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Structural and functional stabilization of bacteriophage particles within the aqueous core of a W/O/W multiple emulsion: A potential biotherapeutic system for the inhalational treatment of bacterial pneumonia

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ABSTRACT

The increase of antibiotic-resistant bacteria is growing every day, most likely associated with the indiscriminate use of these antimicrobials or even with evolutionary adaptability of bacteria to their environment. This situation brings a need to develop new alternatives to conventional antibiotics, and thus the application of strictly lytic bacteriophages has been proposed as an alternative (or complement) to the former, allowing release of the natural predators of bacteria directly where they are needed the most: the infection site. The main advantages of bacteriophages to treat infections is the maintenance of a high concentration of bacteriophage particles in the action site while any viable target bacteria still exist, coupled to the production of enzymes that hydrolyze the polymeric matrix of bacterial biofilms promoting penetration and antibacterial action. In the research effort entertained herein, the potential for protection and stabilization of strictly lytic bacteriophages with broad spectrum capable of infecting *Pseudomonas aeruginosa*, so as to maintain their structure and functionality, was investigated via encapsulation within the aqueous-core of lipid nanodroplets integrating a W/O/W multiple emulsion system, aiming at developing isotonic derivative solutions thereof for administration by nebulization.

1. Introduction

The increasing worldwide awareness related to the appearance of multiple bacterial resistance to conventional chemical antibiotics [1,2] has shed a renewed interest from the scientific community to bacteriophage (or phage) particles, with these inert (i.e., devoid of any metabolic machinery) entities being re-discovered as high-potential candidates for biopharmaceutical (antimicrobial) applications [3–7]. However, like most biological (macro)molecules, phage particles are intrinsically fragile and therefore their full structural and functional stabilization is mandatory [3,4,8–11] prior to any use. Solubilization of these protein-like entities in the aqueous-core of lipid nanodroplets, protecting them from deactivation by the immune system, dilution effects, and from any chemical stress [12–15], will thus promote their structural and functional stabilization [3,4,9,10], a *sine qua non* condition for these particles to be used as (bio)medicines. Colloidal systems such as water-in-oil-in-water (W/O/W) multiple emulsions (MEs) are systems in which dispersions of small water droplets within larger oil droplets are themselves dispersed in a continuous (external) aqueous phase [8,10,16–22]. The smaller the sizes of the aqueous-core lipid nanodroplets the longer their retention times [23,24], and hence such (lytic) phage-encasing nanodroplets could be utilized, e.g. in the formulation of isotonic aerosols to eliminate bacterial pneumonia caused by *Pseudomonas aeruginosa* with one clear advantage: the adhesion of these lipid nanodroplets to the pulmonary mucosa could slowly liberate their bacterial predatory content, reducing both the need for chemical antibiotics and infection elimination time. *P. aeruginosa* is the second pathogen causing nosocomial pneumonia [25,26]. Worldwide, it is found that the antimicrobial resistance rate for *P. aeruginosa* has increased dramatically [27–30]. Quite recently, a newly developed nanocontainment technique for stabilization of protein entities has started to gain momentum [8,10,31–35]. Entropic confinement (physical entrapment) of macromolecular (protein-like) entities such as phage particles within the aqueous core of these lipid nanodroplets, changes

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the water activity in their microneighborhood while reducing molecular motions [9], leading to increased viscosity [9,34,36]. Lipid nanodroplets integrating a W/O/W ME are composed of a (physiologically compatible) lipid matrix [8,10,31,32,37,38], stabilized by emulsifiers such as phospholipids and/or polyoxyethylene ethers [8,10,16,31,32,39]. When producing a W/O/W ME system, the lipid concentration required to produce the lipid nanodroplets must be kept at low levels, since high lipid concentrations further enhance the thermodynamic instability of these systems and may even promote their rupture (following release of the inner aqueous core under shear rate, with concomitant expulsion of the water-soluble protein entities through the oily layer between both water phases) [16,40,41]. In the research effort entertained herein, a stable W/O/W ME system was produced according to the procedure described by Glasser et al. [8,31], integrating small-sized lipid nanodroplets with aqueous cores housing phage particles; such phage-encasing W/O/W ME was then used for developing an isotonic system aiming at the administration via nebulization for the inhalation of lytic (endotoxin-free) phages for the treatment of pulmonary infection by *P. aeruginosa*. Hence, the natural predators of bacteria responsible for provoking lung infections would be released directly where they are needed the most: the site of infection.

2. Materials and methods

2.1. Materials

2.1.1. Biological materials

Bacteriophage: Bacteriophage JG004 (ref. DSM 19871) was purchased from Leibniz-Institute DSMZ (Braunschweig, Germany). Bacterial host: Phage JG004 was propagated in a *Pseudomonas aeruginosa* host (ref. DSM 19880), acquired from DSMZ. Cell lines: The cell lines were purchased from BCRJ (Duque de Caxias/RJ, Brazil): cell line V79-4 (BCRJ code 0244; Chinese Hamster, Lung-Normal); cell line A549 (BCRJ code 0033; Human, Caucasian, Lung-Carcinoma), and cell line 3T3 (BCRJ code 0017; Mus musculus (Swiss albino), Fibroblast/Embrio). Dulbecco's Modified Eagle's Medium (DMEM) was from Gibco Life Technologies (Alto de Pinheiros, São Paulo/SP, Brazil). The Tali® Apoptosis Kit (consisting of Annexin V Alexa Fluor® 488 and Propidium Iodide) was purchased from Invitrogen (Carlsbad CA, U.S.A.).

2.1.2. Chemicals

Lipids: Softisan100™ was a kind gift from Sasol (Sasol Olefins & Surfactants GmbH, Hamburg, Germany) and glycerol was from Fluka (Steinheim, Germany). ***Surfactants***: Tween 80 was from Sigma-Aldrich (St. Louis MO, USA). Kolliphor P188™ (poloxamer 188) was a kind gift from BASF ChemTrade GmbH (Ludwigshafen, Germany). Soybean phosphatidylcholine (lecithin) was from Alamar Tecno-Científica Ltda (Diadema/SP, Brazil). ***Other chemicals***: Commercial HCl (37%, w/w) was from ECIBRA Analytical Reagents (Curitiba/PR, Brazil). Anhydrous Na_2HPO_4, NaH_2PO_4, $CaCl_2$ and NaCl were from Dinâmica Ltda (Diadema/SP, Brazil). Luria Bertani Broth (Miller, LB-Broth) was from HiMedia Laboratories Pvt. Ltd (Mumbai, India) and solid agar was from Gibco Diagnostics (Madison WI, U.S.A.). The sterilizing filtration system (Stericup™-GP, polyethersulphate (PES) membrane with 0.22 μm pore diameter) was a kind gift from Merck-Millipore (Darmstadt, Germany). Nipagin™ (methylparaben) and $MgSO_4$ were from Labsynth (Diadema/SP, Brazil). Tap water was purified in a Milli-Q Plus 185 system (Molsheim, France) to a final conductivity of ca. 18.2 $M\Omega.cm^{-1}$. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and DMSO were from Sigma-Aldrich (St. Louis MO, U.S.A.).

2.1.3. Analytical equipment

Zeta Potential (ZP), Hydrodynamic Size (HS) and Polydispersity Index (PI) were determined in a ZetaPALS system (model NanoBrook 90PlusPALS from Brookhaven Instruments, Holtsville NY, U.S.A.). FTIR spectra were gathered in a Fourier Transform Infrared Spectrophotometer from AGILENT (model Cary 630, Santa Clara CA, U.S.A.). X-ray diffractograms were gathered in an X-ray Diffractometer (XRD) from Shimadzu (model XRD7000, Kyoto, Japan). Nanoparticle Tracking Analyses (NTA) were carried out in a NanoSight device from Malvern Instruments Ltd (model LM14C, Worcestershire, United Kingdom), with NTA software (NanoSight version 3.1). The UV–vis Spectrophotometer was from Perkin Elmer (model Lambda 3s, Waltham MA, U.S.A). TGA analyses were carried out using a thermogravimeter from TA Instruments (model 2050, New Castle, U.S.A.), whereas DSC analyses were carried out using a differential scanning microcalorimeter from TA Instruments (model MDSC 2910, New Castle, U.S.A.). TEM analyses were carried out in a Transmission Electron Microscope from JEOL (model JEM-1400Plus, JEOL, Tokyo, Japan), equipped with a lanthanum hexaboride (LaB_6) filament, operating at 120 kV, and a high-resolution CCD camera from GATAN Inc. (model MultiScan 794, Pleasanton CA, U.S.A.) with a resolution of 1k x 1k pixels for the acquisition of digital images. Cell viability readings via the MTT assays were carried out in an ELISA microplate reader from Robonik India Private Ltd. (model *Readwell PLATE*, Maharashtra, India). All optical microscopy analyses for the Comet™ assays were carried out in a Zeiss Axiovert-60 optical microscope (Carl-Zeiss, Göschwitzer Str., Jena, Germany).

2.2. Experimental procedures

2.2.1. Propagation and purification of bacteriophage JG004

The phage (JG004, ref. DSM 19871) was propagated using a specific host microorganism, namely *P. aeruginosa* (ref. DSM 19880), grown in LB molten agar.

Preparation of a bacterial suspension of *P. aeruginosa*. The lyophilized pellet was dissolved in 4.5 mL of Luria-Bertani Broth with the aid of a sterile loop, and 1 mL of the resulting suspension was plated on the surface of LB solid medium in Petri plates, which were then incubated during 24 h at 37 °C. For preparation of a suitable bacterial suspension, a single CFU was inoculated in a test tube containing 5 mL sterile LB-broth which was then incubated at 37 °C during 18 h.

Propagation of the bacteriophage. For propagation of the phage (maintained at 4 °C as a concentrated suspension), duplicate serial dilutions (1×10^{-1}–1×10^{-10}) (total volume of 1000 μL) in sterile phage buffer (PB) were duly prepared. To test tubes containing 4 mL LB top agar (maintained at 40 °C), 300 μL of a 1:10 dilution of the bacterial suspension in LB-broth were added together with 1000 μL of a given phage dilution. The resulting mixture was gently shaken, immediately poured and spread on top of Petri plates containing 25 mL solid Luria-Bertani agar, which were then incubated overnight at 37 °C.

Purification of the bacteriophage. After the incubation period, all Petri plates were visually inspected for the presence of plaque forming units (PFU) and, for those where complete lysis of the bacterial lawn occurred or where PFU's were visible, 3 mL PB solution were added to each Petri plate, after which they were isolated with Parafilm™ (to prevent them from drying out) and further incubated at room temperature during 3 h. Following this time period, the layer of top agar was fragmented by gently swirling a sterile loop, and the mixture of PB and top agar was collected into a sterile 50-mL Falcon tube. Cell debris and agar were then removed by centrifugation (5500 rpm, 15 min at 4 °C), and the supernatant carefully collected, filtered under vacuum through a sterilizing Stericup™-GP device (equipped with a PES membrane with 0.22 μm pore diameter), and stored at 4 °C until use was in order.

Determination of bacteriophage titer. For determination of phage concentration in the stock-suspension, the propagation procedure was repeated, with small differences, namely the plating of a top agar layer containing 100 μL of a given serial dilution of the phage stock-suspension in PB and 300 μL of the bacterial suspension. This procedure