Design of a lipid nanovesicle system encasing bacteriophages for inhalational therapy: A proof of concept.

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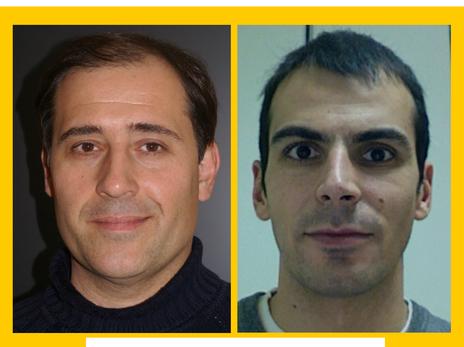

Abstract

Inflammatory diseases that occur in the pharynx and involving both the adenoids and tonsils are important not only for being very frequent, but also because they often require minor surgery for their resolution. These structures have immunological functions leading to production of antibodies, and work in the local immunity of the pharynx and protection of the entire 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 penetration of 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 for the bacteriophage against the immune system) to treat infectious pathologies such as pharyngo-tonsillitis caused by Streptococcus pyogenes. 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 side effects. As a proof of concept for the nanoencapsulation strategy, and since there is not yet available a strictly lytic bacteriophage cocktail for Streptococcus pyogenes, a well-defined and characterized bacteriophage was utilized, viz. bacteriophage T4. 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 biomolecules, such as the ability to carry both polar and non-polar molecules, and a better control over releasing of therapeutic molecules. T4 bacteriophage was entrapped within W/O/W multiple nanoemulsions, aiming at mimicking the multifunctional design of biology, optimized with several lipid matrices, poloxamers and stabilizing layer compositions. Physicochemical 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 10000 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.

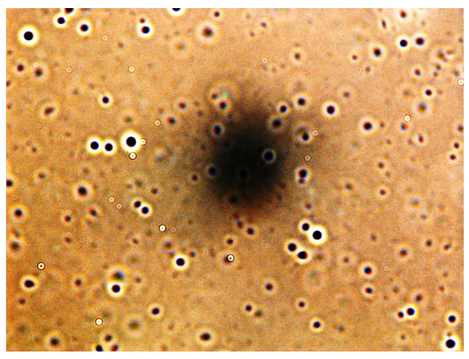

Figure 1. Physical appearance of the lipid nanovesicles (Water-in-Oil-in-Water multiple nanoemulsion), as observed under an optical microscope at maximum resolution (Oil immersion, x1000).

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

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 Lutrol F-127 (replacing Lutrol F-68)	With +50% of Tween and +25% of Lecithin	With +25% of Tween	With +40% of Tween	With +50% of Tween	Best formulation achieved: Initial conditions
Homogenization speed (rpm)		10000	10000	10000	10000	10000	10000	
Temperature (°C)		40	40	40	40	40	40	
Internal aqueous phase	T4 Phage (mg)	1.03	1.03	1.08	1.06	1.14	1.08	
	HCl 0,010 M (mL)	1	1	1	1	1	1	
	Tween 80 (mg)	56.6	56.6	75	67.2	71	75	
Oil phase	Softisan 100 (mg)	510.4	494.9	498.7	511.6	512.5	533.1	
	Soybean Lecithin (mg)	50.5	52.1	66.4	50.3	50.6	50.8	
	Glycerol (mL)	5	5	5	5	5	5	
External aqueous phase	Lutrol F-68 (mg)	406.6	403.9	506.9	502.9	401	506.9	
	Ultrapure H_2O (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 2).

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 2 and 3.

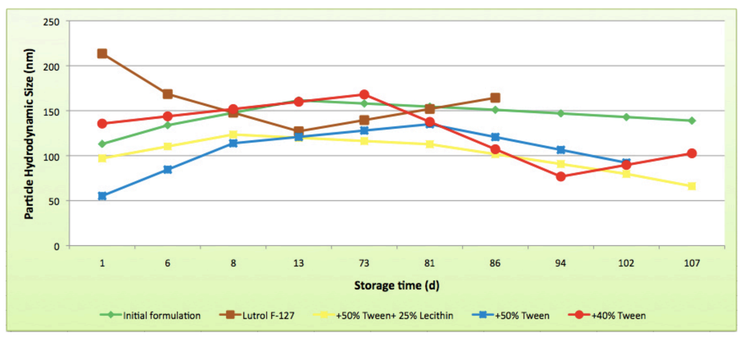

Figure 2. Changes in particle Hydrodynamic Size throughout storage time.

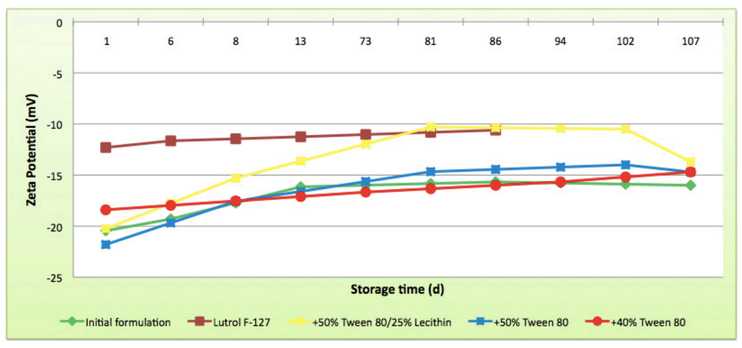

Figure 3. Changes in Zeta Potential values throughout storage time.

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™.

Antimicrobial activity determinations

The preliminary results obtained for the antimicrobial (lytic) properties of the optimized nanoemulsion encasing bacteriophage-T4 are displayed in Figure 4 (see inserted arrow), clearly showing the inhibition halo produced by the whole nanoemulsion.

Figure 4. Petri dish after performance of the "spot" test to the nanoemulsion encasing bacteriophage T4.

Cryo Scanning Electron 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 (during 20s); (vi) finally, the sample was transferred into the SEM chamber for microscopy analysis.

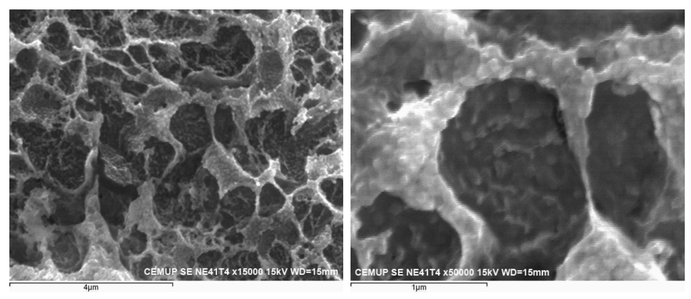

Figure 5. Cryo-scanning electron microphotographs of bacteriophage T4-encasing nanoemulsions.

Microcalorimetric analysis of the nanoformulations

Calorimetric analysis was performed in a differential scanning calorimeter (Shimadzu, Kyoto, Japan). For every calorimetric assay, ca. 10 mg of emulsion were weighed (using a microsyringe) directly into the interior of high-pressure aluminum pans (Shimadzu, P/N 557 201-53090, 64U3670609), and duly sealed by pressure. A reference aluminum pan was also prepared by simply sealing air inside an empty case. The samples were then heated from room temperature to 100 °C at a constant linear scanning rate of 5 °C/min, during which the amount of heat absorbed by the sample was recorded.

The effect of changing the poloxamer was not very notorious in the amount of heat absorbed (melting enthalpy) by the lipid nanoformulation encasing bacteriophage T4, since nanovesicles produced with Lutrol F-68 and Lutrol F-127 exhibited nearly the same melting enthalpy. However, the temperature peak of heat absorption was significantly displaced from 35.5 °C (in the case of nanovesicles produced with Lutrol F-68) to 53.6 °C (in the case of nanovesicles produced with Lutrol F-127) (see Figure 6).

Thermodynamic stability of lipid nanovesicles depends upon their existing lipid modification. Polymorphic transitions after crystallization of triacylglycerol nanovesicles are slower for longer-chain triacylglycerols, as in our case, than for shorter-chain triacylglycerols, whereas these transitions are faster for small size of crystallites. The type of surfactant utilized in lipid nanovesicle formulation and their storage time affects the crystallinity of lipid nanovesicles and, consequently, degradation velocity.

Bacteriophage-T4 lyophilizate alone in ultrapure water showed a melting enthalpy of 164.34 J/$g_{suspension}$, at a temperature peak of 87.8 °C, a value clearly higher than that of the nanoemulsion encasing bacteriophage-T4 after 10 min of homogenization, which probably accounts for the stabilizing effect of the nanoencapsulation procedure.

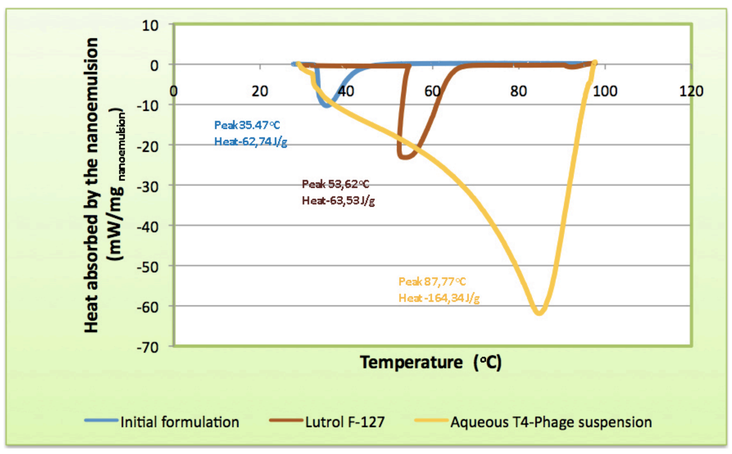

Figure 6. Differencial scanning calorimetry thermograms of multiple nanoemulsions encasing bacteriophage-T4.

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 115-145 nm and Zeta Potential values of ca. -16 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.

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