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Review Article

ARCHAEOSOMES and tetraether lipid Liposomes

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ABSTRACT

Archaea have phytanyl ether lipids which is one of the characteristics that separates them from bacteria and eukaryotes. Some archaea have also unique membrane spanning tetraether lipids (TEL); in Sulfolobus and Thermoplasma species these TELmake up the majority of total membrane lipids. Archaeal lipids are able to form stable liposomal structures, both from membrane factions, mainly the polar membrane fraction, i.e. archaeosomes, or from highly purified TEL (tetraether lipid liposomes). Liposomes of the main polar lipid (MPL) from thermoacidophilic archaeon Thermoplasma acidophilum were thoroughly inevstigated. Archaeosomes and TEL liposomes exhibit extremely low proton permeability and high stability at low pH, which makes them suitable to delivery of therapeutics and vaccines via the gastro-inetestinal route. Liposomes from egg lecithin can be stabilized by incorporation of 11-12 mole% MPL. MPL liposomes show long-term shelf stability even at high temperatures without conservation. The size of MPL liposomes between 100 nm (unilamellar) and several μm (multilamellar) depends mainly on the method of preparation. Toxicity and mutagenicity have not been detected in toxicological screening. This is a review report on the lipid from archaea to be developed as an alternative to conventional liposome for various industrial applications, especially for pharmaceutical industry.

Keywords:*Tetraether lipid, liposomes, archaeosomes, stability, toxicity, vaccine, drug delivery.*

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Abbreviations

MPL, main polar lipid = main phospholipid = main glycophospholipid; TEL, tetraether lipid; EYL, egg yolk lecithin; TLC, thin layer chromatography; BSA, bovine serum albumin; DTA, differential thermo analysis; DSC, differential scanning calorimetry; CF, 6-carboxyfluorescein; PBS, phosphate buffered saline; OD, optical density; DPPC, dipalmitoylphosphatidylcholine; HPLC, high performance liquid chromatogaphy; MPLC, medium pressure liquid chromatography; SDS, sodium dodecylsulfate.

Archaeal lipids

In 1978, Woeseet al. divided the whole formerly dualistic world into tripartide: eukaryotic and prokaryotic cells were now accompanied by the urkingdom of archaebacteria (Woese, Magrum & Fox, 1978). Soon, they were renamed as archaea (plural of Greek archaeon), when prokaryotes were reclassified. Now, they comprise bacteria (also named eubacteria to separate them from archaebacteria) and archaea. This review will only refer to one criterion that separates archaea from all other cells, apart from genetic differences: glycerol sn-2,3-configurated phytanol ether lipids. Common bilayer-forming phospholipids such as lecithin are glycerol-sn-1,2-configurated, which means that two fatty acids are linked via esters to the hydroxyl groups at C1 and C2 of the glycerol backbone, whereas the phosphoester (or another hydrophilic head group) is bound to C3. In archaeal lipids, instead of fatty acids there are phytanol chains (like in the tail of tocopherols), which are linked as phytanylethers to C2 and C3 of the glycerol backbone, whereas the phoshoester (if present) or sugar residues form the polar head group at C1. The ether bonds in archaeal lipids are much more stable towards hydrolysis than the ester bonds in common lipids, so they can better resist extreme environmental conditions, such as high temperatures at very low pH.

Archaea use isoprenoide derivatives to synthesize the hydrocarbon moieties of their lipids and in archaeal membranes, ther are various types of diether(archaeol) and tetraether (caldarchaeol) lipids. Archaeols form bilayers as common phospholipids do in other prokaryiotes or eukaryotes, whereas in caldarchaeols phytanols are covalently omega-linked forming one or two membrane-spanning biphytanyl chains. In the latter case,the tetraether macrocycle contains two glyceryl residues and two membrane-spanning biphytanyl chains linked via four ether-oxygen bonds, with a total number of 72 atoms participating in the cycle (Langworthy & Pond, 1986; De Rosa & Gambacorta, 1986).These tetraether lipids do not form bilayers but monolayers in similar dimension (thickness) as bilayer membranes (Stern, Freisleben, Janku & Ring,1992).

The phytanyl chains are fully saturated and contain methyl side groups, e.g., 3,7,11,15-tetramethyl-hexadecanol. The latter are considered to exert a fluidizing effect to membranes (Blöcher , Six, Gutermann, Henkel & Ring, 1985) as do the links of double bonds in common membranes. Either structure needs fluidizing rotational space within membrane structures; however, the methyl side groups are not sensitive to oxidation (lipid peroxidation) as double bonds in fatty acids. In

other words, we have a second stabilizing factor in archaeal lipids: besides resistance towards hydrolysis, there is high resistance towards oxidation.

Membrane-spanning tetraether lipids exert even an additional stabilizing factor, which is mechanical: tetraether membranes do not break in the middle of the membrane as bilayers do, e.g., in freeze-fracture electron microscopy or in Langmuir-Blodgett experiments and transfer of lipid layers to wafers in ultrathin surface coating (Vidawati , Sitterberg, Bakowsky & Rothe, 2010). In freeze-fracture electron microscopy tetraether lipid membranes break in direction perpendicular to the plane of the membrane (Sternberg & Rudolph, 1992). As already mentioned above, these structural differences have been used to characterize archaeal cells and to differentiate them from (eu)bacteria and eukaryotes (Langworthy & Pond, 1986; De Rosa & Gambacorta, 1986).

However, pentacyclization within the phytanyl chains needs attention, because it appears to be another membrane-stabilizing factor, since the number of pentacycles increases with growth temperature of the respective archaea (Antonopoulos, 2010; Ernst, 1998; Gliozzi, 1983) and varies with external pH (Shimada, Nemoto, Shida, Oshima & Yamagishi, 2008).

Sulfolobus and Thermoplasma

Tetraether lipids are found in several archaeal species, at highest amounts in Sulfolobales and Thermoplasmatales. Hence, we are especially interested to culture these microorganisms from samples isolated from Tangkuban Perahu (Handayani, 2012). Whereas Sulfolobales have a cell wall, Thermoplasmatales do not, that means, their lipid membrane is naked and easily accessible. Therefore, we focus on Thermoplasma species.

Thermoplasma acidophilum is a thermoacidophilic archaeon which had first been isolated by Darland, et al. (1970) from a steamingcoal refuse pile, self-heated to 56°C (Darland , Brock, Samsonoff & Conti, 1970). Oxydative degradation of pyrrite-containing material generated sulfuric acid and an environmental milieu of pH 1-2. Segereret al. (1988) found further species within the genus Thermoplasma in their natural environments, such as volcanoes, hot springs, solfataric soils and mudholes in Italy, Iceland, the Yellowstone Park, on Dieng Plateau and TangkubanPerahu and named them Thermoplasma volcanium (Segerer, Langworthy & Stetter, 1988). All species grow anaerobically and (micro-)aerobically, show pleomorphism and sometimes flagellation. In KawahDomas on TangkubanPerahu, Sulfolobus and Thermoplasma species live autotrophically on sulfur (Handayani, 2012). In the laboratory, heterotrophical-growth optimum of Th. acidophilum is at pH 2 and 59°C in a sulfuric acid-containing Freundt's medium (Freisleben, et al, 1994)

Liposomes and archaeosomes

Conventional liposomes have been widely used since Bangham, et al (1965) had reported on phospholipid lamellae in 1965 and technology developed during the following decades. However, expectations into liposome technology have not been reached, in general, although several preparations have been very suc-

cessful, e.g., liposomal amphotericin B and liposomal doxorubicin. Liposomes of conventional phosphoester lipids are not very stable towards hydrolysis of the esters and oxidation of unsaturated double bonds within the fatty acid chains, which provide fluidity to biological lipid membranes and also to the membranes of conventional liposomes.

Lipid extracts from archaeal membranes yield more stable membranes in liposomes. Archaeosomes use fractions of total membrane extracts from archaeal membranes, mainly the polar lipid fraction (Patel, et al, 2000). Krishnan andSprottdefined archaeosomesas 'liposomes comprised of glycerolipids of archaea'(Krishnan & Sprott, 2008). Such archaeosomal delivery systems are applied for oral vaccines, since bacterial or archaeal membrane components appear to exert potent adjuvant properties in oral vaccination (Krishnan & Sprott, 2008; Li Z, L Zhang, W Sun, Q Ding, Y Hou & Y Xu, 2011). Further applications seem to be possible: González-Paredes et al.incorporated archaeosomes into hydrogels with the aim to apply these 'archaeosomal hydrogels' as novel delivery system for antioxidants (González-Paredes, et al, 2011)

Our own working group made attempts to purify the main polar lipid (MPL), which is the main glycophospholipid of Thermoplasma acidophilum (Sternberg & Rudolph, 1992). MPL is a membrane-spanning tetraether lipid(TEL) which is able to form stable liposomes alone and in combination with other lipid components (Freisleben, 2000).

Preparation of liposomes and archaeosomes

Irrespective of the preparation method the polar lipid fractionor purified tetraether lipidis dissolved in organic solvent, which is subsequently removed by evaporation in arotavapor and dried. For liposome preparation the dry lipid film is suspended in a suitable buffer.

Archaeal lipids can be mixed with egg yolk or soy phosphoesterlipids to form more stable mixed liposomes. For the preparation of mixed liposomes, varying amounts of MPL were admixed to egg lecithin.Archaeosomes, tetraether liposomes and mixed liposomes can be prepared with the classical methods of liposome technology as compiled in (New RRC, 1990). After preparation of the liposomes non-liposomal material can be removed by centrifugatrion.

. Hand -shaken liposomes or archaeosomes

A suitable buffer, normally PBS is added to the dry lipid film and shaken by hand to disperse the lipid film until a homogenous liposome suspension with alipid concentration of 20 mg/mL is formed (Bangham, Standish & Watkins, 1965).

. Sonication

Hand-shaken liposomes or archaeosomesare sonicatedwith a Branson Sonifier at 20 kHz for 5 min on ice (Finer, Flock & Hauser, 1973) or more gently in a sonication bath.

. Detergent solubilization and detergent dialysis

Lipids are dissolved in PBS contain-

ing the detergent octyl-ß-D-thioglucopyranoside. The resultingmicellar suspension is transferred to a Lipoprep® dialysis cell (Diachema AG, Langnau, Switzerland) and dialysed at RT for 24 h (Weder & Zumbühl, 1984). The lipid concentration should be 10-20 mg/mL. Mixed liposomes of archaealdiether and tetraether lipids and soy or egg lecithin can be prepared in the same way as TEL liposomes or archaeosomes.

. *Extrusion through polycarbonate filters*

Hand-shaken TEL liposomes, mixed liposomes or archaeosomes can be freeze-thawed for three times, sonicated, or directly applied and extruded through 800 or 500 nmpolycarbonate filters and finally through 200 or 100 nm polycarbon filters by means of a LiposoFast™Extruder (Avestin Inc., Ottawa, Canada).

. *French pressure cell*

Hand-shaken liposomes or archaeosomes withlipid concentration of 10-20 mg/mLcan be transferred to a French pressure cell and extruded withfour cycles at a pressure of 16000 p.s.i. (Barenholtz, Amselem & Lichtenberg, 1979).

Size distribution and zeta potential of archaeal liposomes

The size distribution of tetraether liposomesand archaeosomes generally depends on the method of preparation handshaken, sonicated, detergent dialysed, polycarbon filter-extruded, and French pressure cell-extruded. Hand-shaken liposomes and archaeosomes are in the range of several µm with a very broad size distribution. They are multilamellar and polymorph, whereas smaller liposomes tend to become unilammellar und round. Sonicated-tetraether liposomes are in the range of 450 to 600 nm, whereas detergent dialysed liposomes (C) are 370 to 450 nm. Extrusion through polycarbonate filters (D) yields a similar size (100-200 nm) as the French pressure cell (E). The latter preparation method exerts very uniform

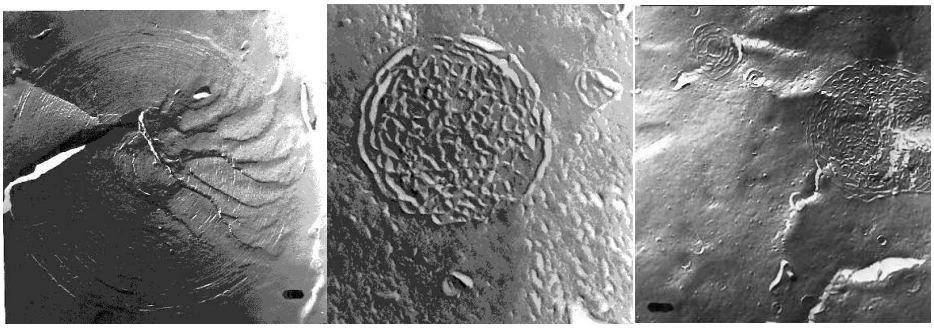

Figure 1. Freeze-fracture electron microscopy of tetraether lipid (TEL) structures, magnification x 54.000, black bars = 100 nm;
(A) giant multilamellar structure; (B, C) stepwise disruption in a French pressure cell; some uni- and bi-lamellar liposomes are already visible in B and C. (Sternberg & Rudolph, 1992; Sternberg, Rudolph & Freisleben, 1992).

liposomes with narrow size distribution (e.g., 150 ± 22,1 nm). Using the various methods, the whole spectrum of tetraether liposomes from a minimum size of 80 nm to 600 nm can be prepared with good reproducibility. Larger archaeosomes and liposomes (hand-shaken) are not uniform and vary over a wide range in size, shape and lamellarity.

Besides the method of preparation, the grade of purity of the lipid is important. Extended studies of differing purification grades of MPL on size and size distribution of the liposomes were accomplished. With the five methods investigated, the particle size decreased in parallel with higher purity of tetraether lipid, i.e. primarily, but not only, with removal of pigments. Hence, efforts were made in our laboratory to achieve a high level of tetraether lipid purity using the purification procedure of (Antonopoulos, et al, 2012), which yields more than 95% purity. The zeta potential of MPL liposomes was determined in a Malvern Zeta Sizer to be -58.2 ± 0.2 mV at pH 7.4 in diluted PBS.

Mixed liposomes of tetraether lipid and egg lecithin

The main glycophospho-tetraether lipid (MPL) is able to form mixed phases with bilayer forming lipids (DPPC, egg lecithin) as demonstrated by DTA measurements (Blöcher, Gutermann, Henkel & Ring, 1985) and forms mixed liposomes with soy and egg lecithin. Homogenicity of the preparations was evaluated from the uniform or inhomogenous size distribution (one or more peaks in the laser particle sizer). Due to the method of preparation, stable mixed liposomes between 143.5 ± 41 nm and 372 ± 35 nm were formed. Size distribution of mixed liposomes showed homogenicity at MPL/egg lecithin 25:75and 50:50 and shelf stability was essentially increased compared to pure egg lecithin liposomes.

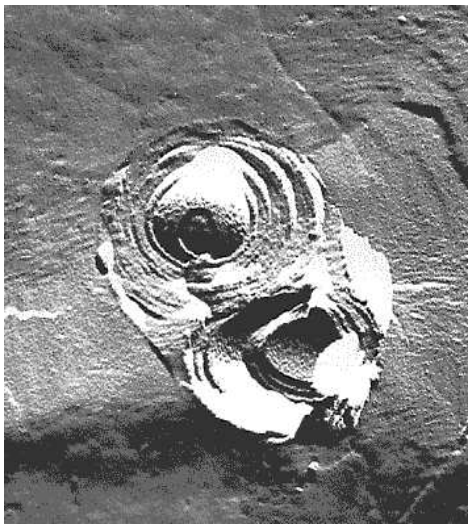

Figure 2. Multilamellar structures of mixed liposomes containing 25% (10-11 mol%) TEL and 75% egg yolk lecithin; magnification x 67500.

Long-term shelf stability and swelling

Tetraether liposomes were stable for 109 weeks when stored in PBS (10 mg lipid/mL) at 37°C, RT, and at 4-8°C, as measured by changes in size distribution (swelling). For comparison, liposomes from egg lecithin were stable only for one to four weeks in the refridgerator (4-8°C), at RT and 37°C only for a few days. At high temperatures (60, 70, 80, 90, and 100°C) TEL liposomes were stable for 10 weeks, but not at 120°C.

Mixed liposomes MPL/egg lecithin 25:75 and 50:50 (w/w) were stable in the refridgerator up to 22 months. Samples with 75:25 (w/w) mixtures separated during storage into two peaks corresponding to the size of pure egg lecithin and TEL liposomes. In general, admixing tetraether lipid to egg lecithin increased the shelf stability of the liposomes, especially at the mixture 25:75 (w/w) which correlates to 11-12 mol% of MPL.

Influence of protons, temperature, alcohols and detergents

Swelling of TEL liposomes by addition of polyols (glycerol, xylitol, mannitol etc.) was less than 5% at RT. The liposomes also resisted several-fold higher detergent and alcohol concentrations than DPPCand egg lecithin liposomes (Freisleben, 2000; Ring, Henkel, Valenteijn & Gutermann, 1986). Proton permeability of liposomal TEL membranes is about two magnitudes lower than in EYL liposomes (Elferink, et al, 1994; Freisleben, 1995). Even at 74°C, the rate constant of proton permeability per second was only 50% of that in EYL liposomes at RT (Freisleben, 1995).

Influence of high concentrations of bile salts on the size of liposomes

Mixtures of bile salts, as they may occur in the small intestine were incubated at total concentrations of 10 and 30 mM. Size distribution of TEL liposomes was not strongly influenced and was stable under varying concentrations (133.1; 109.3; 118.3 nm), whereas egg lecithin liposomes underwent tremendous size variations (140.6 nm; 38.4 nm; 1302.6 nm) under increasing bile salt concentrations (0, 10, 30 mM).

MPL liposomes as vehicles or carriers

Incorporation of compounds into liposomes or archaeosomesfor drug or vaccine transport and delivery depends on the physicochemical properties of both the compound and the lipid. The most effective procedure is the incorporation of sufficiently lipophilic compounds into thearchaeosomal or liposomal membrane, which can be achieved in one step with the preparation of the liposomes, provided the respective compound does not disturb the formation of liposomes, which may be the case if this compound tends to form stable micellar structures by itself or together with the lipid. If so, application of the method using detergents and subsequent dialysis (Weder & Zumbühl, 1984) may be advantageous to solve the problems.

Anyways, if the amounts of lipophilic compound which should be incorporated is not too high, and suitable method for the preparation of liposomes or archaeosomes is applied, the compound will be taken up into the liposomal membrane by approximately 100%. Most success-

ful liposomal drug delivery systems with common phosholipids are of this kind and good results were also achieved with the experimental incorporation of lipid soluble compounds like nile red, thiolytebromobimane and spin labeled retinoic acid into liposomes of archaeal lipids (Michel, 1992).

Interaction of tetraether lipid liposomes with cell membranes and cells

In the above-mentioned cases, delivery, i.e. release of lipophilic compounds to target cells was demonstrated by intermembrane exchange from MPL liposomes to erythrocyte ghosts and membranes of intact cells. Intermembrane exchange does not need the uptake of liposomes into cells, because lipophilic compounds are able to migrate from the liposomal membrane and penetrate into the cell membrane, if the liposomes touch the cell surface or are attached to it (Freisleben, 2000). Many pharmacologically relevant compounds are sufficiently lipophilic to follow this pattern of liposomal incorporation and release.

The problem is quite different for incorporation of water soluble compounds. They are dissolved in the buffer and are enclosed in the liposomes, when they are formed. Since the inner volume of the liposomal lumen is roughly 1% of the outer volume (10-20 mg lipid per ml), this will also be the rate of compound that will be found in the liposomes, about 99% will not be entrapped. Some amount of the compound may be attached to the outer surface of the liposomes. There are possibilities to increase the entrapped amount by osmosis or temporary pores (e.g., with bile salts), but none of these modifications can alter the result in general: the great majority will remain in the outer buffer. This has to be removed via a column and should be regained for economic and other reasons. The liposomal suspension contains the entrapped amount of compound.

Release of water soluble compounds from liposomes may follow different patterns: there is a certain amount of 'contact release', as soon as liposomes touch cell membranes or get attached to them. A second mechanism is membrane fusion: the liposomal membrane fuses with the cell membrane with rapid release of the liposomal load into the interior of the cell; the third mechanism appears to occur more often: liposomes are actively taken up into the cell by endocytosis and the liposomal load will be released into the cytoplasm more slowly.

These are mechanisms and limitations of liposome technology, the application of archaeal lipids to form more stable liposomes or archaeosomesalso follow these principles, in general.

Release of 6-carboxyfluorescein (CF) and 125I-insulin

As a first approach, incorporation and release of CF was studied withsonicated MPL liposomes and compared to liposomes made of DPPC and EYL. Incorporation and release of 125I-insulin was studied with hand-shaken liposomes of MPL and egg lecithin. Release was studied in a gastro-intestinal simulation apparatus, up to 7 days with variation of temperature, pH, electrolytes and osmolarity.

The idea behind these experiments was the oral application of insulin in a gastro-

intestinal delivery system of acid-stable liposomes. Although pH-dependent release experiments were promising with archaeal liposomes(at alkaline pH, release was higher than at acidic pH), incorporation rates were low, only 4% of the applied insulin was entrapped.

The in vitro intestinal absorption model used in these studies was composed of two chambers separated with excised porcine intestinal mucosa. The buffer pH at the intestinal lumen side varied from acidic to alkaline. Uptake was measured in the opposite 'blood side' chamber as a model for absorption through the mucosa.

Gene transfer and vaccines

For delivery of special load, such as DNA or vaccines, special techniques have been applied, including admixture of positively charged lipids to the negatively charged MPL, or chemical modification of MPL itself by introduction of positive head groups.

Similar questions occur for special targeting with introduction of structures, which can dock to specific receptors on cell membranes or in the circulating blood. Membrane-spanning MPL can well serve as an anchor to fix linked structures at the liposomes.

Sterilization of liposomes

Although TEL liposomes are heat stable and can be autoclaved or even heat sterilized (Choquet, Patel & Sprott, 1996), we prefer to use sterile filtration, especially, since this can be done in one run with the extrusion method for liposome preparation, if polycarbonate filters with 100 or 200 nm pores are used.

Penetration into the skin

Penetration of spin-labeled retinoic acid into the skin of nude mice was investigated from liposomal and non-liposomal pharmaceutical preparations. The penetration of the spin-label-loaded liposomes could be followed by electron paramagnetic resonance (EPR) imaging (Michel, 1992). Liposomal systems appear to have higher penetration rates than conventional lipogels or hydrogels; liposomal systems from archeal TEL were more stable than systems from common phosholipids but did not provide essential advantages over common liposomes in terms of penetration into the skin.

Cytotoxicity, mutagenicity, toxicity and distribution in mice

The influence of MPL liposomes on wide range of cell cultures was investigated in cytotoxicity screening including tests on mutagenicity and antimutagenic efficacy. None of the testing showed cytotoxic or mutagenic properties of MPL or MPL liposomes [33].

No toxicity was detected in mice in central nervous screening and the survival rates. The MPL fed mice lived insignificantly longer than the controls (Freisleben, et al, 1993).Distribution screening showed, that TEL liposomes (100-200 nm) - as other negatively charged liposomes of the same size – are rapidly cleared from the circulating blood and appear mainly in the liver and in the spleen within 30 min (Freisleben, et al, 1995).

Degradation of tertaether lipids

Biodegradation of tetraether lipids has not been clarified, so far. Uptake into hepatocytes has been observed; however, degradation products could not be identified.

CONCLUSION

The main tetraether lipid (MPL) from Th. acidophilum forms extremely stable liposomes down to a size of 100 nm. This is the first message of this review. Liposome technology with archaeal lipids does not differ in principle from the technology with common lipids. Archaeal tetraether lipid can be used to stabilize liposomes of common lipids. The stability of MPL liposomes and archaeosomes towards acidity and the low permeability of their membranes for protons provide wide possibilities for gastro-intestinal applications, in the first place for oral vaccination, especially since bacterial and archaeal membrane lipids seem to exert adjuvant properties in the GI mucosa.

The question of liposomes from highly purified archaeal tetraether lipid or archaeosomes from polar membrane fractions should be addressed carefully. Lipid purification in general is very time-consuming and cost-intensive, even if relatively inexpensive lipid sources are used (soy, egg yolk). For certain applications, highly purified lipid components are necessary. However, for pharmaceutical application in oral drug delivery or vaccination, use of membrane fractions may be advantageous. This does not mean that membrane fractions or not purified. They should not contain membrane-associated pigments and lipopolysaccharide complexes, which may have pro-inflammatory effects.The highly purified main glycophospholipid 98% pure MPL (Antonopoulos et al., 2013) does not contain any such components, but the entire procedure from cultivation of Thermoplasma, extraction, fractionation and purification is not an economic way to produce a drug delivery system with a chance for marketing. It is a scientific procedure for scientific application and stereoselsctive total synthesis of tetraether lipids appears still difficult, too. Purified tetraether lipid may be useful to stabilize common liposomes in the way described above for mixed liposomes or to attach surface components. Membrane-spanning tetraether lipid is an ideal anchor to fix such componentsin order to 'spike' the liposomal surface.

The great advantage of higher stability of archaeosomes and tetraether lipid liposomes may be especially useful to applications on the skin and in the gastro-intestinal tract, i.e., for oral administration of acid-sensitive drugs, DNA, peptides and proteins and vaccines. However, in these applications archaeosomes from archaeal membrane fractions, which must of course be purified from pigments and lipopolysaccharide complexes and from any other possibly toxic compounds, will be more cost-effective than highly purified single tetraether lipids. Most stable archaeosomes will be achieved with membrane fractions from archaea which have high amounts of tetraether lipid in their membranes, i.e., Sulfolobus and Thermoplasma species.

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