Design of a lipid nanovesicle system encapsulating bacteriophages integrated in a multiple emulsion formulation: a *proof-of-concept*.

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ABSTRACT

Development of a biotechnological process for the inhalational administration of a bacteriophage was pursued, using strategies of nanoencapsulation within lipid nanovesicles. As a *proof-of-concept* for the nanoencapsulation strategy, a bacteriophage with broad lytic spectrum was entrapped within W/O/W multiple nanoemulsions. Physicochemical characterization of the optimized bacteriophage-encasing nanovesicles encompassed determination of particle hydrodynamic size, size distribution and particle charge via DLS, surface morphology via CRYO-SEM, and thermal analysis via DSC, whereas antimicrobial activity of the nanoemulsions produced was assessed *in vitro* using several bacterial strains. The optimized nanosystems showed no phase separation and encompassed nanovesicles with an average size of ca. 114 nm and an average Zeta Potential of ca. -13 mV, which were maintained stable over a storage timeframe of ca. 3 months.

Keywords: bacteriophages, antibiotherapy, multiple emulsions, nanoencapsulation

1 INTRODUCTION

The emergence of antibiotic-resistant bacterial strains put an emphasis in the need for safe(r) and effective alternatives for antimicrobial treatments. The application of strictly lytic bacteriophages (or cocktails therefrom) has been proposed as an alternative (or complement) to conventional antibiotics, allowing release of the natural predators of bacteria directly to the site of infection. Probably, the major advantage of bacteriophage-based antibiotherapy relative to its chemical counterpart lies in the fact that bacteriophages replicate directly at the site of infection, becoming profusely available where they are most needed, as long as there exists viable hosts. When compared to conventional chemical antibiotics, bacteriophages present many other relevant advantages: (i) strong tissue permeability; (ii) total compatibility (and may act synergistically) with chemical antibiotics; and (iii) isolation and large-scale production of new lytic bacteriophages is much simpler and economical than developing a new antibiotic.

Water-in-oil-in-water (W/O/W) emulsions are examples of multiple emulsions, in which dispersions of small water droplets within larger oil droplets are themselves dispersed in a continuous (outer) aqueous phase [1, 3, 4]. Due to their compartimentalized internal structure, multiple emulsions present advantages for encapsulation of bioentities, such as the ability to carry both polar and non-polar molecules, and a better control over releasing of therapeutic molecules [2, 4, 5].

In this research effort, development of a biotechnological process for the inhalational administration of a bacteriophage was pursued, using strategies of nanoencapsulation within lipid nanovesicles. As a *proof-of-concept* for the nanoencapsulation strategy, a bacteriophage with broad lytic spectrum was entrapped within W/O/W multiple nanoemulsions. Physicochemical characterization of the optimized bacteriophage-encasing nanovesicles encompassed determination of particle hydrodynamic size, size distribution and particle charge via DLS, surface morphology via CRYO-SEM, and thermal analysis via DSC, whereas antimicrobial activity of the nanoemulsions produced was assessed *in vitro* using several bacterial strains.

This method of targeting may have a high potential for the treatment of respiratory bacterial infections because the aerosol is delivered directly at the site of infection, thereby accelerating the action of bacterial predators, whereas protection for the bacteriophage against the immune system is provided by the lipid nanovesicles. The optimized nanosystems showed no phase separation and encompassed nanovesicles with an average size of ca. 114 nm and an average Zeta Potential of ca. -13 mV, which were maintained stable over a storage timeframe of ca. 3 months.

Compatibility between increased amounts of Tween 80 and other components of the internal aqueous phase can be attributed to hydrogen bonding and to the lower crystalline lattice energy of this polymer, which had a notorious impact in the melting profile of the optimized nanoemulsion. Increasing molecular weight of the lipid (such as in Softisan 100™) and decreasing dielectric constant (such as in Tween 80) indicates greater hydrophobicity, leading to a greater impregnation of the interface and therefore to a more stable

(nano)emulsion, which is in clear agreement with the long-term stability observed for our multiple nanoemulsion systems.

2 EXPERIMENTAL METHODS

In the present research work, the potential of nanoencapsulating a broad lytic spectrum phage able to infect enteric *Salmonella* and *E. coli* has been investigated. Phage phi-PVP-SE1 was entrapped within W/O/W multiple nanoemulsions, aiming at mimicking the multifunctional design of biology, with several lipid matrices, poloxamers and stabilizing layer compositions. Physicochemical characterization of the optimized phage-encasing nanovesicle formulations encompassed determination of particle size, size distribution and particle charge, via Zeta potential analysis, surface morphology via SEM, encapsulation efficiency, and thermal analysis via DSC. The antimicrobial activity of the nanoemulsions produced was also assessed *in vitro*, using several microbial strains.

The experimental methodology encompassed three phases: (i) Development and optimization of a multiple nanoemulsion W/O/W encompassing lipid nanovesicles for phage (nano)encapsulation; (ii) Evaluation of stability and physicochemical characterization of the formulated and optimized multiple nanoemulsion; and (iii) *In vitro* assessment of viability of nanoencapsulated bacteriophages. For preparation of the multiple emulsion systems, an oily phase (O) was prepared by melting together 0.5 g Softisan 100™ and 0.05 g lecithin on a thermostatted bath set at ca. 40 °C and maintained at this temperature. In a separate beaker, 5 mL glicerol was heated up to ca. 40 °C in the same thermostatted bath. For the internal aqueous phase (Win), 5 mL HCl 0.01 M and 0.05 g Tween 80™ were heated together up to ca. 40 °C in the same thermostatted bath, and added with 5 mg lyophilized bacteriophage. When Softisan 100™ and lecithin were melted down, both glicerol and 1 mL of Win were added and thoroughly mixed with an UltraTurrax IKA T25 for 10 min, set at 10000 RPM, in the thermostatted bath, thus forming an Win/O emulsion. The external aqueous phase (Wext) was prepared by dissolving 0.4 g Lutrol F68 in 40 mL water. 20 mL Wext was heated up in a thermostatted bath set at ca. 40 °C, added to the Win/O emulsion, and thoroughly mixed with the UltraTurrax for 10 min at 10000 RPM. The remainder 20 mL of Lutrol was added to the emulsion thus produced and homogenized using a magnetic stirrer until room temperature.

	Phage amount (mg)	Tween 80 (mg)	Softisan 100 (mg)	Soy Lecithin (mg)	Glicerol (mL)	Lutrol F-68 (mg)	RPM	Macroscopic characteristics
Initial formulation	5.3	54.9	520	51.8	5	399.7	10000	Clear
+ 25% Lecithin	5.3	54.9	500.1	70.7	5	399.7	10000	Precipitate
+25%Tween/Lecithin	6.2	68.6	509.9	65.3	5	506.9	10000	Clear
+ 25% Tween 80	4.4	69.9	508.9	51.6	5	411.0	10000	Clear

Table 1: Formulation and optimization of the Multiple W/O/W nanoemulsion system, with bacteriophage Phi-PVP-SE1 encased.

Full physicochemical characterization of the nanoemulsion produced proceeded via determination of the Zeta Potential, differential scanning calorimetric analysis, hydrodynamic size and polydispersion index, SEM and Cryo-SEM analysis.

As a *proof-of-concept* to test the bacteriophage nanoencapsulation procedure, a newly isolated bacteriophage (from poultry production sewage water), phi-PVP-SE1 (2/2) (a broad lytic spectrum phage able to infect *Salmonella* enteric and *E. coli*) was utilized in all nanoencapsulation procedures. Phage 2/2 was produced in a Salmonella enteric Enteritidis host, grown in Luria-Bertani molten agar. A total volume of 40 mL of SM- buffer containing 10^9 PFU (plaque-forming units) / mL was lyophilized, and subsequently utilized in all multiple nanoemulsion formulations. Dynamic Laser Scattering analysis of an (ultrapure) aqueous suspension of this bacteriophage revealed an average particle size of ca. 65.2 nm.

Optimized nanoemulsions were assessed for antimicrobial (lytic) activity, following a simple laboratory procedure: (1) Lipid nanoparticles encasing bacteriophage (1000 µL) were added with 20 µL of chloroform in a test tube, to extract the encased bacteriophages; (2) The resulting suspension was gently vortexed for a short period of time (5 s), and was subsequently centrifuged at 9000 x g for 10 min; (3) Following centrifugation, (aqueous) supernatant was immediately recovered and submitted to the "spot" test as follows (4.-7.); (4) 100 µL of bacterial suspension (Salmonella enteric Enteritidis) grown overnight at 37 °C were added to 3 mL of top-agar; (5) 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; (6) A 5 µL drop of the recovered sample supernatant (3.) was then applied and allowed to dry; (7) Incubation of the Petri dish was then allowed at 37 °C, overnight.

3 EXPERIMENTAL RESULTS

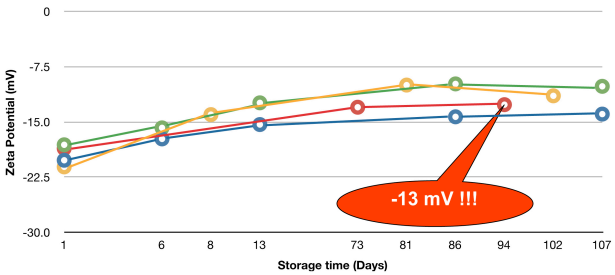

Figure 1: Changes in nanoparticle Zeta Potential values throughout storage time.

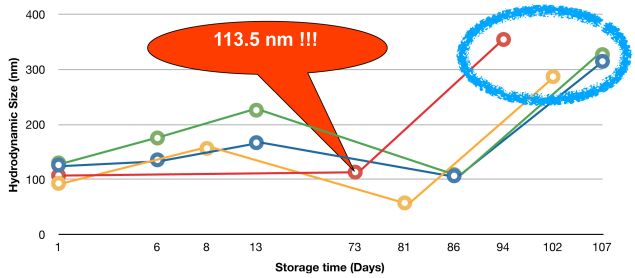

Figure 2: Changes in nanoparticle Hydrodynamic Size throughout storage time.

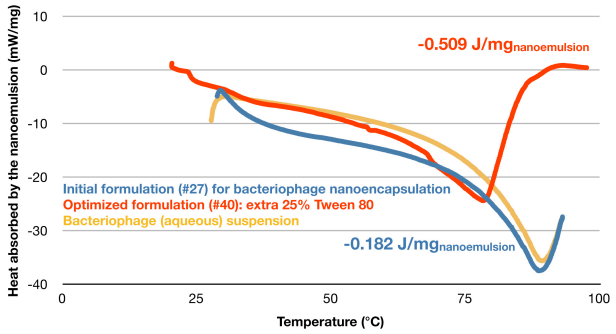

Figure 3: Thermograms of optimized bacteriophage-encasing nanoemulsion and plain bacteriophage suspension.

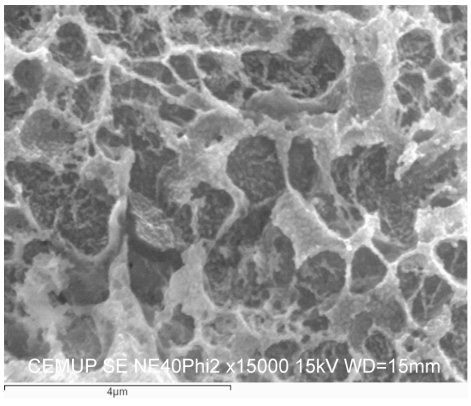

Figure 4: Nanoemulsion structure, with bacteriophage Phi-PVP-SE1 encased, via CRYO-SEM.

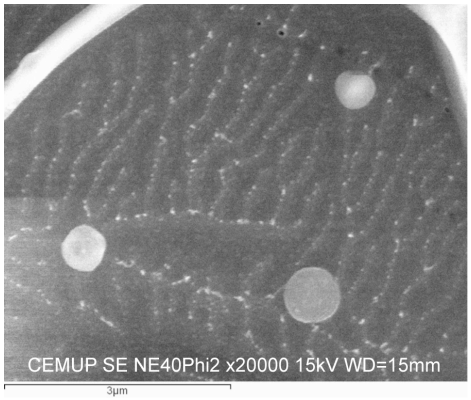

Figure 5: Nanovesicles encasing bacteriophage Phi-PVP-SE1, via CRYO-SEM.

4 DISCUSSION AND CONCLUSIONS

Bacteriophage lyophilizate reconstituted in SM-buffer displayed lytic activity, as expected. The first bacteriophage-encasing nanoemulsions did not exhibit lytic activity, probably due to one (or more) of the following: (i) low pH in the inner aqueous phase (pH ≈ 2.31)? most likely, since the bacteriophage utilized demonstrated to be sensitive to a pH value of 2.0 (but not to pH values of 4.0-5.0); (ii) homogenization stirring speed? not likely, since several researchers reported no loss of viability at stirring speeds of 14000 rpm. Our procedure encompassed homogenization at 10000 rpm; (iii) thermolability of bacteriophages? not likely, since the bacteriophage utilized in the nanoencapsulation trials thrives in the gastrointestinal tract of chickens, where normal temperature reaches ca. 41 °C (105 °F). The temperature utilized in our nanoencapsulation trials was always below 39 °C (melting temperature of SOFTISAN 100™ is ca. 35 °C); (iv) low bacteriophage concentration? yes, this was most likely the cause for not detecting lytic activity of the bacteriophage-encasing nanoemulsions... New nanoemulsions were produced using amounts of bacteriophage 5 and 10 times higher, which displayed positive antimicrobial activity.

Macroscopic observations of optimized nanosystems with increased amount of the semicrystalline polymer (Tween 80™, i.e. poly(oxyethylene) sorbitan monooleate) showed no visible phase separation, and absence of adherence to the container walls even after a prolonged storage at room temperature. Optimized nanosystem encompassed nanovesicles with an average size of ca. 114 nm and an average Zeta Potential of ca. -13 mV, which were maintained stable over a storage timeframe of nearly 3 months. The sudden increase in particle size after storage at room temperature for ca. 3 months was attributable to particle aggregation in the nanosuspension. Inclusion of Tween 80™ in higher amounts led to a significative decrease in the peak temperature (from 88 °C and absorption of 0.182 J/mg in departing nanoemulsion to ca.

79.2 °C and absorption of 0.509 J/mg in optimized counterpart), denoting a widening of the melting profile in the optimized nanoemulsion, with an increase in peak area of over 64 %.

Compatibility between increased amounts of Tween 80™ and other components of internal aqueous phase can be attributed to hydrogen bonding and to the lower crystalline lattice energy of this polymer, which had a notorious impact in the melting profile of the optimized nanoemulsion.

In water-in-oil emulsions (such as our nanovesicles), there is a positive correlation between emulsion stability and fatty acid chain length and a negative correlation with the dielectric constant of the emulsifier. Increasing molecular weight (such as in Softisan 100™, with C10-C18 fatty acid moieties) and decreasing dielectric constant (such as in Tween 80™) indicates greater hydrophobicity, leading to a greater impregnation of the interface and to a more stable (nano)emulsion, which is in clear agreement with the long-term stability observed for our multiple nanoemulsion systems.

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