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A multiple emulsion formulation of bacteriophage encapsulated in lipid nanovesicles

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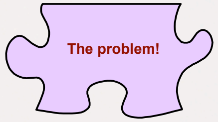

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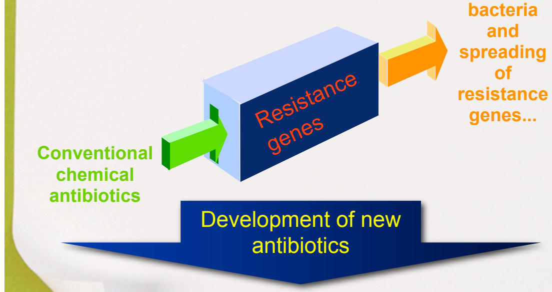

- Complex
- Expensive
- Time consuming
- etc...

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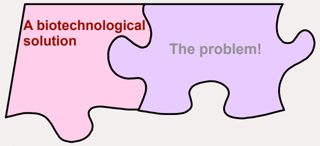

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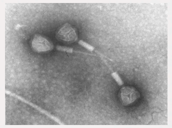
An intelligent alternative



Electron micrograph of bacteriophages that kill *Salmonella* [source: www.intralytix.com]

Advantages of Bacteriophage-based antibiotherapy:

- If bacteria develop resistance to phages, isolating new lytic phages is simpler and more economical than developing a new chemical antibiotic;
  - Phages replicate directly at the site of infection;
  - High concentrations of phages achieved only at the site of infection;
  - Phage elimination occurs only after eradication of infeccious bacterial host;
  - Specificity against target bacterial host;
  - Compatible with conventional chemical antibiotics;
  - High penetration capability in bacterial biofilms;
    - Phages are completely harmless to humans.

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1. Development and optimization of a multiple nanoemulsion W/O/W encompassing lipid nanovesicles for phage (nano)encapsulation
2. Evaluation of stability and physicochemical characterization of the formulated and optimized multiple nanoemulsion
3. *In vitro* assessment of viability of nanoencapsulated bacteriophages

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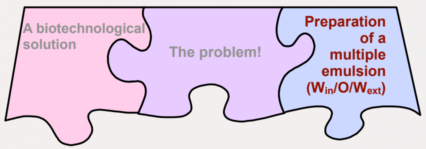

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Oily phase (O): 0.5 g Softisan 100 + 0.05 g lecithin were melted together on a thermostatted bath set at ca. 40 ºC and maintained at this temperature

↓

In a separate beaker, 5 mL glicerol was heated up to ca. 40 ºC in the same thermostatted bath

↓

Internal aqueous phase (W_{in}): 5 mL HCl 0.01 M + 0.05 g Tween 80 were heated together up to ca. 40 ºC 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 W_{in} were added and thoroughly mixed with an UltraTurrax IKA T25 for 10 min, set at 10000 RPM, in the thermostatted bath, thus forming an W_{in}/O emulsion

→

External aqueous phase (W_{ext}): 0.4 g Lutrol F68 was dissolved in 40 mL water

↑

20 mL W_{ext} was heated up in a thermostatted bath set at ca. 40 ºC, added to the W_{in}/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

↑

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

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As a *proof-of-concept* to test the bacteriophage nanoencapsulation procedure...

Bacteriophage phi-PVP-SE1 (2/2) is a broad lytic spectrum phage able to infect *Salmonella* enteric and *E. coli*, and was isolated from poultry production sewage water...

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...

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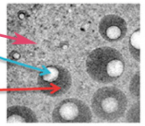
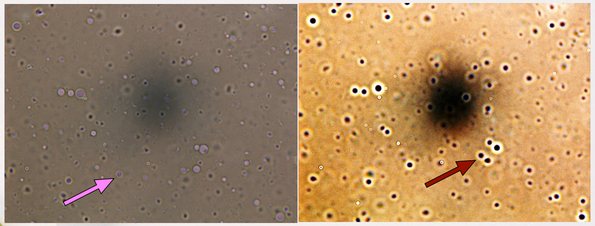
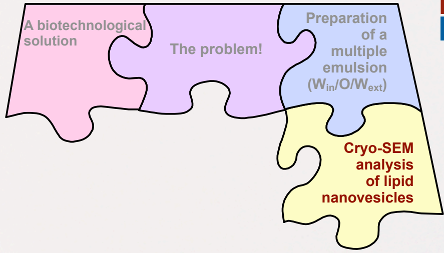
Formulation
Optimization
Production
Characterization

Multiple emulsions "Water-in-Oil-in-Water"

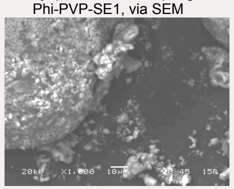


Aspect (observation under optical microscope) of the nanoemulsions produced, displaying the lipid nanovesicles (see arrow)...

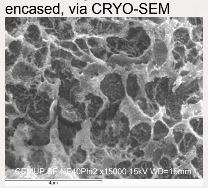




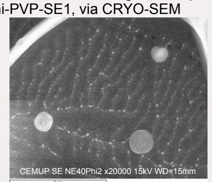
Lyophilized bacteriophage Phi-PVP-SE1, via SEM

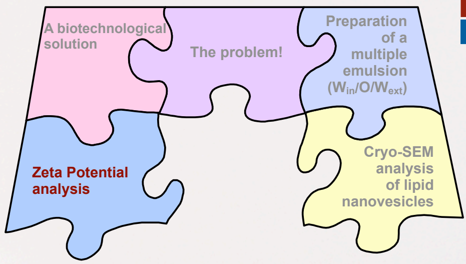


Nanoemulsion structure, with bacteriophage Phi-PVP-SE1 encased, via CRYO-SEM



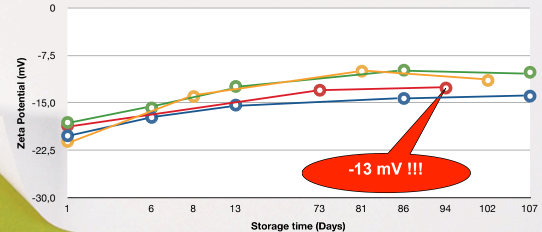
Nanovesicles encasing bacteriophage Phi-PVP-SE1, via CRYO-SEM

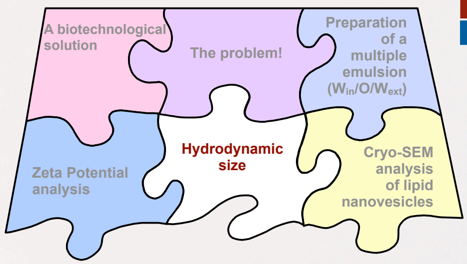




	Phage amount (mg)	Tween 80 (mg)	Softisan 100 (mg)	Soy Lecithin (mg)	Glicerol (mL)	Lutrol F-68 (mg)	RPM	Macroscopic characteristics
Initial formulation	5,3	54,9	520	51,8	5	399,7	10000	Clear
+ 25% Lecithin	5,3	54,9	500,1	70,7	5	399,7	10000	Precipitate
+25%Tween/Lecithin	6,2	68,6	509,9	65,3	5	506,9	10000	Clear
+ 25% Tween 80	4,4	69,9	508,9	51,6	5	411,0	10000	Clear

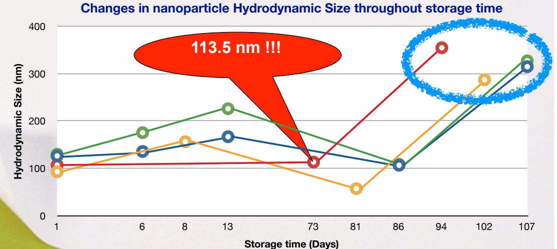
Changes in nanoparticle Zeta Potential values throughout storage time

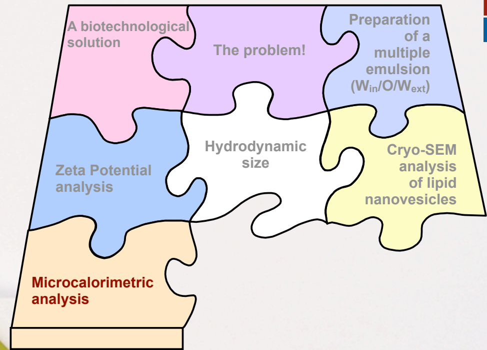




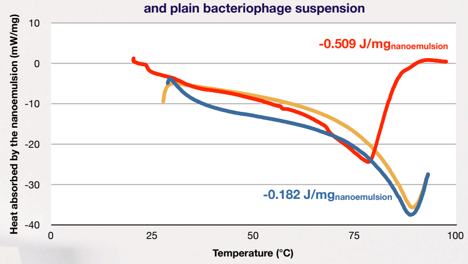
	Phage amount (mg)	Tween 80 (mg)	Softisan 100 (mg)	Soy Lecithin (mg)	Glicerol (mL)	Lutrol F-68 (mg)	RPM	Macroscopic characteristics
Initial formulation	5,3	54,9	520	51,8	5	399,7	10000	Clear
+ 25% Lecithin	5,3	54,9	500,1	70,7	5	399,7	10000	Precipitate
+25%Tween/Lecithin	6,2	85	509,9	65,3	5	506,9	10000	Clear
+ 25% Tween 80	4,4	69,9	508,9	51,6	5	411,0	10000	Clear

Changes in nanoparticle Hydrodynamic Size throughout storage time





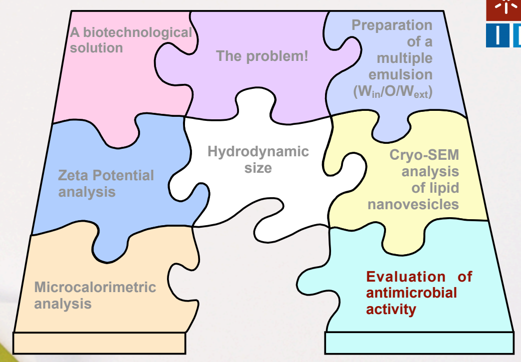
Thermograms of optimized bacteriophage-encasing nanoemulsion and plain bacteriophage suspension



Initial formulation (#27) for bacteriophage nanoencapsulation

Optimized formulation (#40): extra 25% Tween 80

Bacteriophage (aqueous) suspension



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

Preliminary results regarding antimicrobial activity...

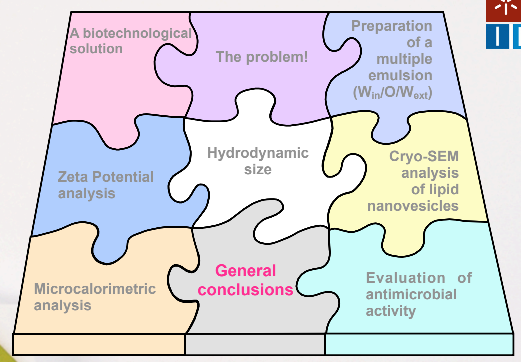
- Bacteriophage lyophilizate reconstituted in SM-buffer displayed lytic activity, as expected
- Bacteriophage-encasing nanoemulsions did not exhibit lytic activity, probably due to one (or more) of the following:

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)...

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

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)

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



General conclusions...

- **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...**

General conclusions...

- **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...**

General conclusions...

- **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 C_{10}-C_{18} 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...**

Thank you for your attention...