P-077 **A lipid nanovesicle system encasing bacteriophages for inhalational therapy.**

Balcão V.[1,2*,#]; Castro L.[1]; Azevedo, A.[1]; Moura A.[2,3]; Moutinho, C.[1]; Teixeira J.[2] and Azeredo J.[2]

[1] Bioeng. Biopharm. Chem. Res. Group (GIBQB-CIAGEB), Univ. Fernando Pessoa, Porto, Portugal. [2] Inst. Biotech. Bioeng. (IBB), CEB – Univ. Minho, Braga, Portugal. [3] Jean Piaget Health College, Vila Nova de Gaia, Gulpilhares, Portugal.

* Supervisor # vbalcao@ufp.edu.pt

INTRODUCTION AND OBJECTIVES

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 (Pitrez, 2003). These anatomophysiological structures have immunological roles leading to production of antibodies, working in the local immunity of the pharynx and protection of the entire 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 (Bisno, 1997). The emergence of antibiotic-resistant bacterial strains and the poor penetration of conventional chemical antibiotics in bacterial biofilms raise the need for safe and effective options of antimicrobial treatment (Gonzalez, 2001). 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 (Sulakvelidze 2001). 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 (Sulakvelidze, 2001). 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 (Ragoonanan 2007) 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 (Bibette 1999; García-Fuentes 2002). 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 (Srinivas 2010; Rawat 2008; Pays 2002; Davis 1987; Hanson 2008; Ficheux 1998; Wang 2006; Gutiérrez 2008). 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.

MATERIALS AND METHODS

Preparation of multiple bacteriophage T4-Encasing lipid nanoemulsions. Production of multiple emulsions encompassing lipid nanovesicles with encased bacteriophage T4 was carried out using an Ultra Turrax (model T25D from IKA) under heating (ca 40 ºC). Lyophilized bacteriophage T4 was suspended in the (inner) aqueous phase (Win) 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 of 10 min. The inner aqueous phase encompassed HCl 10 mM, Tween 80 and non-purified lyophilized bacteriophage T4 preparation (5 mg); the intermediate oily phase encompassed glycerol, Softisan™ 100 (from Sasol Olefins & Surfactants GmbH, Hamburg, Germany) and soy-

bean phosphatidylcholine; finally, the outer aqueous phase encompassed Lutrol™ F68 (poloxamer 188 from BASF, Germany) and ultrapure water.

Optimization of the nanoformulation. Optimization of the multiple lipid nanoemulsion proceeded via preparation of different emulsions with differdifferent concentrations of Tween 80 (50 to 75 mg) and different stabilizing layer compositions.

Determination of hydrodynamic size (HS) and Zeta potential (ZP). Determination of 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.

RESULTS AND DISCUSSION

Several variables were studied, viz. lipid nature, poloxamer nature, soy lecithin concentration and Tween 80 concentration. Replacement of the poloxamer (Lutrol™ F68) 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% more 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. 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.

Both the Hydrodynamic size and Zeta Potential of the several nanoemulsions produced were evaluated and followed throughout a prolonged storage at room temperature. 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™.

The preliminary results obtained for the antimicrobial (lytic) properties of the optimized nanoemulsion encasing bacteriophage T4, showed an inhibition halo produced by the whole nanoemulsion.

CONCLUSIONS

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.

REFERENCES

- Srinivas P.R. *et al*. (2010) *Nanotechnology research: applications in nutritional sciences*, J. Nutr. 140: 119-124.
- Rawat M. *et al*. (2008) *Lipid carriers: a versatile delivery vehicle for proteins and peptides*, Yakugaku Zasshi (The Pharmaceutical Society of Japan) 128: 269-280.
- Ragoonanan V. *et al*. (2007) *Protein stabilization*, Transfusion Medicine and Hemotherapy 34: 246-252.
- Bibette J. *et al*. (1999) *Emulsions: Basic principles*, Rep. Prog. Phys. 62: 969–1033.
- Ficheux M.F. *et al*. (1998) *Some stability criteria for double emulsions*, Langmuir 14: 2702–2706.
- García-Fuentes M. *et al*. (2002) *Design of lipid nanoparticles for the oral delivery of hydrophilic macromolecules*, Colloids and Surfaces B: Biointerfaces 27: 159-168.
- Wang Y.F. *et al*. (2006) *Structural evolution of polymer-stabilized double emulsions*, Langmuir 22: 67–73.
- Gutiérrez J.M. *et al*. (2008) *Nano-emulsions: New applications and optimization of their preparation*, Curr. Opinion Colloid & Interface Sci. 13 (4): 245-251.
- Pays K. *et al*. (2002) *Double emulsions: how does release occur?* J. Control. Release. 79: 193-205.
- Davis S.S. *et al*. (1987) *Multiple emulsions as targetable delivery systems*, Methods Enzymol. 149: 51–64.
- Hanson J.A. *et al*. (2008) *Nanoscale double emulsions stabilized by single-component block copolypeptides*, Nature 455: 85-88.
- Pitrez P.M.C. *et al*. (2003) *Infecções agudas das vias aéreas superiores: diagnóstico e tratamento ambulatorial.* Jornal de Pediatria 79(1): S77-S86.
- Bisno A.L. et al. (1997) *Diagnosis and management of group A streptococcal pharyngitis: a practice guideline. Infectious Diseases Society of America*, Clin. Infect. Dis. 25(3): 574-583.
- Sulakvelidze A. *et al*. (2001) *Bacteriophage therapy*, Antimicrob. Agents Chemother. 45: 649-659.