4 MATERIALS AND METHODS

4.1 Materials

The following chemicals and instruments/equipment were used during the formulation development and evaluation.

Table 4.1 List of chemicals

MATERIALS	SOURCE
Muller Hinton Agar	Himedia, Maharashtra, India
Agar	Himedia, Maharashtra, India
Luria Bertini	Himedia, Maharashtra, India
Tris hydrochloride	Himedia, Maharashtra, India
Magnesium sulfate	Himedia, Maharashtra, India
Gelatine	Himedia, Maharashtra, India
Antibiotic discs	Himedia, Maharashtra, India
Chitosan oligosaccharides	Sisco Research Laboratories, Mumbai, India
Sodium tripolyphosphate	Sisco Research Laboratories, Mumbai, India
Phosphotungustic acid	Merck, Germany
Glycerol (extra pure)	Thermo Fisher, Waltham, MA
DiD dye (1,1′-Dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate)	Thermo Fisher, Waltham, MA
Triton™ X-100	Thermo Fisher, Waltham, MA
Trehalose dihydrate	Thermo Fisher, Waltham, MA
Filmtracer® Live/Dead Biofilm Viability Kit	Thermo Fisher, Waltham, MA

Table 4.2 List of equipments

EQUIPMENT	SOURCE
Digital Electronic Balance	Schimadzu A×200, Electronic Balance
pH meter	Eutech Instruments, Germany
Magnetic Stirrer	Eltek Elecrocraft, Mumbai, India
Zetasizer	Malvern Zetasizer Nanoseries, S90
Brookfield viscometer DVE	AMETEK Brookfield
Benchtop Scanning electron microscopy	Jeol 6000, Germany
Cooling centrifuge	REMI NEYA C-24 plus
Scanning electron microscope	EVO-SEM, MA15/18, Carl Zeiss microscopy ltd
Microplate reader	Thermo Fisher Scientific - IN
Cryo-Transmission electron microscopy	TALOS S, Thermo scientific
Confocal microscope	CLSM 900, Carl Zeiss Microscopy GMBH
Lyophilizer	Decibel Electronic Technology
UV-C germicidal lamp (UV T8/25W, 253.7nm)	Philips, Ved group, India
Ultracentrifuge	Optima XPN-100 Ultracentrifuge
Ultrasound/Photoacoustic (USG/PA) imaging	VisualSonics, Vevo F2 LAZR-X PA scanner
Micropipette	Thermo Fisher Scientific - IN
6 well Multidish	Thermo Fisher Scientific - IN

4.2 Methods

4.2.1 Bacterial strain collection and antibiotic sensitivity test

A. baumannii (BHU/AB/39), *K. pneumoniae* (BHU/KP/657), *S. aureus* (BHU/SA/4193), and *P. aeruginosa* (BHU/PA/1956) (**Fig. 4.1**) bacteria were isolated from the pus sample of the wounded patient, provided by Prof. Gopal Nath, Department of Medical Microbiology, Institute of medical sciences (IMS), Banaras Hindu University, Varanasi. The obtained samples were sub-cultured in Mueller Hinton agar (MHA) media at 37°C overnight. The isolated bacterial strains were then maintained on nutrient agar slants at 4.0±1°C for future use. To assess the susceptibility of bacteria to antibiotics, antibiotic sensitivity tests (AST) were conducted. In this study, antibiotic discs (**Table 4.3**), pure colistin, and vancomycin were used to test the antibiotic sensitivity of bacterial strains (*A. baumannii, K. pneumoniae, S. aureus, and P. aeruginosa*) using the Kirby-Bauer technique. Briefly, antibiotic disks were placed in bacterial lawned MHA petri plates and overnight incubated (Thermo Fisher Scientific, India) at 37℃. Muller-Hinton agar has the advantage of allowing antibiotics to diffuse better than most other media. Inhibition zones are more accurate when diffusion is better (Nassar *et al.,* 2019). The diameter of the zone of inhibition was measured in millimeters (mm), and isolates were scored as susceptive or resistant by comparing the values with the recommended standard charts. In this experiment, *P. aeruginosa* ATCC 27853 strain and *S. aureus* ATCC 25923 strains were taken as controls.

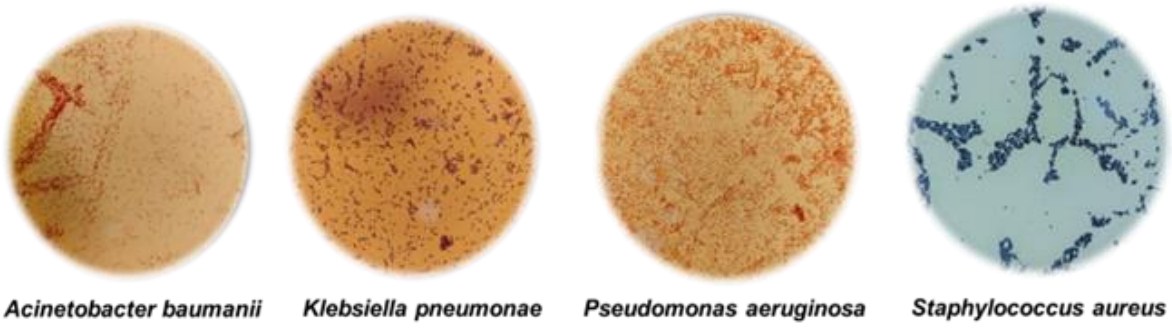

Figure 4.1 Gram staining image of bacteria

Table 4.3 List of antibiotic discs used for antibiotic sensitivity test

S. No	***A. baumannii***	***K. pneumoniae***	***S. aureus***	***P. aeruginosa***
a)	Piperacillin/ Tazobactam (100/10 mcg)	Cefepime (30 mcg)	Ampicillin (10 µg)	Ofloxacin (5 µg)
b)	Cefepime (30mcg)	Ertapenem (10 mcg)	Amikacin (30 µg)	Amikacin (10 µg)
c)	Co–Trimoxazole (25mcg)	Imipenem IPM (10 mcg)	Cefotaxime (30 µg)	Norfloxacin (10 µg)
d)	Amikacin (30mcg)	Oxacillin (1 mcg)	Clotrimazole (30 µg)	Piperacillin (100 µg)
e)	Ceftriaxone (30mcg)	Amikacin (10 mcg)	Gentamycin (10 µg)	Ceftazidime (30 µg)
f)	Polymyxin–B (300 units)	Piperacillin/ Tazobactam (100/10 mcg)	Ampicillin (10 µg) + Sulbactam (10 µg)	Gentamycin (10 µg)
g)	Gentamicin (10mcg)	Meropenem (10 mcg)	Cefepime (30 µg)	Polymyxin B (300 unit)
h)	Cefalexin (30 mcg)	Ceftazidime (30 mcg)	Penicillin (10 µg)	Piperacillin + Tazobactam (100 µg/10 µg)
i)	Ampicillin (10mcg)	Ofloxacin (5 mcg)	Linezolid (30 µg)	Aztreonam (30 µg)
j)	Ertapenem (10mcg)	Gentamicin (10 mcg)	Erythromycin (15 µg)	Cefepime (30 µg)
k)	Nitrofurantoin (300mcg)	Cefalexin (30 mcg)	Clindamycin (2 µg)	Imipenem (10 µg)
l)	Co–Trimoxazole (25mcg)	Nitrofurantoin (300 mcg)	Ciprofloxacin (5 µg)	Meropenem (10 µg)
m)	Ofloxacin (5mcg)	-	Vancomycin (30 µg)	-

4.2.2 Bacteriophage isolation, amplification, and purification

The BPs against the multidrug-resistant bacteria *A. baumannii, K. pneumoniae, S. aureus, and P. aeruginosa* were isolated from the Ganga River and Sunder Lal Hospital sewage water. The collected water samples were centrifuged at 9408 X g (REMI NEYA

C-24 plus, India) for 15 min at 4℃, and supernatants were collected. The process was repeated thrice to remove settled bacteria and debris.

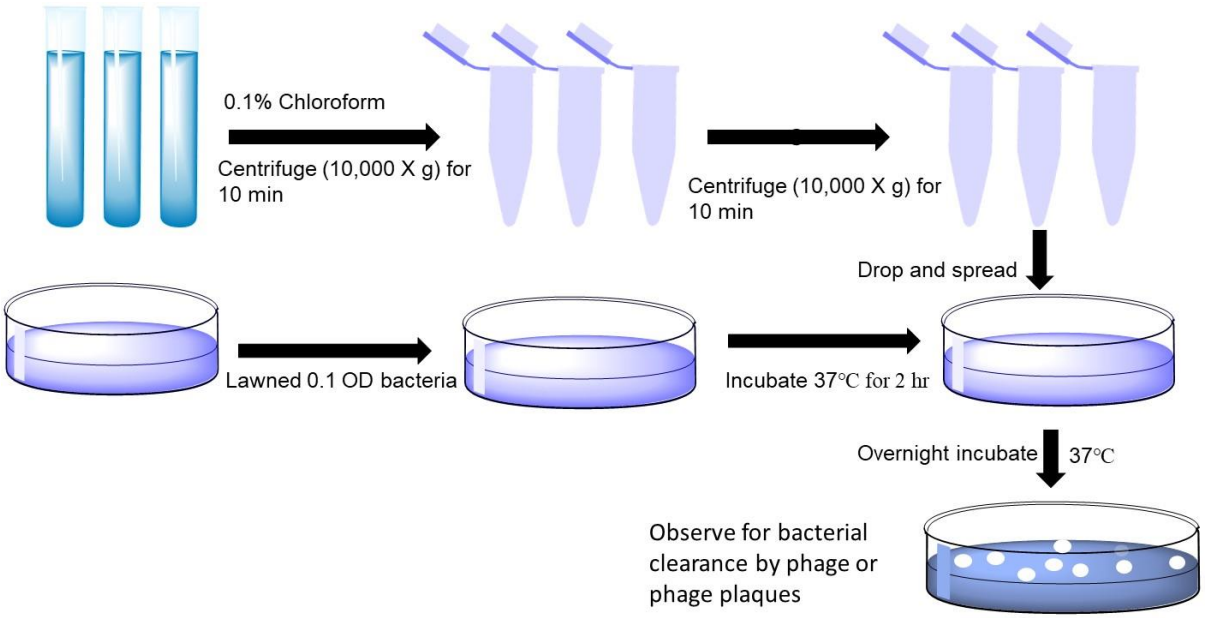

Figure 4.2 Method for isolating bacteriophages for use against specific microorganisms from diverse water sources

Finally, obtained supernatants were filtered using a 0.45 µm syringe filter. Further, processed water samples were dropped in a separate lawned bacterial MHA petri plate and incubated overnight at 37±1℃. After incubation petri plates were checked for plaques and harvested by using TMG buffer. The culturing and harvesting process was repeated until we got the complete bacterial clearance with an increased BP titer (**Fig. 4.2**) (Hyman, 2019; Singh *et al.*, 2022a).

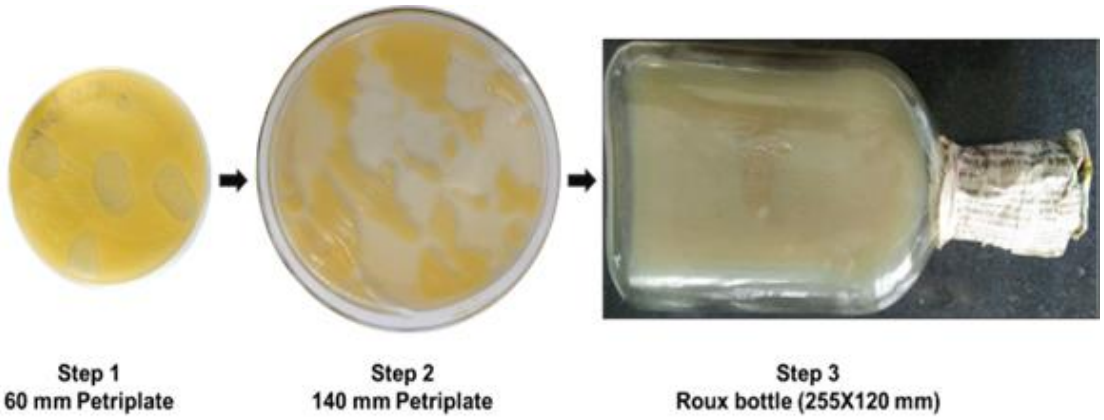

Figure 4.3 Amplification process of bacteriophages

The BP concentration was enhanced by amplification, which included three steps (**Fig. 4.3**); firstly, the isolated BPs were dropped on lawned bacteria in a small Petri plate (60 mm) and incubated at 37℃ for 16-18 h and harvested. This process was repeated until complete bacterial clearance of the entire plate was obtained. Secondly, the procedure followed in step 1 was scaled up using a bigger size petri dish (140 mm). Finally, BP concentration was amplified using a culture Roux bottle (255×120 mm). The foreign materials (bacteria, agar media, endotoxin, etc.) were separated from BP by 0.22 µm syringe filtration followed by polyethylene glycol (PEG) purification method (Luong *et al.*, 2020).

4.2.3 Bacteriophage characterization

4.2.3.1 Quantification of bacteriophage with plaque morphology

BPs were quantified by the double-layer agar overlay (DLAO) method and reported in terms of the phage titer value (**Fig. 4.4**). Briefly, 1 mL of each BP sample and bacterial host (200 µL of 1.0 optical density (OD)) was added to the separate soft agar (0.75% agar) in molten condition. The phage-host suspension in soft agar was overlaid on solidified bottom agar in 90 mm Petri plates. Plates were swirled gently, dried at room temperature for 10 min, and incubated overnight at 37°C. The next day, the BP titer was enumerated by the following equation (Kropinski *et al.*, 2009).

$$Bacteriophage\ titer\ (PFU/ml) = \frac{Numberof\ plaques}{Dilution\ x\ Amount\ plated}$$

Plaques obtained from the DLAO assay were analyzed based on plaque diameter, the presence of halo zones around plaques, and the bacterial clearance rate (El-Atrees *et al.*, 2022; Jurczak-Kurek *et al.*, 2016).

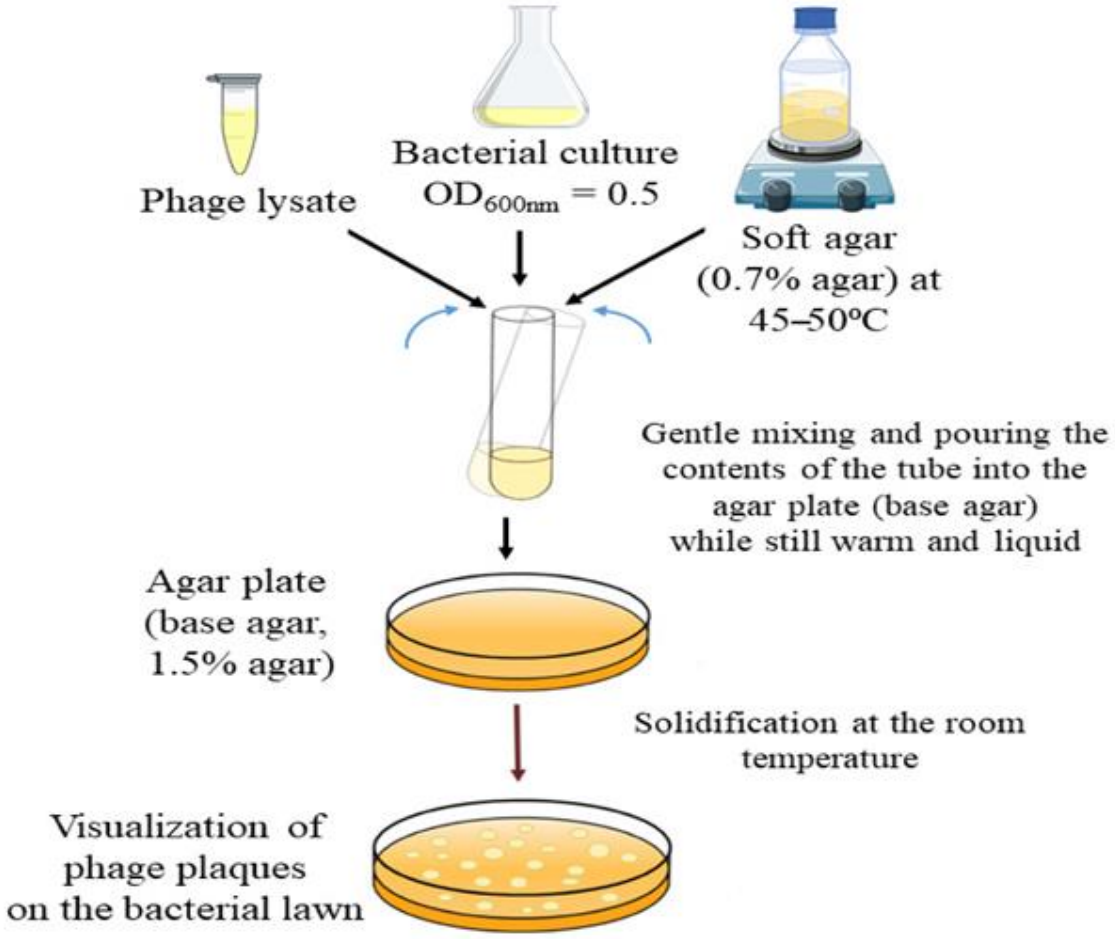

Figure 4.4 Double-layer agar overlay (DLAO) method

4.2.3.2 Morphological evaluation of bacteriophage

Transmission electron microscopy (TEM) was used to classify isolated BPs according to their size and morphology. BPs of 13.6 mL (~10^9-10^{10} pfu/mL) were centrifuged at 30,000 X g for 60 min, and the obtained pellet was washed thrice with 0.1 M ammonium acetate (13.6 mL, pH 7.0). Finally, the pellet was redispersed in 200 µL of ammonium acetate. Prepared samples (5 µL) were dropped onto a carbon-coated TEM grid, and Phosphotungustic acid (5 µL) as a negative stain was applied to the BPs (Singh *et al.*, 2022b). The BP morphology was examined by Cryo-TEM (TALOS S, Thermo scientific at SAIF-AIIMS Delhi).

4.2.3.3 Bacteriophage lytic range with phagogram

The BP lytic range (host specificity) was determined by using a modified phagogram spot test (Askoura *et al.*, 2021; Ferry *et al.*, 2022) and verified by using the DLAO method on 46 bacterial isolates **(Annexure Table 9.1-9.4**). In brief, 5 µL (10X serial diluted) of BPs (BPABΦ1, BPKPΦ1, BPSAΦ1, and BPPAΦ1) were dropped serially from high to low concentration on separate dry petri plates (90 mm, diameter) containing 0.75% w/v soft agar mixed with 200 µL (1 OD) of each bacterial species. The petri plates were incubated at 37°C overnight after the drops were dried at room temperature. The following day, petri plates were visually examined for a clear spot. The lysis activity was confirmed by performing the DLAO method of lowest lytic active dilution and observed for the plaques. Each spot test was performed three times.

4.2.3.4 Singular step growth curve

One (singular) step growth curve was performed to monitor the growth of BP in a respective bacterial host (*A. baumannii, K. pneumoniae, S. aureus, and P. aeruginosa*). This study helps to identify the burst size of BPs (BPABΦ1, BPKPΦ1, BPSAΦ1 and BPPAΦ1). The one-step growth curve was performed by the traditionally used method, which was reported by Ellis E.L. and Delbruck M. (Chang *et al.*, 2015; Ellis *et al.*, 1939). Briefly, freshly grown *A. baumannii, K. pneumoniae, S. aureus, and P. aeruginosa* ($1x10^6$ CFU/mL) were centrifuged (10 mL, 6000 X g for 10 min) and mixed with 10 mL of respective phage BPABΦ1, BPKPΦ1, BPSAΦ1, and BPPAΦ1. The multiplicity of infection (MOI=0.01) was maintained and incubated for 10 min (permitting phage adsorption at the bacterial surface) at 37°C with aeration (150 rpm). Centrifugation at 10,000Xg for 5 minutes was used to eliminate any non-adsorbed phages that were present in the mixture. The pellet was redispersed in 10 mL of LB broth and incubated. The bacteria and BP complex (100µL) were collected after different time intervals (5, 10, 15, 20, 25, 30, 35, 40, 50, 60, 55, 60, 65, and 70 min).

The sample set was diluted instantly and plated for BP titration by the DLAO method. The time series data were plotted by using GraphPad Prism 8.0, and the average burst size per infected host and average latent period was determined from the sigmoidal curves.

4.2.3.5 pH, temperature, and UV light stability studies of bacteriophage

To determine the pH stability, LB broth was prepared at different pH ranges (1.5, 3.5, 6.8, 7.4, and 8.5) with 6M NaOH and HCl solution. Phage solution (1 ml) was added to each tube corresponding to each pH value and incubated at 37°C for 24 hours (Thermo Fisher Scientific, India). To determine the thermal stability of the Bacteriophage, 1 mL of its filtrate was incubated for 60 minutes at various temperatures (37, 50, 55, 60, 65, and 70°C). The UV sensitivity of the bacteriophages was tested by placing the 1 mL solution under a UV-C germicidal lamp (UV T8/25W, 253.7 nm, Philips, Ved group, India) for the specified duration (0, 5, 10, 15, and 20 min). At the end of the experiment, the double agar overlay method was employed to examine the phage titer and all the experiments were performed in triplicate (Akhwale *et al.*, 2019; Allué-Guardia *et al.*, 2012; Vitzilaiou *et al.*, 2022).

4.2.3.6 Hemocompatibility assay of bacteriophages

Hemocompatibility testing is an integral part of biocompatibility testing. The developed formulations should be hemocompatible and not show any adverse effects when used for *in vivo* applications. The hemocompatibility assay of BPs was performed by a previously reported method with slight modifications (Pan *et al.*, 2017).

Fresh human blood was obtained from the blood bank, collected in an EDTA tube, and centrifuged at 135 X g for 15 min to separate RBCs. The settled RBCs were washed thrice with PBS (pH 7.4), and a 2 % suspension of RBCs was prepared in PBS (pH 7.4). Further, 100 µL (~10^4 PFU/mL) of each sample (phage BPABΦ1, BPKPΦ1, BPSAΦ1,

and BPPAΦ1) were mixed separately, with 500 µL of prepared 2 % RBCs suspension and incubated at 37°C for 1.5 h with gentle shaking at every 15 min. Further, each tube was centrifuged (212 X g for 5 min), the supernatant (200 µL) was placed in 96 well plates, and the absorbance was recorded at a wavelength of 545 nm. The PBS (pH 7.4) and Triton™ X-100 (1% v/v) treated RBCs samples were used as the negative and positive control groups, respectively. The hemolysis ratio (HR%) was calculated by using the following equation:

$$Hemolysis\ ratio\ percentage\ (HR\ \%) = \frac{(At - Anc)}{(Apc - Anc)} * 100$$

Where A*t*, A*nc*, and A*pc* are the absorbance of test samples, negative control, and positive control, respectively.

According to the American Society of Testing and Materials (ASTM, 2000), biomaterials are divided into three broad categories based on the degree of hemolysis, i.e. (a) hemolytic if hemolysis (%) is > 5%, (b) slightly hemolytic if hemolysis (%) is between 2 and 5%, (c) non-hemolytic if hemolysis (%) is <2%. Additionally, the hemolysis was also qualitatively confirmed on microscope visualization by Leishman stanning technique (Sareen *et al.*, 2018).

4.2.4 Formulation development

To ensure the suitability of excipients (chitosan, sodium tripolyphosphate, trehalose, and glycerol) for formulation development, a compatibility assessment with BPs was conducted. The experimental procedure involved incubating BPs (BPABΦ1, BPKPΦ1, BPSAΦ1, and BPPAΦ1) in LB (LB broth is a rich nutrient medium, originally developed for bacteriophage studies) (MacWilliams *et al.*, 2006) broth at 37°C for 24 h, with each excipient present in a 1:1 ratio. Finally, the BP titer was determined using the DLAO method (Dehari *et al.*, 2022).

4.2.5 Bacteriophage microparticle preparation

Chitosan microparticles (CHMPs) loaded single bacteriophage (BPABΦ1 or BPKPΦ1) and mixed bacteriophage (BPSAΦ1 with BPPAΦ1) were prepared for targeting single bacterial and polybacterial infections. CHMPs of BPABΦ1, BPKPΦ1, BPSAΦ1, BPPAΦ1, and mixed BPs (BPSAΦ1 and BPPAΦ1) denoted as BPABΦ1-CHMPs, BPKPΦ1-CHMPs, BPSAΦ1-CHMPs, BPPAΦ1-CHMPs, and MBP-CHMPs, respectively, were formulated by using the ion gelation method (**Fig. 4.5**).

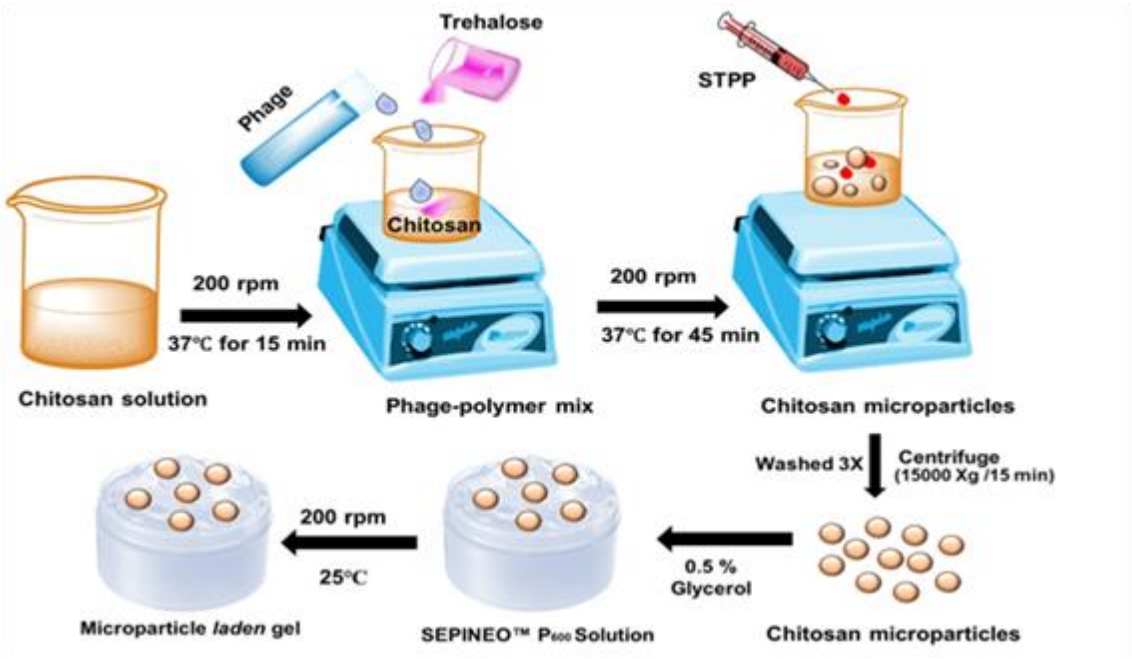

Figure 4.5 Schematic representation of bacteriophage microparticle gel preparation

Briefly, blank CHMPs were prepared by dissolving chitosan and D (+)-Trehalose dihydrate (T.D., 0.5 % w/v) in PBS (50 mL) and passed through 0.45 µm sterile syringe filter. Under sterile conditions with continuous magnetic stirring (200 rpm) at room temperature (25°C), the aqueous solution of STPP was added by syringe (gauge size 28) dropwise in the above-prepared solution. To acquire the phage-loaded CHMPs, 1.0 mL of the phage solution (Single phage (BPABΦ1, BPKPΦ1, BPSAΦ1, BPPAΦ1) or mixed phages MBP (BPSAΦ1 with BPPAΦ1)) were added separately to the polymeric solution, and similarly, STPP solution was added dropwise with continuous stirring.

Prepared microparticles (MPs) were centrifuged at 3387 X g for 10 min, and pellets were washed twice with PBS (3 mL) (Ilomuanya *et al.*, 2022; Rahimzadeh *et al.*, 2021). Further, the microparticles were lyophilized for incorporation into the gel. The composition of the various CHMPs has been presented in **Table 4.4.**

Table 4.4 Composition of the various microparticle formulations

Group	Chitosan (mg)	Phage (CFU/mL)	Trehalose % (w/v)	STPP (mg)
Blank CHMPs	650	-	0.5	12.0
BPABΦ1-CHMPs	650	8.2E+08 (BPABΦ1)	0.5	12.0
BPKPΦ1-CHMPs	750	1.3E+08 (BPKPΦ1)	0.5	14.0
BPSAΦ1-CHMPs	650	4E+08 (BPSAΦ1)	0.5	12.0
BPPAΦ1-CHMPs	650	1.3E+09 (BPPAΦ1)	0.5	12.0
MBP-CHMPs	650	2E+04 (BPSAΦ1) 1.3E+04 (BPPAΦ1)	0.5	12.0

Blank CHMPs: Blank chitosan microparticles
BPABΦ1-CHMPs: Bacteriophage against *A. baumannii* chitosan microparticles
BPKPΦ1-CHMPs: Bacteriophage against *K. pneumoniae* chitosan microparticles
BPSAΦ1-CHMPs: Bacteriophage against *S. aureus* chitosan microparticles
BPPAΦ1-CHMPs: Bacteriophage against *P. aeruginosa* chitosan microparticles
MBP-CHMPs: Mixed bacteriophage chitosan microparticles

4.2.6 Microparticle incorporation into gel

The developed microparticles were loaded into the gel for topical application on the wound. Microparticles-laden gel was prepared under aseptic conditions in laminar flow. Briefly, filtered SEPINEO™ P 600 (2.5% v/v of total formulation) was taken and mixed with 1.0 gm of individual phage microparticles (BPABΦ1-CHMPs or BPKPΦ1-CHMPs or BPSAΦ1-CHMPs, or BPPAΦ1-CHMPs) and mixed phage microparticles (MBP-CHMPs) (1.0 gm of the lyophilized microparticles), and sterilized glycerol (0.5% of total gel) by using magnetic stirring (200 rpm for 30 min) to make a uniform gel. Later, the prepared gel was kept overnight for proper swelling of gel and named mixed BPs

microparticles laden gel (MBP-CHMPs-gel) (Baghel *et al.*, 2020a). The blank gel was prepared without incorporating MPs. Further, blank-CHMPs-gel was prepared by using the above method with the incorporation of 1.0 g blank CHMPs.

4.2.7 Formulation characterizations

4.2.7.1 Analysis of particle size, polydispersity index, and zeta potential

Particle size, polydispersity index (PDI), and zeta potential of prepared MPs were determined using a zeta sizer (Malvern Zetasizer Nanoseries, S90) by dynamic light scattering and electrophoretic mobility method. Before analysis, samples were dispersed in Millipore water with a dilution of 0.1 mL of microdispersion to 1 mL (Li *et al.*, 2008).

4.2.7.2 Determination of entrapment efficiency

Entrapment efficiency was determined by the indirect method. The MPs dispersion was centrifuged at 21168 X g in a cooling centrifuge at 4°C for 15 min. The supernatant was collected and serially diluted (Anjum *et al.*, 2021). The amount of BPs present in the supernatant was determined by the DLAO method. Percentage entrapment efficiency (EE%) was determined by using the following formula.

$$EE\% = \frac{\text{Total amount of incorporated BP} - \text{free BP}}{\text{Total amount of incorporated BP}} x\ 100$$

4.2.7.3 MBP-CHMPs-gel characteristics

Organoleptic appearance (clarity, odor) and homogeneity of gel were determined by visual inspection. The viscosity of the prepared BP-CHMPs-gel was measured with a viscometer (Brookfield viscometer DVE, LV Spindle no. 61). Briefly viscometer spindle was dipped in a beaker containing 20 gm of gel and rotated at 50 rpm under room temperature, and viscosity of the samples was recorded in centipoise (Proniuk *et*

al., 2002). Additionally, the prepared gel was screened for spreadability test. Briefly, 0.5 g of the gel was transferred on a 2 cm circle-marked glass palate. The spreadability of the gel was then examined by placing a second glass plate on the top, with 500 g of weight for five minutes. The circle's increasing diameter was measured after the gel spread (Dantas *et al.*, 2016). Every measurement was made three times, with a fresh sample each time.

4.2.7.4 *In vitro* release study

In vitro release studies of phage-loaded microparticles and microparticles laden gel were carried out by a previously established method by Jamaledin *et al.*, 2023 (Jamaledin *et al.*, 2023; Patel *et al.*, 2019). Briefly, phage microparticles and phage-loaded MPs gel were suspended in 100 mL of phosphate-buffered saline (PBS) (pH 7.4) contained in a glass beaker, and maintained at 37°C, with continuous stirring at 100 rpm. Samples (1 mL) were periodically removed, and the volume of each sample was replaced with an equal volume of fresh medium. The amount of released BPs was analyzed by using the DLAO method. The *in vitro* release studies were performed in triplicate for each BP sample.

4.2.7.5 Surface morphology

Surface morphology of the prepared BPABΦ1-CHMPs, BPKPΦ1-CHMPs, BPSAΦ1-CHMPs, BPPAΦ1-CHMPs, and MBP-CHMPs was examined by scanning electron microscopy (SEM; EVO-SEM, MA15/18, CARL ZEISS MICROSCOPY LTD). A drop of diluted (50X) microparticle was casted on a coverslip, uniformly spread, and left overnight for drying under a vacuum. The samples were carbon-coated and imaged using SEM. In addition, the microparticle-laden gel (BPABΦ1-CHMPs-gel, BPKPΦ1-CHMPs-gel, BPSAΦ1-CHMPs-gel, BPPAΦ1-CHMPs-gel, and MBP-CHMPs-gel)

was lyophilized, and BT SEM (JEOL 6000, Germany) pictures with carbon coating were taken (Patel *et al.*, 2019; Winkelhausen *et al.*, 2010).

4.2.8 *In vitro* antibacterial studies

4.2.8.1 Minimum inhibitory concentration & minimum bactericidal concentration

Minimum inhibitory concentration (MIC) values of different groups (BPABΦ1-CHMPs, BPKPΦ1-CHMPs, BPSAΦ1-CHMPs, BPPAΦ1-CHMPs, and MBP-CHMPs) were determined against *A. baumannii, K. pneumoniae, S. aureus, P. aeruginosa* and a mixture of *S. aureus* and *P. aeruginosa* host respectively as per CLSI guidelines on planktonic cultures by the microbroth dilution method. In brief, bacteria were separately grown in LB broth ($1X10^8$ CFU/mL), and 100 µL of bacterial suspension was filled into 96 well plate. Then serially diluted 100 µL of MPs (ranging from 0.1-10 mg/mL) were added to the separate well. Corresponding bacterial controls (LB-grown bacteria), phage controls (phage stock solution), and media controls (LB broth) were maintained. The microtiter plates were incubated at 35°C for 24 h with gentle shaking at 20 rpm. The lowest concentration of BP at which no turbidity was seen was regarded as the MIC (Patel *et al.*, 2019).

Minimum bactericidal concentration (MBC) was also assessed by using the CLSI protocol. A sample (100 µL) was obtained from wells of microplates, where no apparent growth was seen after 24 h of MPs treatment and inoculated onto the surface of MHA plates. The resultant sample was maintained at 37°C overnight, with MBC indicating the minimum concentration of the substance at which no colonies formed. The lack of growth on the MHA plate indicated that the concentration was below the limit of detection for this method, which is 10 CFU/mL (Rodríguez-Melcón *et al.*, 2021).

4.2.8.2 Antibiofilm assay

The ability of phage to eradicate mature biofilms is much more important than their ability to inhibit the formation of biofilms. A previously established quantitative microtiter plate assay (crystal violet) was used to assess the antibiofilm efficacy of MPs (**Fig. 4.6**) (Liu *et al.*, 2021; Plota *et al.*, 2021).

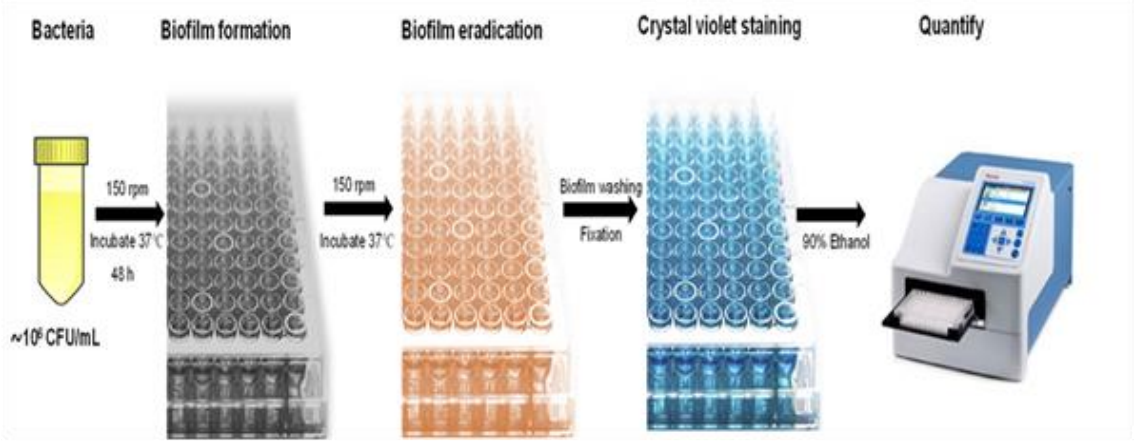

Figure 4.6 Microtiter plate antibiofilm eradication study

In brief, 180 µL of LB growth media with 20 µL of bacteria *A. baumannii, K. pneumoniae, S. aureus, P. aeruginosa,* and a mixture of *S. aureus* and *P. aeruginosa* (0.5 OD_{600nm}) were separately (20 µL) transferred in a sterile 96-well plate and incubated at 37℃ for 48 h. Further, planktonic bacteria were aspirated, and specific MPs (200 µL, 2X dilution of MIC) were added to the respective well, followed by incubation for 24 h. The sample of the microplate wells was removed after incubation and thoroughly rinsed with PBS (pH 7.4) to eliminate free-floating non-adherent bacteria. The microplate wells were then air-dried for 60 minutes. Following drying, adhering "sessile" bacteria in the wells were fixed with 2% w/v sodium acetate before being saturated with 0.1% w/v crystal violet dye and placed in a dark room for 30 minutes. The wells were then carefully cleansed with deionized water to eliminate any remaining color. After drying the plate, 200 µL of ethyl alcohol (95%, v/v) was introduced to each well, and absorbance at 570 nm was recorded (Multiscan plate

reader, Thermo Fisher Scientific). Wells containing only growth media inoculated with the tested bacterial isolates were used as control. The mean value of the three measurements was reported. The percentage of inhibition of biofilm formation was calculated using the following equation.

$$Inhibition\ \% = \frac{Optical\ density\ of\ control - Optical\ density\ of\ treatment}{Optical\ density\ of\ control} * 100$$

4.2.8.3 Microscopy of Biofilm

The overnight cultures of *A. baumannii, K. pneumoniae, S. aureus, P. aeruginosa,* and a blend of *S. aureus with P. aeruginosa* bacteria were distributed into a 12-well culture plate equipped with sterile round coverslips. Subsequently, the plate was placed in an incubator and left undisturbed for a period of 48 h. After incubation, the formed biofilm was treated with BPABΦ1-CHMPs, BPKPΦ1-CHMPs, BPSAΦ1-CHMPs, BPPAΦ1-CHMPs, and MBP-CHMPs against *A. baumannii, K. pneumoniae, S. aureus, P. aeruginosa*, and a mixed bacteria (*S. aureus with P. aeruginosa*) respectively in a concentration of (~10^9 PFU/mL) and then incubated further for 24 h. Next, the glass slide was washed using PBS and dried. The biofilm eradication was analyzed by SEM (Carl Zeiss Microscopy Ltd.).

The polybacterial biofilm matrix eradication was also studied by using confocal microscopy followed by the treatment with MBP-CHMPs. Filmtracer Live/Dead Biofilm Viability Kit was used for staining cells inside the extracellular matrix grown on the coverslip. Glass slides were submerged in a 6 μM SYTO9 and propidium iodide (30 μM) staining solution for 30 min. After staining, the glass discs were washed with PBS, and images were captured by confocal microscope (CLSM 900, Carl Zeiss Microscopy GMBH) at 40X magnification (Guo *et al.,* 2019). Further, the complexity and eradication of poly bacterial biofilm were also analyzed by Atomic Force Microscopy (AFM, NTEGRA Prima, NT-MDT) (Gomes *et al.*, 2017).

4.2.9 *In vivo* studies

All animal experiments were approved by IAEC, Dept. of Pharm. Eng. & Tech., Indian Institute of Technology (BHU), Varanasi, U.P., India. Formulation efficacy was evaluated in female/male Wistar rats (200-250 g). The animals were randomly divided into different groups (n=5). *In vivo* efficacy was studied against both mono-bacterial infections (*A. baumannii* or *K. pneumoniae or S. aureus or P. aeruginosa*) and polybacterial infection (*S. aureus with P. aeruginosa*) on burn wounds.

4.2.9.1 *In vivo* burn wound healing study by using Ultrasound and Photoacoustic (PA) imaging system

For developing the burn wound, animals were first anesthetized with an intraperitoneal injection of ketamine and xylazine (80 mg/kg and 20 mg/kg). A cylindrical stainless-steel rod (1.5 cm diameter) was heated to 100 °C in boiling water and placed in the dorsal region of the rat for 20 s. After 6 h of wound creation, different bacterial suspensions (100 µL of 1 OD) (*A. baumannii*, *K. pneumoniae*, *S. aureus, P. aeruginosa, and mixed bacteria*), were swabbed on the wound area, and 20 µL injected subcutaneously for two successive days to induce different bacterial infections and robust biofilm formation in the wound. After the development of burn-induced wounds, the animals were randomly divided into different groups (n = 5) Group 1: control group (no treatment), Group 2: treatment with marketed formulation, Silvadene® cream (silver sulfadiazine (SSD 1.0%), Group 3: treatment with prepared phage microparticle gel specific against each bacterial infection. Specific Phage formulation (0.5 g) was applied twice daily, and the wound healing potency of prepared gel was evaluated by measuring the wounded area following the different treatments by a scale on different days until complete re-epithelialization. The wound healing was quantified using a scale:

$$\%\ \text{wound retraction on day X} = \frac{(\text{wound area on day zero} - \text{wound area on day X})}{(\text{wound area on day zero})}$$

Simultaneously, the wound volume and oxygen saturation were assessed by ultrasound/photoacoustic (USG/PA) imaging system (VisualSonics, Vevo F2 LAZR-X PA scanner, UHF 48 transducer, FUJIFILM VisualSonics Inc., Toronto, Canada) (Kim *et al.*, 2018; Suda *et al.*, 2022). Additionally, angiogenesis, wound volume, and saturated oxygen percentage were also validated using ultrasound and photoacoustic imaging. The scanning of the wound was performed in B-mode (ultrasound imaging) and oxy-hemo mode at 750/850 nm laser for photoacoustic imaging. The following imaging parameters were set for photoacoustic imaging: PA gain = 37 dB, step size 0.33 mm, power 100%, and sensitivity was kept high. During imaging, rats were anesthetized by using 3% isoflurane (induction dose) and 1.5–2% maintenance dose. The animals were then laid prone position on an operating table maintained at 37 °C to perform the ultrasound and photoacoustic imaging. All the images were processed by using Vevo LAB software (FUJIFILM VisualSonics, Toronto, ON, Canada). Moreover, angiogenesis, wound volume, and saturated oxygen percentage were validated using ultrasound and photoacoustic imaging. The time required for re-epithelialization was calculated as the number of days needed for wound healing to the number of days required for the eschar to come off without leaving any raw wounds. The time required for complete re-epithelialization of the wound was considered the endpoint of the experiment.

4.2.9.2 Histopathological examination

After the animal undergoes complete re-epithelization, the wound skin is removed and fixed in formalin before being processed and encased in paraffin. Sections were cut, where 5 µm sections were stained with hematoxylin and eosin. The optical microscope was used to examine the re-epithelization of wound skin and organ damage. Epithelialization, fibroblast proliferation neovascularization, and collagen deposition

were also observed under a light microscope (Olympus BX51; Olympus, Tokyo, Japan; magnification: ×100) (Li *et al.*, 2016).

4.2.10 Gel occlusion and bioimaging study

Microparticles and gels loaded with DiD dye were formulated to enable fluorescence imaging of the wound area following their application. The Photon Imager Optima System (Biospace Lab, France) was employed for capturing the fluorescence images. Animals were anesthetized under a continuous flow of 3 % isoflurane and fluorescence signals were captured at excitation and emission wavelengths of 620 and 710 nm, respectively at 0, 3, 4, 9, and 12 h post-application of the free DiD and DiD loaded formulation. The radiant efficiency (measured as fluorescence intensity/area/time) was analyzed using the Biospace Lab imaging software, and the region of interest (ROI) tool was used for circling the wound area.

4.2.11 Stability studies

BPs solutions, BPs microparticles, and BPs microparticles gel were sealed and stored at 4℃ for 8 months. After each month, it was placed in a beaker containing 50 mL of PBS (pH 7.4) and kept for continuous stirring for 24 h, and liveability and lytic activity against host bacteria was checked by the DLAO method (Puapermpoonsiri *et al.*, 2009).

4.2.12 Statistical analysis

Data for the *in vitro* and *in vivo* experiments were presented as the mean ± SD (n = 3). GraphPad Prism 5.0 was used for statistical calculation. One-way ANOVA was used to analyze the statistical significance among the groups. The following statistically significant levels were considered as non-significant (ns) ($p \geq 0.05$) and significant: * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$).