of lipids from archaeal samples.

To determine the best protocols to extract archaeal lipids, two papers compared 3 and 13 extraction protocols, respectively (Huguet et al., 2010; Lengger et al., 2012). According to Huguet et al., the best extraction method was Soxhlet, when the purpose was to obtain intact polar lipids from cultures (Huguet et al., 2010). This was mainly due to the fact that the very polar intact lipids were difficult to recover from the aqueous phase in the M-B&D method. By using Soxhlet this step was avoided. In contrast, the study of Lengger et al., showed that the M-B&D method is the preferred method for yielding intact polar lipids from environmental samples because Soxhlet extractions showed a bias for certain lipid species, (Lengger et al., 2012).

Obviously, it depends on the desired application, which extraction method to choose. However, to obtain intact polar lipids any acid hydrolysis step should be avoided as this will result in a loss of the head groups (Huguet et al., 2010).

3.2.6. Liposome preparation

Generally, liposomes including archaeosomes and mixed vesicles were prepared by lipid hydration followed by filter extrusion giving unilamellar vesicles (ULV), or bath sonication giving multilamellar vesicles (MLV) (Benvegnu et al., 2005a; Parmentier et al., 2011a; Parmentier et al., 2011b; Patel et al., 2000). For a protocol using sequential filter extrusion see (Jensen et al., 2015a), with factors of importance for the outcome of filter extrusion are described in (Hinna et al., 2016). In a recent study liposomes were prepared by dual asymmetric centrifugation (Parmentier et al., 2014).

3.3. In vitro stability screening of liposomes containing archaeal lipids including archaeosomes and mixed vesicles

As stated earlier, the stability of liposomal formulations in the presence of certain stress factors including low pH, bile salts and lipases is limited. Liposomal formulations containing archaeal lipids generally show increased stability compared to conventional liposomes. However, the stabilizing effect varies. In this section results from different in vitro studies investigating the stability of liposomal formulations containing archaeal lipids in the presence of stress factors including low pH, bile salts and lipases are discussed (see Table 1). For comprehensibility, Table 1 only shows excerpts of the presented studies.

To investigate liposomal stability in vitro, liposomes loaded with a marker (14C-sucrose, 5(6)-carboxyfluorescein (CF) and fluorescein isothiocyanate (FITC)-dextran) are used as described in Section 3.2. Non-encapsulated marker was removed from the prepared liposomes by centrifugation, ultracentrifugation, Sephadex® G25 column chromatography or size exclusion chromatography (Benvegnu et al., 2005a; Jensen et al., 2015a; Li et al., 2010; Parmentier et al., 2011a; Patel et al., 2000). Experiments normally are conducted at 37 °C. After the incubation of the liposomal formulation in the presence of a stress factor, the amount of released marker is determined. Since CF and FITC-dextran are fluorescent compounds, the amount of released CF or FITC-dextran will be determined using a fluorometer (Benvegnu et al., 2005a; Parmentier et al., 2011a; Patel et al., 2000). One drawback of the method is that the fluorescence of CF is pH sensitive (Benvegnu et al., 2005a), complicating the stability investigation at low pH. To avoid pH influencing the results, pH can be increased to 7.4 after incubation using a saline NaOH buffer with a pH of 11.9 (Benvegnu et al., 2005a) or a Tris buffer with pH 10 (Parmentier et al., 2011a), respectively. In the study by G.B. Patel et al., this challenge was bypassed by using radioactive 14C-sucrose. The amount of released 14C-sucrose was determined by measuring radioactive decay using a liquid scintillation counter (Patel et al., 2000).

As can be seen from the results presented by G.B. Patel (2000), MLV liposomes generally are more stable than ULV liposomes at low pH. Furthermore, all formulations prepared from M. mazei total polar lipid fraction (TPL) were less stable at low pH than corresponding formulations prepared from M. espanolae and T. acidophilum TPL. Comparing liposomes prepared from M. espanolae and T. acidophilum TPL, liposomes prepared from T. acidophilum TPL exhibit the greatest stability. MLV liposomes prepared from T. acidophilum TPL are the most stable, with a leakage under 10% after 90 min. There are differences in membrane lipid composition between the three investigated archaeal species. The membrane of M. mazei consists entirely of archaeol and hydroxyarchaeol lipids (DELs), in contrast the membrane of M. espanolae and T. acidophilum contain caldarchaeol lipids (TELs), 65% and 90% respectively (Patel et al., 2000). It could be seen that the stability of liposomes prepared from TPL from the three different archaeal species correlates with the content of TELs in the archaeal membrane.

In the study by T. Benvegnu et al. (2005b) the stability of liposomes containing various synthetic TELs with identical core structure but with different head groups were investigated. From the results it could be

concluded that not only the TEL core structure is important for the membrane stabilizing properties of TELs but also the nature of the head group. Synthetic TELs with neutral head groups (hydroxyl or lactose) did not contribute to the stability of the liposomes at low pH compared to conventional egg-phosphatidylcholine (EPC) liposomes in contrast to synthetic TELs with an amphiphilic phosphocholine (PC) head group, which increased liposome stability compared to the conventional EPC liposomes. It has to be noticed that incubation time was considerably shorter in this study compared to the other studies summarized, making direct comparison difficult.

In the study by J. Parmentier et al., the stability of mixed vesicles containing glycerylcaldityl tetraether (GCTE), PL, Chol and various bioenhancers (cholysarcinose (CS), octadecanethiol (OT) and TPGS 1000) was investigated. In contrast to the studies discussed earlier, no stabilizing effect of TELs at low pH could be demonstrated compared to the conventional liposome formulation (EPC:Chol 1:1) (Parmentier et al., 2011a). Still, important conclusions can be drawn when comparing the leakage of the low molecular weight marker CF and the larger FITC-dextran. After 60 min of incubation 100% CF was released in contrast to only 12% FITC-dextran indicating that, small molecules are subject to considerably greater permeability than larger molecules. This may seem favorable at first glance, since peptides, which are large molecules, potentially can be delivered by this technology. Nevertheless, as indicated by the results, the permeability for protons may be high and may thus result in denaturation of the encapsulated peptides.

The investigation of archaeosome stability in the presence of simulated human bile (SHB) presented in the study by G. Patel et al. shows the same trends as seen for the stability of archaeosomes in low pH (Patel et al., 2000). Generally, MLV archaeosomes are more stable than ULV archaeosomes, even though ULV and MLV archaeosomes from T. acidophilum TPL showed very similar stabilities. Similar to low pH, archaeosomes from M. mazei TPL were least stable compared to archaeosomes from M. espanolae and T. acidophilum and archaeosomes from T. acidophilum TPL were comparably more stable than archaeosomes from M. espanolae in the presence of bile salts. As stated above, these results can be explained by considering the membrane lipid composition i.e. archaeosome stability correlates with the content of TELs in the membrane.

The stabilizing effect of the synthetic TELs investigated by T. Benvegnu et al. were dependent on the nature of the head groups, similarly to low pH (Benvegnu et al., 2005b). TELs with amphiphilic PC head groups did not contribute to the stability of the liposomes no matter in which ratio they were used. In contrast, TELs with neutral head groups (hydroxyl or lactose) were able to increase liposome stability. It is possible that the positive charge of the PC head groups contributes to an increased interaction with the negatively charged sodium cholate,
thereby destabilizing the liposomal formulation. The results from the investigation of liposome stability at low pH and in presence of bile salts are inverted, making it difficult to assess which formulation will be advantageous in an in-vivo situation, as the liposomal formulation would meet both stress factors, low pH and bile salts, when used as an oral formulation.

GTCE studied by J. Parmentier et al. is a TEL with neutral head groups as are some of the synthetic TELs studied by T. Benvengiu et al., which had a stabilizing effect on the liposomal formulation in the presence of bile salts when compared to conventional EPC liposomes. The same is true for mixed vesicles containing GTCE, which had an increased stability in the presence of sodium taurocholate (10 mM) compared to liposomes only containing Chol and EPC (1:1) (Parmentier et al., 2011a). Furthermore, a remarkable stabilizing effect could be observed when comparing mixed vesicles containing bioenhancers and GTCE to conventional liposomes containing bioenhancers (85–90% CF and 40–65% FITC-dextran release) (Parmentier et al., 2011a). This indicates a promising formulation strategy as a combined application of archaeal lipids, increasing liposome stability, and bioenhancers, enhancing the uptake of drug compounds, may effectively increase bioavailability in vivo after oral administration of peptides.

Since both DELs and TELs contain ether bonds instead of ester bonds as in PLs, a stabilizing effect against lipases of both DELs and TELs is expected, as lipases naturally cleave only ester bonds. Generally, all formulations investigated by G.B. Patel et al. showed a good stability in the presence of pancreatic lipase (Patel et al., 2000). Stability differences between the formulations from the different archaea were not as pronounced as in the experiments at low pH and in the presence of bile salts. However, archaeosomes from T. acidophilum still represent the most stable formulation.

As for the formulations investigated by G.B. Patel et al., the mixed vesicles studied by J. Parmentier et al. generally showed a good stability against pancreatin. However, compared to the conventional liposome formulation (EPC:Chol 1:1), no additional stabilizing effect of TELs in the presence of pancreatin could be demonstrated, which may be due to the content of PLs (36%) in the mixed vesicles (Parmentier et al., 2011a).

### 3.4. In vivo investigation of liposomes containing archaeal lipids including archaeosomes and mixed vesicles

Archaeal lipids have been found non-toxic upon administration by both, the intravenous and oral route (Omri et al., 2003). Parmentier et al. (2011b) demonstrated that only a minute fraction of radiolabeled lipid was absorbed after oral administration of archaeosomes.

In vivo studies investigating the performance of liposomal formulations containing archaeal lipids in oral drug delivery were conducted by Z. Li et al. (2010), J. Parmentier et al. (2011b, 2014) and P. Uhl et al. (2016) and will be discussed in this section. Results for the best performing formulations are summarized in Table 2. For the in vivo study investigating the bioavailability of octreotide a 4-fold increase in bioavailability of octreotide could be observed for two mixed vesicle formulations compared to free drug, indicating that the mixed vesicles offer some protection from premature degradation in the GI tract. Also when compared to the performance of conventional liposomes (EPC:Chol 2:1, 2.5 fold increase in octreotide bioavailability), the mixed vesicles containing TELs performed better indicating that TELs may increase the stability of liposomes in the GI tract (Parmentier et al., 2011b). Still, it has to be mentioned that not all mixed vesicle formulations did perform better than the conventional liposome formulation (Parmentier et al., 2011b). This may indicate the importance of the TEL-to-PL-ratio used in the investigated formulation.

In the study investigating the bioavailability of hGH a 337-fold increase in bioavailability could be observed compared to the free drug. Here the experimental animals were pretreated with omeprazole, a proton-pump inhibitor, leading to an increased gastric pH (Parmentier et al., 2014). This may explain the larger increase in bioavailability compared to the study investigating the bioavailability of octreotide because low pH is an important stress factor for liposomal formulations as discussed in Section 3.3. Additionally, cetylpyridinium chloride (CpCl) was used as permeation enhancer (Parmentier et al., 2014), which also is likely to play a role in the large increase in hGH bioavailability. When considering the potentially beneficial effect of the permeation enhancer CpCl and the pretreatment with omeprazole, contradictory results were achieved in a recent study by P. Uhl et al. (2016). Here the performance of mixed vesicles containing GTCE for the oral administration of the investigational drug Myrcludex B was compared to conventional liposomes and free drug. No significant differences between the performances of the formulation only containing GTCE, the GTCE formulation after pretreatment with omeprazole or the formulation containing additionally 1 or 10 mol% CpCl could be observed (Uhl et al., 2016). It has to be mentioned that in the study by Parmentier et al.

### Table 2

<table>
<thead>
<tr>
<th>Drug</th>
<th>Bioavailability of free drug</th>
<th>Bioavailability of liposomal drug</th>
<th>Species</th>
<th>Formulation</th>
<th>Enhancers</th>
<th>Author</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin</td>
<td>–</td>
<td>–</td>
<td>Mice</td>
<td>Archaeosomes from S. acidocaldarius PLFE</td>
<td>–</td>
<td>(C)</td>
</tr>
<tr>
<td>Octreotide</td>
<td>&lt;0.3%</td>
<td>1.23%</td>
<td>Rats (♂)</td>
<td>Mixed liposome (DPPC:GTCE 3:1)</td>
<td>–</td>
<td>(A)</td>
</tr>
<tr>
<td>hGH</td>
<td>0.01%</td>
<td>3.37%</td>
<td>Wistar Rats (♂)</td>
<td>Mixed liposome (DPPC:GTCE:LE 1:2:3:1)</td>
<td>–</td>
<td>(B)</td>
</tr>
<tr>
<td>Myrcludex B</td>
<td>–</td>
<td>–</td>
<td>Wistar Rats (♂)</td>
<td>Mixed liposome (EPC:Chol:GTCE) 10 mol% Chol, 5 mol% GTCE</td>
<td>–</td>
<td>(D)</td>
</tr>
</tbody>
</table>

*AF-1 = A cationic semi-synthetic DGTE analog, (A) J. Parmentier (2011), (B) J. Parmentier (2014), (C) Z. Li (2010), (D) P. Uhl (2016).*
and the pretreatment with omeprazole may be drug compound specific. Clearly, additional research is required to resolve this. Still, as seen in the earlier studies, P. Uhl et al. could show that the performance of mixed vesicles containing 5 or 10 mol% GCTE was significantly better than the performance of conventional liposomes. However, no significant difference between the 5 and 10 mol% GCTE mixed vesicles could be observed (Uhl et al., 2016). Additionally, P. Uhl et al. could show that long-term storage of mixed vesicles containing GCTE is possible which was achieved by freeze-drying (Uhl et al., 2016). For future research and possibly the commercial application of archaeal lipids in pharmaceutical formulations, this may be of great importance.

4. Conclusion

New pharmaceutical formulation strategies for the oral administration of labile compounds are of great relevance, especially as more protein and peptide drugs are reaching the market. Liposomal formulations stabilized with archaeal lipids may potentially be used in this context and hence different studies investigating archaeal lipids especially in the context of pharmaceutical applications with emphasize one the oral route of administration were reviewed.

In vitro studies discussed in this work generally showed that archaeal membrane lipids are able to stabilize liposomal formulations. However, which structural feature(s) of archaeal membrane lipids primarily are the cause for the stabilizing effect needs further investigation. For example, the importance of the head group for the stabilizing effects of archaeal membrane lipids remains unclear, even though the study by T. Benvenegu et al. indicated that the head groups might have an important role (Benvenegu et al., 2005a). Nevertheless, it seems clear that TELs, which arrange in a monolayer, have a significantly larger stabilizing effect than DELs, as indicated by the study by G.B. Patel et al. However, what the actual mechanism behind this observation is needs further investigations for understanding the membrane physical effects involved.

In vivo studies discussed in this work generally showed that liposomal formulations containing archaeal lipids could increase the amount of drug delivered orally compared to oral administration of the free drug. This highly indicates the potential of this formulation for oral administration.

In a study by J. Parmentier et al. (2011) a remarkable stabilizing effect of archaeal membrane lipids could be seen for liposomal formulations containing permeation enhancers, which per se lead to increased instability of liposomal formulations. Permeation enhancers represent an attractive formulation possibility when considering the oral delivery of therapeutic peptides as their application may lead to an increased bioavailability. A combined application of archaeal lipids and permeation enhancers may thus be of particular interest. However, in vivo studies by J. Parmentier et al. (2014) and P. Uhl et al. (2016) showed contradicting results, indicating that further work is needed on this subject.

It appears advisable to combine liposomes with other mechanisms for permeation enhancement to further improve absorption of peptides. Liposomes containing archaeal lipids appear particularly promising in this respect, as they may allow for delivery of protein/peptide drugs and enhancers together within one construct to/near the enterocytes. This may allow for a reduction of the amount of permeation enhancer needed and thus side effects.

Overall it is clear that at the present stage the application of archaeal lipids in the pharmaceutical and/or biotechnological industry would need improvements in both the production and formulation of archaeal lipids. When these will be implemented by future research and development in this area then the application of archaeal lipids can reach its already visible potential.

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References


