This is a pre-copyedited, author-produced PDF of an article accepted for publication in *Clinical Infectious Diseases* following peer review. The version of record Danish J Malik, Henrique Goncalves-Ribeiro, Dirk GoldSchmitt, Joe Collin, Aouatif Belkhiri, Diogo Fernandes, Henry Weichert, Anya Kirpichnikova, Advanced Manufacturing, Formulation and Microencapsulation of Therapeutic Phages, *Clinical Infectious Diseases,* Volume 77, Issue Supplement_5, 1 November 2023, Pages S370–S383 is available online at: https://doi.org/10.1093/cid/ciad555

Advanced manufacturing, formulation and microencapsulation of therapeutic phages

Danish J. Malik[1*], Henrique Goncalves-Ribeiro[1], Dirk GoldSchmitt[2,3], Joe Collin[1], Aouatif Belkhiri[1], Diogo Fernandes[4], Henry Weichert[5], Anya Kirpichnikova[2]

[1] Chemical Engineering Department, Loughborough University, UK

[2] Division of Computing Science and Mathematics, University of Stirling, UK

[3] Department of Psychology, University of Sheffield, UK

[4] Malvern Panalytical, UK

[5] Sartorius Stedim Biotech GmbH, Germany

*Correspondence: +44 (0)1509 222507; d.j.malik@lboro.ac.uk

Abstract

Manufacturing and formulation of stable, high purity and high dose bacteriophage drug products suitable for clinical usage would benefit from improved process monitoring and control of critical process parameters. Chemistry, Manufacturing and Controls (CMC) for both upstream (USP) and downstream processes (DSP) need mapping of critical process parameters (CPP) and linking these to critical quality attributes (CQA) to ensure quality and consistency of phage drug substance (DS) and drug product (DP) development. Single-use technologies are increasingly becoming the go-to manufacturing option with benefits both for phage bioprocess development at the engineering run research stage and for final manufacture of the phage drug substance. Future phage drug products under clinical development will befit from implementation of process analytical technologies (PAT) for better process monitoring and control. These are increasingly being used to improve process robustness (to reduce batch-to-batch variability) and productivity (yielding high phage titres). Precise delivery of stable phage drug products that are suitably formulated as liquids, gels, solid-oral dosage forms etc., could significantly enhance efficacy of phage therapy outcomes. Pre-clinical development of phage drug products must include at an early stage of development, considerations for their formulation including their characterisation of physiochemical properties (size, charge etc.), buffer pH and osmolality, compatibility with regulatory approved excipients, storage stability (packaging, temperature, humidity etc), ease of application, patient compliance, ease of manufacturability using scalable manufacturing unit operations, cost, and regulatory requirements.

Keywords: phage delivery, upstream and downstream processing, phage therapy;

Introduction

Bacteriophage drug substances (DS) and products (DP) undergoing clinical trials are typically manufactured using cGMP (current Good Manufacturing Practice) manufacturing processes prior to evaluation in clinical investigations. Regulatory bodies across the world such as the FDA (US Medicines regulatory agency) undertake rigorous evaluation of applications for clinical trials and assessment of the manufacturing processes. A thorough understanding of the relationship between the Critical Quality Attributes (CQA) and the clinical performance of the phage drug product is essential. Identification of the product attributes that relate to the safety profile and efficacy (the dosage form and route of administration, dose delivery and pharmacokinetic aspects are critically important) and the design of the manufacturing process to deliver these attributes through control of Critical Process Parameters (CPP) and associated QC/QA (Quality Control/Quality Assurance)

analytical data package as part of the CMC (Chemistry, Manufacturing & Controls) submission is a critical element of the dossier of information submitted in the filing application to obtain permission to carry out clinical trials.

Bacteria-bacteriophage interactions at the site of infection are not well understood and complex in nature. Bacterial refugees in biofilms growing in competition in polymicrobial communities are difficult to replicate in the laboratory during pre-clinical evaluation of phage drug substances. Dosing and delivery strategies developed using *in vitro* studies seldom translate well into effective treatment outcomes *in vivo*. Product formulation and the mode of administration are important aspects related to the Target Product Profile (TPP) including intended use and indication of the product (treatment of a specific infection caused by specified strains of bacteria against which a single phage or cocktail of phages are active) [1]. Formulation of phages is a significant part of CMC activities aimed at improving the shelf-life of phage drug products. Stability studies may also help in other areas of phage development such as for phage-based diagnostics. Phage susceptibility testing (also called phagograms) is used to determine the susceptibility of a bacterium to infection by a specific phage; this may subsequently inform selection of the candidate phages for treatment of a patient. Formulation and encapsulation of phages in controlled release formulations may allow targeted delivery of phages at the site of infection and maintenance of therapeutic dose levels over a period of time accounting for natural clearance rates e.g. due to the immune system [2].

The pre-clinical development pathway of phage drug candidates includes selection of phages and manufacturing hosts with identification of appropriate product safety and characterization profiles. This includes phages that are obligately lytic, fully sequenced (essential attributes include absence of antibiotic resistance genes, virulence factors and transduction genes) [3]. QC/QA assays (for the product impurity profile) need to be developed alongside the manufacturing process with mapping of CPP to CQA [4]. Banks of bacterial hosts used for manufacturing phages and engineering runs for Upstream Processing (USP) of phages need to be carried out to ensure production of genetically and phenotypically consistent batches and identification of any strain drift during production cycles [5].

Stirred-tank bioreactors are available from different vendors and are well-suited for research and development as well as for cGMP production. They are well-characterised in terms of scale-up criteria (based on dimensional similarity principles), allow for controlled mixing, gassing strategies, and process monitoring and control of CPP including temperature, pH, OD (optical density), OUR (oxygen uptake rate), CER (carbon dioxide emission rate), RQ (respiratory quotient) etc [6]. Standard ports (6mm/12mm) allow ease of integration of sensors available from a variety of different vendors (e.g. Hamilton and Mettler-Toledo (pH, OD, dissolved oxygen), BlueSens (O_2 and CO_2 for off-gas)). On-line process monitoring allows control of the timing of phage inoculum addition to the fermentation vessel. This can be critical in terms of the final phage production titres achieved and needs to be linked to online process monitoring of the bacteria growth rates and bacteria concentration during batch production (see below). Feedback (and more advanced) control strategies can be easily implemented to maintain growth rates with control of limiting nutrient concentration (glucose concentration can be controlled using fed-batch production strategies), dissolved oxygen through set-point control etc., thereby allowing control of host growth rates, timing of phage inoculation at the optimum bacteria concentration and monitoring of subsequent phage replication and determination of culture harvest time. Batch production allows flexibility during production with online process monitoring allowing accommodation of slight variability in batch times during production.

This manuscript provides an overview of unit operations that are suitable for a standardised phage manufacturing process flowsheet. It identifies suitable scalable manufacturing unit operations

covering both Upstream (USP) and Downstream Processing (DSP) operations typically used for cGMP manufacture of phage drug substances. CPP and linking these to CQA and the Target Product Profile (TPP) for the application of phages for therapeutic use is discussed. Potential suitable process analytical technologies (PAT) are highlighted and discussion of approaches for online/at-line process monitoring of CPP to improve phage production and to reduce batch-to-batch variability and improve volumetric productivity of phage drug substances are presented. Process modelling approaches and model-based control strategies are briefly discussed. Relevant analytical methods useful for the characterisation of the phages during manufacture that could be part of QC/QA testing are discussed. Considerations for optimisation of liquid formulations and approaches for producing solid/semi-sold dosage forms are presented. Encapsulation technologies suitable for phages to improve storage stability and improve delivery are also briefly covered.

Materials and Methods

Escherichia Coli Culturing and Bacteriophage Propagation

E. coli strain ATCC11303 and its lytic phage T3 (ATCC11303-B3) or strain ATCC BAA-1025 and T7 phage (BAA-1025-B2) were sourced from LGC Standards (Teddington, Middlesex, UK). Single colonies of *E. coli* were selected by streaking onto LB agar (25 g/L LB broth Miller, Fisher Scientific U.K., Loughborough, UK) with 1.5 *w/v*% Bacteriological Agar No. 1 (Oxoid, Basingstoke, UK) and incubated overnight at 37 °C. A single *E. coli* colony was added to a sterile 200 mL flask containing either 25 mL of LB broth or TSB and the flask was incubated in a shaking incubator (Thermoscientific MazQ 6000) overnight at 37 °C at 150 RPM. Optical density measurements were recorded using a spectrophotometer (Thermoscientific Genesys 50 UV-Vis Spectrophotometer) at a set wavelength of 600nm. At fixed time points, 1ml of sample was withdrawn from the culture flask for OD measurement and viable cell counts (colony forming units, CFU/ml) by serially diluting the culture in PBS (phosphate buffer saline) using a sterile 96 well plate using dilution factors 10^{-1} to 10^{-8}. 10 µL of each dilution sample was spotted in quadruplicate and incubated for overnight at 37 °C.

For the bioreactor runs, after overnight culture (as above), 5ml of overnight culture was added to 5 litres of fresh medium (LB/TSB) in a 5 litre stirred tank Univessel operated using a Biostat B Plus controller (Sartorius), The Univessel was equipped with a double six-bladed Rushton impeller. Agitation rate was set at 300rpm, air gassing rate at 2 vvm using a mass flow controller (Alicat). The bioreactor was equipped with the following on-line sensors for monitoring pH (Easyferm Plus pHI ARC, Hamilton), dissolved oxygen (Oxyferm FDA Arc, Hamilton), optical density (Fundalux, 10mm, Sartorius), off gas carbon dioxide and oxygen concentration measurement (BioPAT Xgas, Sartorius). The bioreactor was equipped with an aseptic sampling port to allow withdrawal of culture medium for off-line analysis of viable cell counts, OD, PFU etc.

Final phage titres were enumerated using the standard plaque assay. For each plate, 4 mL of LB top agar (LB broth, Fisher Scientific U.K. with 0.5% Bacteriological Agar No. 1, Oxoid, Basingstoke, UK) was combined with 1 mL salt solution (400 mM $MgCl_2$ and 100 mM $CaCl_2$) in a sterile 15ml Falcon centrifuge tube. 100 µL of overnight *E. coli* culture was added to the molten agar solution (temperature maintained at 50°C) and poured over an LB agar plate. The phage sample was serially diluted in LB broth using a sterile 96 well plate using dilution factors 10^{-1} to 10^{-8}. 10 µL of each dilution sample was spotted in quadruplicate and incubated for 4 h at 37 °C.

Dynamic light scattering and zeta potential measurements

Off-line measurement of phage containing samples involved centrifuging 1ml of phage containing culture (x 13500 g, 3 min, Eppendorf Centrifuge 5425R) to remove lysed and unlysed bacteria. The clear supernatant was diluted 1:10 in PBS and added to clear 10mm pathlength fluorimeter

polystyrene cuvettes and measurements of multiangle dynamic light scattering were acquired using a Zetasizer Ultra Blue (Malvern Panalytical Ltd., Malvern, UK) equipped with a He-Ne laser at a wavelength of 633 nm. All measurements were performed at 25 °C using a sample volume of 1ml. The instrument setting were optimised automatically using the ZS Xplorer software (Panalytical Ltd., Malvern, UK). Refractive index for protein (n = 1.42) and refractive of suspension medium as water were selected.

Zeta potential measurements of samples were undertaken using the Zetasizer Ultra Blue (Malvern Panalytical Ltd., Malvern, UK) employing a disposable folding capillary cuvette cell (DTS 1070) with built-in electrodes. The sample should be homogenous and transparent. T3 phage samples at concentration between 10^{10}-10^{11} PFU/ml were washed in de-ionised distilled water (Millipore, Elix) using Amicon Ultra 100 kDa MWCO centrifugation membrane filters (Millipore, UK) with ionic strength adjusted using sodium chloride (Sigma-Aldrich, UK).

Downstream purification using microfiltration and ultrafiltration for buffer exchange and phage concentration

Harvesting of 5 litres of crude phage lysate from the upstream production step using stirred tank bioreactors was followed by centrifugation at x 17000g (Beckman, Model J2-21M/E, Rotor JA-10) for 60 min at 4 °C. The clarified lysate was filtered using a Repligen Krosflo KR2i TFF filtration unit equipped with either 0.2µm Sartocon 200 (Sartorius, Germany) microfiltration cassettes or 300kDa MWCO Midikros (Repligen, USA) hollow fiber units for diafiltration and phage concentration. Transmembrane pressure was typically between 0.1-0.2 bar.

Transmission electron microscopy of phages

An 8µl aliquot of sample was pipetted onto a carbon coated copper grid (HC300Cu, Holey Carbon film on Copper 300 mesh, EM Resolutions, UK). Excess liquid was removed by blotting using filter paper (Whatman No. 1), and the grid was frozen by plunging into a liquid of ethane/propane cooled with liquid nitrogen. The sample was kept at liquid nitrogen temperature throughout analysis to keep it frozen. TEM imaging was undertaken using a JEOL 2200FS TEM at 200 keV using a Gatan K2 Summit and Gatan 914 Cryo-holder.

Energy Dispersive X-Ray Spectroscopy (EDAX) and X-ray diffraction (XRD) analysis

0.1g of the phage containing powder samples were mounted on a metal stub using double-sided sticky carbon tape. The discs were placed on a sample holder and coated with a thin layer of Gold-Palladium for 90 seconds using a sputter coater. The metal coating was required to prevent charge build-up on the specimen surface. EDAX analysis was conducted using a Carl Zeiss Leo 1520 FEGSEM system equipped with an Oxford Instruments X-max 80mm^2 EDX detector.

X-ray diffraction patterns were obtained using a Bruker D2 Phaser diffractometer fitted with a 1-dimensional LynxEye detector. A copper X-ray source (K_α = 1.54184Å) was used which was run at 30 kV and 10mA, with K_β radiation suppressed by means of a 0.5mm thick nickel filter. Approximately 0.1g of sample particles were packed to a flat surface into a sample holder. This holder was then placed on a mount in the analysis chamber and the sample measurement was acquired. Patterns were recorded over a range of 5° – 70° (2θ) with a step size of 0.02° and an equivalent step time of 0.2 seconds per step. Sample rotation was set at 15 rpm. Bruker's proprietary Eva 2.0 software was used to evaluate the crystal structure of the sample.

Mathematical modelling of phage-bacteria dynamics during batch production

The aim of the model was to capture essential bacteria-phage interaction dynamics and fit experimental data obtained during batch manufacture of phages and undertaken hypothesis testing to optimise phage production. Using a dynamical systems approach, the model represents bacteria

(B) and phage (P) concentrations in a continuously stirred bioreactor, allowing for the application of mass-action principles and assuming spatial homogeneity due to mixing.

$$\frac{dB}{dt} = gB\left(1 - \frac{B}{K}\right) - P\left(1 - e^{-\delta B}\right) \quad (1)$$

$$\frac{dP}{dt} = bP_t\left(1 - e^{-\delta B_t}\right) - nP\left(1 - e^{-\delta B_t}\right) \quad (2)$$

Bacterial growth is included by a logistic growth function, with growth parameter (g) and carrying capacity (K) representing lean (small K) and rich (large K) media conditions. Phage adsorption is modelled using a Poisson growth function with interaction parameter δ. For each successful interaction, a fixed number of phage (burst size, b) are added to the phage population with time delay (t). Additionally, an adsorption coefficient (n) is introduced to model the removal of infected phage due to deactivation upon binding to bacteria.

The model was evaluated during the absence of phage (before phage inoculation) and during the presence of phage (after phage inoculation). Before inoculation, the phage concentration (P) was set to zero, and only bacterial growth was modelled. The model was numerically solved using Matlab software with the dde23 solver. To obtain growth rates, Eq. (1) was fitted first in the absence of phage (P=0) to experimental observations of *E.coli*. We systematically varied remaining model parameters to achieve best model-observation fits. Initial values were chosen based on experimental starting conditions.

Towards a standardised phage manufacturing process flowsheet

Industrial scale production of bacteriophages typically involves the following unit operations: 1) Host strain propagation and infection with a host susceptible phage to amplify the bacteriophages. At commercial scale, this can typically be done using stirred-tank bioreactors; 2) Clarification of the phage lysate using high speed centrifugation can be done instead of or prior to depth or tangential flow microfiltration to remove un-lysed cells and cell debris; 3) Removal of soluble contaminants (including host cell proteins (HCP) and host cell DNA and endotoxins etc) in the lysate, buffer exchange and concentration of phages using ultrafiltration and diafiltration operations; 4) Polishing of bacteriophages using anion exchange or mixed-mode chromatography to further reduce soluble impurities; 5) Buffer exchange and re-formulation of phages in storage buffer using diafiltration; 6) Sterile microfiltration (0.22μm) prior to the final fill-and-finish step.

The host propagation and phage amplification USP can be done using batch, fed-batch or continuous mode [6,7]. Seed culture for the host and phage are prepared from working cell (WCB) and phage banks (WPB) [8]. Selection of media and process monitoring and control of CPP includes maintaining the host in the mid-exponential growth phase prior to phage infection. Selection of the growth medium impacts on the ability of the medium to sustain bacterial growth in the exponential log phase for longer impacting phage yields (Figure 1). Growth of an *E. coli* strain in LB (Luria Bertani) versus TSB (Tryptone Soya Broth) clearly demonstrates the significant difference in achievable cell densities. The media plays an important role in providing the bacterial host suitable nutrients for growth which makes a significant impact on the final phage titres e.g. during batch production (Figure 1). The richer media (TSB) resulted in higher host concentration and in higher final phage titres (Figure 1). The concentration of bacteria at the time of inoculation and the initial concentration of added phage can significantly impact on phage titres. Mathematical modelling of phage-host interaction using well-described mass action laws [7,9] can be used to optimise the timing of phage addition and may be used to develop model based control strategies (Figure 2). Addition of phage too early in the exponential growth phage can reduce the amplification of phage resulting in lower

final phage titres (Figures 1, 2). Online monitoring of the optical density (e.g. Fundalux, Sartorius) allows precise timing of phage inoculation and can help reduce batch-to-batch variability (Figure 3). On-line monitoring of the host metabolic state using OUR/CER/RQ (e.g. Bluesens) allows close monitoring of the infection process and more precise control over identifying the time of harvest (Figure 3). Off-line nanoparticle concentration measurements allow at-line monitoring of phage amplification using dynamic light scattering (e.g. MADLS Ultra Blue, Malvern Panalytical) (Figure 4). In the future, where demand for phage products is well-established and larger volumes of phage may be needed, continuous production methods would allow the manufacture of phages using steady-state approaches with high volumetric productivity and reduced process footprint allowing process intensification and reduction in manufacturing costs [7].

Typically, the crude phage lysate is clarified by centrifugation (e.g., 1 litre lysate, 30min, x 16,000 g) and/or depth/crossflow micro-filtration (e.g. Sartobran or Sartocon, Sartorius) to remove cell debris and un-lysed cells (Figure 5). Membrane fouling during microfiltration is the main challenge and results in significant reduction in filtered phage fluxes (Figure 5). Centrifugation and microfiltration of crude lysate is a potential bottleneck in the processing of phages. Loss of phages during the microfiltration/depth filtration step is a common problem. Using specific primers and PCR/qPCR, the phage lot produced can be tested for confirmation of phage identity and/or presence of any contaminant phages previously processed in the facility. Phenotypic assays (e.g. plating the phage out on different host range strains) is also typically used to confirm phage identity. Addition of endonuclease (Benzonase, 4000 U/L, 2-3 hours) to remove host DNA and RNA in the lysate can help to reduce lysate viscosity and facilitates the ultrafiltration downstream purification step (Nucleic acid quantification can be done using fluorescence based assays such as Quant-iT™ PicoGreen®, ThermoFisher/invitrogen™ limit of detection 25 pg/ml).

DSP process development features the tangential flow filtration (TFF) ultrafiltration (UF) unit operation as the workhorse. In this unit operation, the phage lysate solution is recirculated under pressure tangentially across the membrane surface. A differential pressure is maintained across the membrane allowing selective passage of contaminants present in the lysate (HCP, HC-DNA, endotoxin etc) through the membrane as permeate but not the phage nanoparticles. Bacteriophage lysate purification focuses on selection of a suitable membrane and membrane molecular wight cut off (MWCO, typically testing 100kDa and 300 kDa are good starting points). Single-use flat sheet cassettes (e.g. Sartocon, Sartorius) and hollow fiber units (e.g. Repligen) are available with a variety of different MWCO (membrane molecular weight cut-off) and module sizes (membrane areas). Scale-up is based on membrane area and pilot-scale testing (typically 1-10 litre batch filtration runs). CPP include the shear rate at the membrane surface (10^3-10^4 s^{-1}, controlled by manipulating the feed flow rate which affects the cross-flow velocity), transmembrane pressure (TMP 0.1-1 bar) and the number of diafiltration volumes (typically between 5-10 per batch). The flux (l m^{-2} h^{-1}) increases with TMP thereby reducing batch processing time, until concentration polarization effects begin to dominate and the flux no longer increases with TMP. High initial flux rates decline over time due to membrane fouling and negatively influences the purification of the phage lysate (Figure 6). Typically, constant volume diafiltration is performed which could be done before or after a concentration ultrafiltration step (Figure 6). Online process monitoring includes TMP, permeate flux and measurement of conductivity and absorbance (surrogate for residual protein concentration) can be done online (Konduit, Repligen) or via offline sample measurement using fixed pathlength UV-Vis spectrometers.

Quarternary amine and mixed-mode chromatography media suitable for phage polishing e.g. reduction of endotoxin include membrane adsorbers (e.g. Mustang, Pall Life Sciences; Sartobind, Sartorius) and monolith columns (e.g. PrimaS, Sartorius BIA, hydrogen bonding and anion exchange

chemistries). The open structure of the monolith media allow high flowrates 1-5 CV/min (CV = column volume) and linear scale-up and high dynamic binding capacities ~10^{13} PFU/ml. CPP for the chromatography unit operation include loading buffer and pH, column media, equilibration buffer and salt concentration and gradient or step elution protocol and flow rates during column loading and elution. pH, conductivity and addition of excipients such as arginine and divalent metal ions (Ca^{2+}/Mg^{2+}) can improve phage recoveries. Typically binding using a low salt (150mM) buffer (Tris or PBS) and gradient elution using high salt (1M NaCl, again in Tris or PBS) is a good starting point for development. High salt in the solution buffer can result in phage aggregation (observed with DLS or TEM) so ultrafiltration to swap elution buffer for final formulation buffer is recommended as soon as possible following the chromatography step. Zeta potential measurements of purified phage as a function of buffer pH and ionic strength can aid development of the chromatography step (Figure 7). A final sterile filtration step using 0.2μm filters may lead to loss of phage drug substance particularly if the phage are present in an aggregated state (Figure 8). Both TFF and chromatography unit operations can be fully automated with target specifications on residual protein levels (BCA assay, ThermoFisher, limit of detection ~25 μg/ml) and endotoxin (LAL assay, Pierce, Thermofisher, limit of detection ~0.1 EU/ml, linear range 0.1-1 EU/ml). Host specific ELISAs, Time of Flight Mass Spectrometry (TOF-MS) and cell-based assays (cytokine response e.g. Monocyte Activation test, Merck) may be used for assessing and quantifying the impurity profiles and guide acceptance specifications for the DSP.

Product release testing of the phage drug substance and formulated drug product (e.g. cocktail of phages) require well-defined acceptance criteria e.g. purity levels of endotoxin and potency in terms of PFU/ml. In early pre-clinical development, these can have wider ranges which can be refined as the product is developed e.g. as more experience is gained during manufacturing and during pre-clinical evaluation [1]. IND (Investigational New Drug application) submissions need details of the acceptance criteria and release specifications that cover the identity, purity and potency of the phage drug substance and drug product. Biochemical and genotypic methods are used for identity. Assessment of product potency e.g. through measurement of phage viability assays (PFU/ml) require accurate and reproducible measurements which are also used to evaluate the performance of unit operations e.g. DSP TFF or chromatography units. Surrogate measurements such as qPCR and DLS may augment these data sets. Detection of subpotent batches depend on robust validated QC assays. Phage cocktails present challenges in terms of accurately accessing the potency of each phage making-up the cocktail in the final formulation. Plating out on a common host and use of PCR to evaluate identity of individual plaques may provide some validation of the potency of the different phages in the cocktails. Automation of such time-consuming assays is needed and may also help to improve robustness of such analytical methods.

Stability testing is an important consideration and requires setting-up of a stability testing plan demonstrating that the phage drug substance and phage drug products manufactured in a specific way remain within the acceptance specifications in terms of purity and potency under specified storage conditions and for the specified duration of storage. Examples of changes to liquid formulated phages include phage aggregation over time and precipitation of salts. Using XRD (X-ray diffraction), TEM (Transmission Electron Microscopy) and EDAX (Energy Dispersive X-ray analysis) (Figure 8), early detection of such problems allows reformulation of the drug product e.g. by changing the buffer pH or composition.

Well-defined SOP (standard operating procedures) and adequately controlled equipment and manufacturing environment are essential pre-requisites. SCADA (supervisory control and data acquisition) systems help automate the collection and analysis of data related to the production of

biopharmaceuticals in a cGMP environment. These data can help demonstrate that the manufacturing process is under control and producing phage drug products that meet quality standards. Single-use equipment helps reduce risk of cross contamination and can help avoid the complication of time consuming and costly CIP (cleaning-in-place)/decontamination and validation protocols where multiple phages are being produced in the same manufacturing facility. Manufacturing processes such as spray drying of phages which generate phage-laden dust are an example of a particularly challenging process for phage production from a containment and cleaning of facilities perspective. Sterility of the dry powder products is also a challenge.

Formulation development of phage biologics

Liquid and lyophilised formulations currently constitute the most common phage dosage forms undergoing clinical evaluations. Reduced molecular mobility and degradation kinetics in the dried state favours dry powder formulations [10]. Liquid formulations favour ease of use from a clinical point of view. A fundamental challenge in developing phage-containing therapeutic formulations is ensuring their stability in liquid aqueous buffered suspensions or as dry powders. Bacteriophages are often exposed to a variety of environmental stresses throughout their product life cycle. These include suboptimal buffer composition during purification (e.g. chromatography), freeze-thaw cycles (osmotic shock) and mechanical stresses (e.g. shear during ultrafiltration) during manufacturing and fill-finish, temperature deviations and light exposure during transportation and storage, and interaction with the surfaces of a variety of container-closure systems (polymer, glass etc). Their large size, structural complexity and compositional variability results in phages being prone to undergoing a variety of physical and chemical degradations resulting in loss of activity. Phages have a propensity to aggregate over time during storage (Figure 8). Conformational changes in tail proteins can affect the crucial phage-host binding event and hence loss of activity. Rational liquid formulation design and optimisation requires understanding of the routes of degradation to protect phages against physical and chemical damage. Temperature of storage, pH, buffer excipients and container type may affect the stability of the phage drug substance and drug product. Typically, formulation studies involve screening of the buffer type, pH, ionic strength and excipients (tonicity, surfactants, metal ions, amino acids, sugars and polyols, proteins, antioxidants, chelators etc). The route of administration and interaction of the formulation components with the dosing device e.g. metal parts of a spray dosing pump (e.g. nasal sprays used for upper respiratory tract applications) needs consideration. Impurities within pharmaceutical excipients including trace metal ions and bacterial endotoxin may affect the purity and stability of the formulated phages. A general universal approach to stabilising phages in liquid formulations isn't clear at present. Adsorption of phages at the solid-liquid (e.g. to container wall material) or gas-liquid interface needs consideration. Formation of aggregates or precipitation of buffer components resulting in presence of sub-visible and visible aggregates is controlled by regulatory agencies. Characterisation of the size range of particles in suspension from nm - μm can be done using tools such as SEM and DLS are part of QC testing and formulation stability studies. Characterisation of aggregates using electron microscopy, XRD (X-ray diffraction) and EDAX (Energy-dispersive X-ray analysis) can be illuminating in terms of which formulation components may need attention (Figure 8).

Freezing of aqueous liquid phage suspensions (e.g. for phage banking or during freeze drying) results in two phases: ice and freeze concentrated solution (FCS) consisting of concentrated buffer components and other excipients. Solidification of FCS occurs below its glass transition temperature typically below -35°C. Thus, storage in conventional freezers (-20°C) exposes phages to greatly increased concentration of the formulation components, pH changes and exposure to ice/solution interface which may accelerate aggregation (Figure 8). The rate of cooling (affects whether

cryoprotectants e.g. sugars have time to crystallize in the FCS or not), final freezing temperature and primary and secondary drying conditions are CPP [11]. The moisture content in the dry powder affects the material glass transition temperature and therefore storage conditions (temperature, relative humidity etc) affect the stability of the phages in the dry powder form [10]. The reconstitution process to rehydrate the freeze-dried phage formulation is a relatively unexplored area worthy of investigation.

Recent development in phage encapsulation strategies

Encapsulation of phages protects them from environmental and processing stresses, improves storage stability and it can make a significant difference in terms of improving targeted phage delivery and controlled release at the site of infection [2]. Encapsulation of phages has previously been evaluated in a variety of materials including hydrogels using different biopolymers e.g. alginate, pectin and chitosan [12–14], synthetic polymers e.g. PLGA and acrylic polymers [15,16] and liposomes [17,18]. Techniques used to encapsulate phages in powder form or as small micro- or nano- particles include spray drying [19], freeze drying [11], membrane emulsification [20], extrusion [21] and microfluidics [12]. Phages have been 3D-printed [22] and extruded into nanofibers [23] and as core-shell capsules [21]. Spray dried phages have been compressed to produce tablets for oral delivery [24]. Phages have been encapsulated to avoid the need to have a cold supply chain [2,25]. Formulations with pH-responsive triggers (responding to pH changes along the gastric tract) or in an encapsulation matrix that is degraded by enzyme action (e.g. hydrolases such as β-glucosidase or pectinase in the colon) or polymers that respond to local virulence factors (hyaluronidase produced by *S. aureus*) potentially allows targeted delivery and controlled release of phages [26,27]. A recent study for delivery of phage-based vaccines reported slow release (over a period of several days) of phage encapsulated in poly (lactic-co-glycolic-acid) (PLGA) microparticles prepared using a $W_1/O_1/W_2$ double emulsion [16]. Phages have also been immobilised on surfaces e.g. for food packaging biocontrol applications and for diagnostic purposes [28–30].

Thermo-responsive poloxamer (P407) gels having a low viscosity at 4°C and high viscosity at 25°C and above were used to encapsulate *E. faecalis* phages with *in vitro* data showing sustained release at 37°C over a period of one month [31]. An *acinetobacter baumannii* phage was found to be stable in the poloxamer formulation for up to 24 months stored at 4°C [32]. The effect of autoclaving (to prepare sterile injectable hydrogel formulations) on the rheological and thermo-responsive properties of the poloxamer formulations was evaluated and found not to result in significant changes [33]. However, interaction between the poloxamer and salts in the wound bed (in a rat osteomyelitis femoral defect model) were found to prevent gelation and the poloxamer dissolved in the blood pooling around the wound [33]. Phages incorporated in an alginate matrix containing hydroxyapatite (HA) nanoparticles were tested in *in vivo* implanted in rabbits to prevent infections following orthopaedic surgery [34]. Alginate matrix swelled at pH7 and released phages over time whilst the HA was incorporated to help facilitate bone regeneration (no evidence present in the paper to support this). Shear thinning hydrogels (containing a *S. aureus* bacteriophage) made from binary gels formed between beta-glucans or arabinogalactan and a variety of gums were tested as hydrogels and dried xerogels obtained using freeze drying. The rationale for the combinations studied was not clear and is quite usual for many such empirical studies. Variable phage stabilities were noted over a 6-month storage period stored at 4°C [35]. This study (and many others like it) highlight the lack of robust formulation design rules yielding stable matrices for encapsulating phages. Very few studies evaluate the effect of sterilisation and long-term storage stability of encapsulated phages even under refrigerated conditions. Storage at ambient conditions typically yield worse stability outcomes. There are also very few published studies evaluating the response of

cells and tissues to components in phage formulations and upon release of encapsulated phages. This may be important for intravenous application of phages or bladder irrigation to treat urinary tract infections. *In vitro* cell and tissue culture based assays may be helpful in this regard [15,36–38].

Conclusions and Future Perspectives

The process flow sheet for scalable production of phages is coalescing around a set of well understood unit operations. Documenting the critical process parameters for a variety of bacterial host-phage combinations and mapping the design space for production needs further work to allow reduction in batch-to-batch variability. Most commercially valuable phages can be produced using existing off-the-shelf technologies developed for manufacturing other complex biologics such as vaccines and monoclonal antibodies however, there are specific features of phages that require additional know-how and specialist expertise. Sensors for process monitoring and control coupled with modelling approaches can help in improving the quality and yield of phage bioprocesses. Formulation rules for production of stable phage drug products remain underdeveloped with different phages responding in an unpredictable fashion having undergone similar processing conditions. More advanced encapsulation strategies are being evaluated to improve delivery of high titres of phages at the site of infection or for microbiome modulation, but evidence of the success of these strategies remains to be shown in future clinical studies.

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