A lipid nanovesicle system encasing bacteriophages for inhalational therapy

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Abstract

Inflammatory diseases that occur in the pharynx, involving both adenoids and tonsils, are important not only for being very frequent but also because they often require minor surgery for their resolution. These anatomophysiological structures have immunological roles leading to production of antibodies, working in the local immunity of the pharynx and protection of the en- tire human body. The most common etiologic agent of sore throats is *Streptococcus pyogenes*, an important pathogen of the beta-hemolytic group A which causes streptococcal pharyngitis. The emergence of antibiotic-resistant bacterial strains and the poor pen- etration of conventional chemical antibiotics in bacterial biofilms raise the need for safe and effective options of antimicrobial treatment. The application of bacteriophages (or cocktails therefrom) has been proposed as an alternative (or complement) to conventional chemical antibiotics, allowing the release of natural predators of bacteria directly on these biofilms. The major advantage of bacteriophage- based antibiotherapy relative to its conventional chemical counterpart is that bacteriophages replicate at the site of infection, being available in abundance where they are needed the most. When compared with chemical antibiotics, bacteriophages have other important advantages: (i) strong tissue permeability, (ii) bacteriophage concentration remains high at the focus of infection, continuously increasing with bacterial (host) presence, (iii) elimination of the focus of infection occurs only after eradication of the host bacterium, (iv) bacteriophages are fully compatible with antibiotics and may act synergistically, (v) they are specific against the target bacteria, (vi) have a superior ability to penetrate bacterial biofilms, inducing production of enzymes that hydrolyze the biofilm polymeric matrix, (vii) although bacteria can develop resistance to bacteriophages, isolation of new lytic bacteriophages is much simpler and cheaper than developing a new chemical antibiotic. In this research effort, development of a biotechnological process for the inhalational administration of a bacteriophage cocktail (endotoxin free) was pursued, using strategies of nanoencapsulation within lipid nanovesicles (as forms of protection and stabilization of the bacteriophage against the immune system) to treat infectious pathologies such as pharyngo-tonsillitis. This method of targeting may have a high potential for the treatment of bacterial infections of the respiratory tract, since inhalation therapy is considered to be favorable to certain respiratory infections because the aerosol is delivered directly at the site of infection, accelerating the action of bacterial predators. Additionally, a smaller amount of bioactive substance is needed, thus preventing or reducing possible deleterious side effects. As a *proof- of-concept* for the nanoencapsulation strategy, and since there is not yet available a strictly lytic bacteriophage (or cocktail of lytic bacteriophages) for *Streptococcus pyogenes*, a well-defined and characterized bacteriophage was utilized, viz. bacteriophage T4. In this context, water-in-oil-in-water (W/O/W) multiple emulsions are nanosystems in which dispersions of small water droplets within larger oil droplets are themselves dispersed in a continuous aqueous phase. Due to their compartimentalized internal structure, multiple emulsions present important advantages over simple O/W emulsions for encapsulation of bio- molecules, such as the ability to carry both polar and non- polar molecules, and a better control over releasing of therapeutic molecules. Bacteriophage T4 was entrapped within lipid nanovesicles integrating W/O/W multiple nanoemulsions, aiming at mimicking the multifunctional design of biology, optimized with several lipid matrices, poloxamers and stabilizing layer compositions. Physico-chemical characterization of the optimized bacteriophage-encasing nanovesicle formulations encompassed determination of particle size, size distribution and particle charge, via Zeta potential analysis, surface morphology via Cryo-SEM, and thermal analysis via DSC, whereas antimicrobial activity of the nanoemulsions produced were evaluated via the "spot-test" using appropriate bacterial cultures.

Experimental procedures

PREPARATION OF MULTIPLE BACTERIOPHAGE T4-ENCASING LIPID NANOEMULSIONS.
Production of multiple emulsions encompassing lipid nanovesicles with encased T4-bacteriophages was carried out using an Ultra Turrax (model T25D from IKA) under heating (ca 40ºC). T4-bacteriophages were suspended in the (inner) aqueous phase (W_{in}) and then dispersed in the melted oil phase, via high-speed homogenization (10 min at 9000 rpm). The resulting W/O emulsion was further dispersed in the outer aqueous phase, via another homogenization cycle. The inner aqueous phase encompassed HCl 10 mM (from Vaz Pereira), Tween 80 (from Sigma-Aldrich) and impure lyophilized T4-bacteriophages (5 mg); the intermediate oily phase encompassed glycerol (from Merck, Darmstadt, Germany), Softisan™ 100 (from Sasol Olefins & Surfactants GmbH, Hamburg, Germany) and soybean phosphatidylcholine (from Acofarma, Spain); finally, the outer aqueous phase encompassed Lutrol™ F68 (poloxamer 188 from BASF, Germany) and ultrapure water.

Figure 1. Experimental setup utilized for the preparation of lipid nanovesicles encasing T4-bacteriophages.

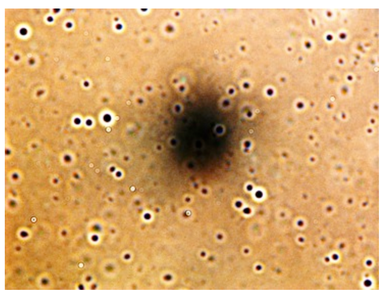

Figure 2. Physical appearance of the lipid nanovesicles (Water-in-Oil-in-Water multiple nanoemulsion), as observed under an optical microscope at maximum resolution (x100).

OPTIMIZATION OF THE FORMULATION PARAMETERS.
Optimization of the multiple lipid nanoemulsion proceeded via preparation of different emulsions with different Tween 80 concentrations (50 to 75 mg) and different stabilizing layer compositions (see Table 1).

DETERMINATION OF HYDRODYNAMIC SIZE (HS) AND ZETA POTENTIAL (ZP).
Determination of the HS of the lipid nanovesicles produced, of the polydispersion index and of their ZP were carried out in a Zetasizer (model Nanoseries Nano-ZS) from Malvern Instruments.

THERMAL ANALYSIS BY DIFFERENTIAL SCANNING CALORIMETRY (DSC).
Calorimetric analyses of the nanoemulsions produced were performed in a differential scanning calorimeter (Shimadzu, Kyoto, Japan), which comprised a detector (DSC-50) and a thermal analyzer (TA-501). The samples were heated from room temperature to 100 ºC at a constant linear rate of 5 ºC/min, during which the amount of heat absorbed by the samples was recorded.

EVALUATION OF ANTIMICROBIAL ACTIVITY BY THE "SPOT" METHOD.
Optimized nanoemulsions were assessed for antimicrobial (lytic) activity, following a simple laboratory procedure. Whole nanoemulsions were submitted to the "spot" test as follows; 1. 100 µL of bacterial suspension (*Escherichia coli*) grown overnight at 37 °C were added to 3 mL of top-agar; 2. Following a gentle homogenization, the top agar added with bacterial suspension was poured into a 90 mm Petri dish previously prepared with 10 mL bottom-agar and allowed to dry; 3. A 5-µL drop of the whole lipid nanoemulsion was then applied and allowed to dry; 4. Incubation of the Petri dish was then allowed at 37 °C, overnight.

Experimental results and discussion

ANTIMICROBIAL ACTIVITY DETERMINATIONS

The preliminary results obtained for the antimicrobial (lytic) properties of the optimized nanoemulsion encasing bacteriophage-T4 are displayed in Figure 5 (see inserted arrow), clearly showing the inhibition halo produced by the whole nanoemulsion.

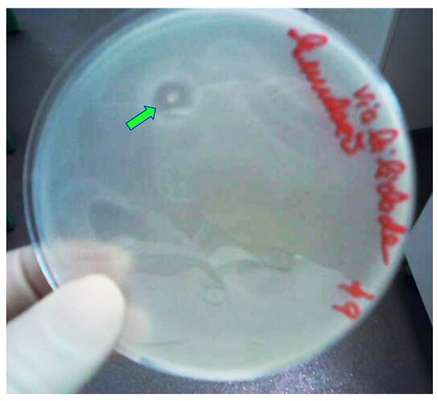

Figure 5. Petri dish after performance of the "spot" test to the nanoemulsion encasing bacteriophage T4.

OPTIMIZATION OF THE NANOFORMULATION

Several variables were studied, viz. lipid nature, poloxamer nature, soy lecithin concentration and tween 80 concentration.

Table 1. Optimization of processing conditions leading to an optimal nanoformulation encasing T4-bacteriophage.

Lipid nanoformulation parameters		Designed starting conditions	With +25% Softisan	With +25% Lecitina	With +25% Lutrol	With +25% Tween	With +25% of Tween and +25% Lecitina
Homogenization speed (rpm)		9000	9000	9000	9000	9000	9000
Temperature(ºC)		40	40	40	40	40	40
Internal	T4 Phage (mg)	10.14	10.14	10.14	10.14	10.06	10.06
aqueous	HCL 0,010 M (ml)	1	1	1	1	1	1
phase	Tween 80 (mg)	59.4	59.4	59.4	59.4	63.3	63.3
	Softisan 100 (mg)	501.7	639.9	504.5	511.9	497.1	503
Oil phase	Soybean Lecithin (mg)	49.9	50.6	63.6	50.2	50.6	63.4
	Glycerol (ml)	5	5	5	5	5	5
External	Lutrol F68 (mg)	403.18	403.18	403.18	500.6	403.18	403.18
aqueous phase	Ultrapure H2O (ml)	40	40	40	40	40	40

Replacement of the poloxamer by Lutrol F-127 led to a substantial decrease (from more negative towards less negative values) in the negativity of the Zeta Potential of the lipid nanovesicles.

Increasing Tween 80 concentration, up to 40% of the departing concentration, led to more negative Zeta Potential values, but the lipid nanovesicles seemed to be unstable over storage time, with notorious disaggregation (see Figure 3).

The effect of simultaneously increasing the amounts of Tween 80 and lecithin were implicit in the high increase of Zeta Potential (from more negative towards less negative values), presumably due to accumulation of adsorbed ions at the particle surfaces. A lower concentration of Tween 80 proved to be suitable in producing lipid nanovesicles with stabler Zeta Potential and higher hydrodynamic sizes, throughout storage time.

HYDRODYNAMIC SIZE AND ZETA POTENTIAL DETERMINATIONS

Both the Hydrodynamic size and Zeta Potential of the several nanoemulsions produced were evaluated and followed throughout a prolonged storage at room temperature. The results obtained are displayed in Figures 3 and 4.

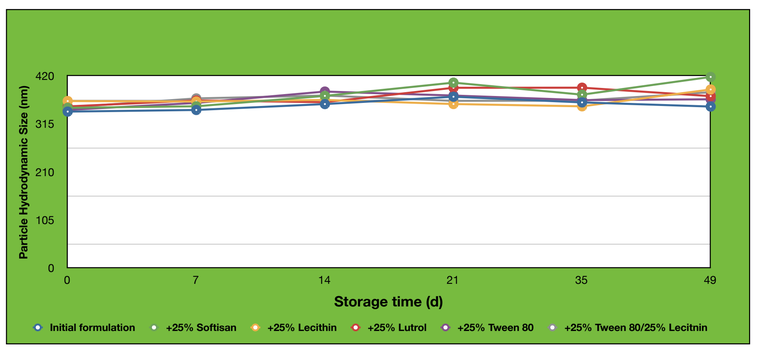

Figure 3. Changes in particle Hydrodynamic Size throughout storage time.

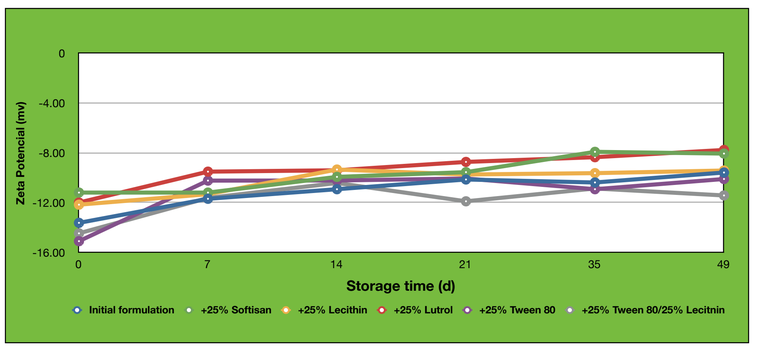

Figure 4. Changes in Zeta Potential values throughout storage time.

Since we aimed at entrapping a bioactive lytic phage within the lipid nanovesicles, a lipid was chosen so as to melt down at a lower temperature, viz. Softisan 100™.

MICROCALORIMETRIC ANALYSIS OF THE NANOFORMULATIONS

We have made two T4-bacteriophages suspensions one with and one without homgenization and we evaluated the changes in Intensity throughout Particle Hydrodynamic Size(see Figure 3 and 4). It was found in both case the existence of particles with smaller size (28,2-78,8nm) and greater (255-396nm) than the T4-phage size (200nm).The weighted average was 164.5 and 219.3, respectively, of phage suspension with and without homogenization. The smaller size, is derived from the decay of phages and cellular debris from bacterial lysis during production and purification of phage.The bigger size, is due to the inclusion of several phages in same nanoparticles or from the phage particle fragmented.It should be noted that homogenization causes the larger sizes of the two curves becomes smaller, which gives an idea of disaggregating the various impurity of phage particles.

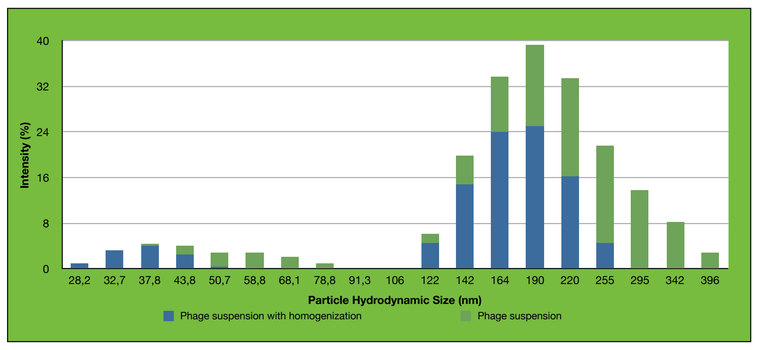

Figure 8. Changes in Intensity throughout Particle Hydrodynamic Size.

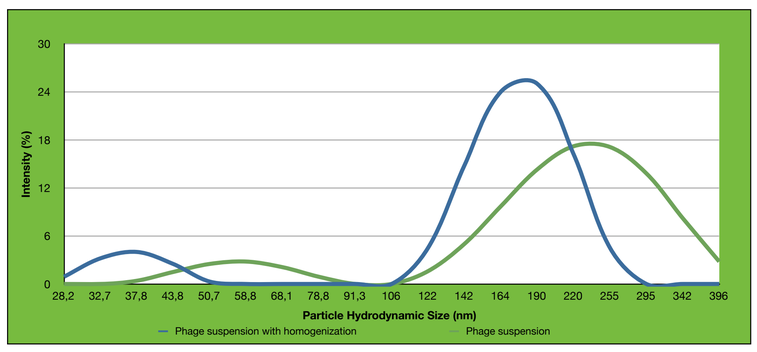

Figure 9. Changes in Intensity throughout Particle Hydrodynamic Size.

ELECTRON SCANNING MICROSCOPY ANALYSES

Lipid nanoemulsions were analyzed for microstructural and morphological characteristics via Cryo-SEM. Briefly, nanoemulsion samples were prepared for analysis as follows: (i) samples were mounted in an appropriate (aluminum) support inserted into a gold-coated plate; (ii) the gold-coated plate was then duly fixed in a transfer stick; (iii) the support containing the sample was immersed in liquid nitrogen (slush nitrogen); (iv) the sample was then transferred under vacuum into the SEM preparation chamber (also under vacuum and maintained cold via addition of liquid nitrogen); (v) inside the chamber, the deep-frozen sample was fractured, undergone sublimation (during 90 s to 300 s) by gently increasing the temperature from -140 °C to ca. -90 °C, and was coated with Au/Pd; (vi) finally, the sample was transferred into the SEM chamber for microscopy analysis.

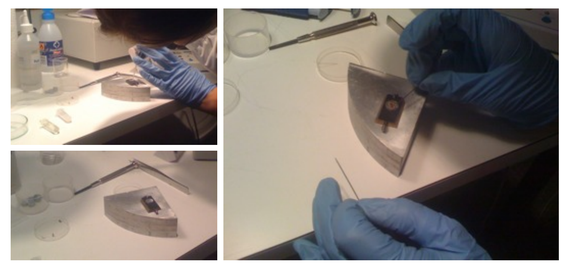

Figure 6. Preparation of nanoemulsion samples for cryo-SEM analysis.

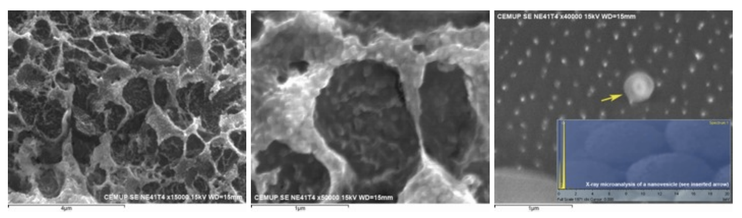

Figure 7. Cryo-electron scanning microphotographs of bacteriophage T4-encasing nanoemulsions.

Conclusions

In this research effort, development and optimization of lipid nanovesicles encasing bacteriophage-T4 was pursued. A lipid with a mild melting temperature, encompassing medium-to-long chain fatty acid moieties was found most appropriate for the discontinuous oily phase. A homogenization timeframe of 10 min, the use of a low concentration of Tween 80, and low bacteriophage concentrations were found to be critical processing variables for producing stable nanovesicle dispersions with diameters ranging from 350-417 nm and Zeta Potential values of ca. -15 mV. Inclusion of these multiple nanoemulsions in isotonic formulations for inhalational therapy of pharyngo-tonsillitis would possess inherent advantages, when compared with the current chemical antimicrobial approach, if bacteriophage-T4 were to be replaced by a lytic phage specific for *Streptococcus pyogenes*, in that bacteriophages are naturally harmless entities with bacteriostatic activity, without any toxicological risk for humans.