Title: Chitosan-based microparticle encapsulated Acinetobacter baumannii phage cocktail in hydrogel matrix for the management of multidrug resistant chronic wound infection

Short title: Encapsulated A. baumannii phage for chronic wound

Margaret O. Ilomuanya¹, Nkechi V Enwuru², Emmanuella Adenokun¹, ABIGAIL FATUNMBI¹, Adebowale Adeluola², Cecilia I IGWILO¹
¹Department of Pharmaceutics and Pharmaceutical Technology, Faculty of Pharmacy. University of Lagos, PMB 12003, Surulere, Lagos, Nigeria
²Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmacy. University of Lagos, PMB 12003, Surulere, Lagos, Nigeria

Corresponding Author Information
Margaret O. Ilomuanya
milomuanya@unilag.edu.ng
+234 8033295077
https://orcid.org/0000-0001-8819-1937
22.05.2021
16.08.2021
24.08.2021

Abstract
Objectives: Multi-drug resistant bacteria has been implicated in various debilitating infections that have led to life loss. This study developed an approach to tackle multi drug resistant Acinetobacter baumannii infection in a chronic wound model through Acinetobacter baumannii phage encapsulation with resuspension in hydrogel.

Materials and Method: Two isolates of A. baumannii specific lytic phages, φAB140 and φAB150 alone, in combination (cocktail) encapsulated within a chitosan microparticle was suspended in chitosan hydrogel and evaluated for their therapeutic efficacy to ensure bacterial clearance in Acinetobacter baumannii induced diabetic wound infection. Microencapsulation of the phage was carried out using ionic gelation technique. Biological characterization via cell cytotoxicity, in vivo wound healing, histology and histomorphometry were carried out.

Results: Two characterized A. baumannii phages (φAB140 and φAB150), specific to twenty A. Baumannii isolates were isolated. The encapsulated chitosan microparticle hydrogel exhibited pH of 5.77 ± 0.05. Wound size reduction was most pronounced in formulation C2 which showed statistically significant wound seize reduction at day 4 and day 7, 56.79% ± 2.02 and 62.15% ± 5.11, respectively. The optimized concentration of C2 was not toxic to the cells as it adequately supported cell growth with a proliferation rate of 215% ± 7.89% compared to control (107.32% ± 4.55%).
**Conclusion:** Micro particle carrier technology was utilized to show the lytic activity against multi drug resistant *Acinetobacter baumannii*. *In vivo* results showed significant wound size reduction which was most pronounced in formulation C2 at day 4.

**Key words:** φAB140 and φAB150 phage, microparticle hydrogel, chronic wound, cytotoxicity.

**INTRODUCTION**

Wound healing requires collagen synthesis, cell migration, angiogenesis, blood clotting, extracellular matrix adhesion, and immune/ inflammatory response, amongst other complex biological processes to ensure the complete restructuring of the skin in the area where the wound occurred. Diabetic wound healing is however mitigated by both systemic and local factors. Systemic factors include the use of steroids, antineoplastic agents and NSAIDs, deficiencies of vitamins A, C & E, magnesium, zinc and copper; nutritional status, associated illnesses/immunity as well as patient age. Blood glucose level, diabetic neuropathy, immune system deficiencies, infections, mechanical stress, chemical stress and pressure are some of the local factors affecting diabetic wound healing. Diabetic wounds are also promoted by the presence of microorganisms that are resistant to antimicrobials.

Infection of diabetic wounds impairs the inflammatory phase of natural wound healing. This is due to pathogenic microbes competing with macrophages and fibroblasts for limited resources, hence impairing neutrophil function, chemotaxis, phagocytosis and decreased t-cell response, leading to necrosis in the wound bed; sepsis and perhaps death. In a bid to combat antimicrobial resistance, encapsulation of bacteriophage has been used to tackle systemic infections caused by *Staphylococcus* sp and *Mycobacterium* sp. Liposome entrapment of bacteriophages has been used as a veritable approach for treating bacterial associated infections after encapsulation of phage cocktail facilitated tissue healing in a diabetic excision wound infection associated with *Staphylococcus* sp.

The prevalence of antibiotic resistant infections in individuals living with diabetes was measured and compared against non-diabetics and found to be 63.4% in diabetic patients as against 50% in non-diabetics. Multidrug resistant organisms (MDRO) were more prevalent in the diabetic population and co-infections were also higher. In a more recent study by Lee et al., high levels of antibiotic resistance and MDRO were observed to limit the treatment options of individual with diabetic wound ulcers. Chitosan based hydrogels have been used as carriers for other therapeutic molecules in enhancing diabetic wound healing. They are ideal to promote wound healing as they serve as both wound dressings and drug delivery systems. Different polymeric and oil-based drug delivery platforms have been examined for delivery of bacteriophages however these drug delivery platforms for bacteriophage delivery of φAB140 and φAB150 have not been documented. A simplified stepwise system for drug delivery involving bacteriophages for translation to bedside use in management of chronic life threatening and debilitating infections is currently not available. The present study presents a novel attempt to encapsulate φAB140 and φAB150. This study developed an approach to tackle multi drug resistant (MDR) *Acinetobacter baumannii* infection in a chronic wound model through *Acinetobacter baumannii* phage (φAB140 and φAB150 phage) encapsulation with resuspension in hydrogel.

**MATERIAL AND METHODS**

**Chemicals and reagents**

Chitosan (MW 80,000 g/mol, degree of deacetylation 80%), polyether sulfone, sodium chloride, polyethylene glycol (PEG), sodium tripolyphosphate (TPP), Alloxan monohydrate and DNase (were obtained from Sigma-Aldrich, St. Louis, USA), glutaraldehyde, acetic acid, Tween 80 and
hydrochloric acid were obtained from Fluka, Steinheim, Germany, pepsin, KH2PO4, sodium hydroxide, pancreatin. bicinechonic acid solution were obtained from (Macklin Biochemical Co. Ltd, Pudong China). Ketamine and xylazine were obtained from Tocris Bioscience Bio-Technne Corporation MN, USA. HPLC Acetonitrile and phosphate buffered saline (Merck Darmstadt, Germany). The water used in formulation development was Milli-Q water. All other chemical reagents were of analytical grade and were used without further purification.

**Bacteria strains**

Fifty wound samples were collected from Medical Microbiology laboratory of Lagos University Teaching Hospital, Nigeria. The samples were sub-cultured on varying media including MacConkey and blood agar at 37°C with time equals 24 hrs. Gram staining and bio-chemical tests was carried out on all bacterial isolates. The identified *A. baumannii* isolates were confirmed using Microbact 12A and B system.

**Antibiotic susceptibility test**

Kirby-Bauer method was utilized to undertake antibiotic susceptibility screening of the *A. baumannii* isolates. Seventeen antibiotics in various concentrations including but not limited to 10 μg imipenem, 30 μg cefepime, 10 μg levofloxacin, 10 μg meropenem, 30 μg amikacin etc were used in this study in line with the procedure from Tanner et al.

**Phage isolation and Purification**

Ten multidrug resistant *A. baumannii* strains were selected for isolation of *A. baumannii* specific phage. Sewage and canal water samples were used for phage isolation. Sewage water was collected from sewage treatment plant of the Department of Works and Services, University of Lagos, while canal water sample were collected from canal behind Lagos University Teaching Hospital. The phage was isolated using enrichment protocol as previously described. Two clonal differences of the *A. baumannii* isolates (ϕAB140 and ϕAB150) that showed very distinct and clear plaques were picked for further purification test. The phage was purified through optimization and biokinetic measuring process as previously described. The phage was amplified against their specific host bacteria stains and titre quantified; this was followed by storage at 4°C.

**Chitosan microparticle encapsulating Acinetobacter baumannii phage cocktails (ϕAB140 & ϕAB150) in hydrogel matrix**

Chitosan (0.1%w/w) was dissolved in 10 mL acetic acid (4% v/v). Fifty microliter of *Acinetobacter baumannii* phage cocktails (1 x 10¹¹ PFU/mL) was added to the chitosan solution under magnetic stirring and continuous sonication to obtain a homogenised fluid at 25°C ± 0.5°C. Utilizing a gauge 25 needle, 20 mL of the phage-chitosan dispersion was then added drop wise into 100 mL of 2% w/v TPP with continuous sonication. The droplets instantaneously gelled into discrete Phage-CS microcapsules upon contact with the crosslinking agent. The microparticle suspension was subsequently centrifuged for 25 min (2750 rpm). The pellet was resuspended in Milli Q water to wash the microparticles and centrifuged again. This washing procedure was repeated twice and the encapsulated phage was stored overnight in an amber glass vial. To prepare the plain gel to be utilized as the control, chitosan (0.1% w/v) was dispersed in Milli-Q water and 0.1% v/w acetic acid. The gel was homogenized at 200 rpm for 1 h and 50 μL of the phage cocktail (1 X10¹¹ PFU/mL) was mixed with 20 mL of the gel and homogenized at 100 rpm for 30 minutes and then stored in a glass vial at 25°C ± 0.5°C.
Formulation C1 contained 20 mL of hydrogel alone whilst formulation C2 contained 20 mL of hydrogel and 50 µL of bacteriophage component comprising *Acinetobacter baumannii* phage cocktail.

**Physical evaluation and Morphological Characterization of the gels**

The hydrogels were evaluated for homogeneity. The pH of the gel was recorded in triplicate and the viscosity was also examined with the use of a DV-E Digital viscometer, Brookfield viscometer at 25 °C, 20 rpm using spindle 04. Scanning electron microscopy (SEM) using JEOL JSM-6360L instrument was used to determine approximate shape, size, and uniformity of the microparticles.

**Microparticle entrapment and yield determination.**

Microparticles suspension (10 mL) was centrifuged (20,000×g / 30 min), followed by supernatant decantation from the microparticle pellets. The pellets were freeze dried to ensure that all residual moisture was removed. Dried microparticle powder was weighted and percentage microparticle yield determined by dividing the weight of the microparticles by the cumulative weight of total solids including chitosan (CS), triplyphosphate (TPP). The weight of the phage particles was excluded from the microparticle yield calculation (Eq. 1)

\[
\text{microparticle yield (\%) = } \left( \frac{\text{microparticle weight}}{\text{Total solids (CS + TPP - 1)} \text{ weight}} \right) \times 100 \quad \text{Eq. 1}
\]

*Acinetobacter baumannii* phage microparticle suspension was evaluated for entrapment efficiency. under centrifugal force of 21700×g for 15 minutes. aided the concentration in the supernatant was determined by bicinchoninic assay where the supernatant from chitosan nanoparticles is utilized as the blank sample. Utilizing bicinchoninic assay the phage entrapment efficiency was determined using Eq. 2.

\[
\% \text{ Entrapment efficiency} = \left( \frac{\text{Total phage} - 1 - \text{free phage} - 1}{\text{total phage} - 1} \right) \times 100 \quad \text{Eq.2}
\]

**FTIR-Fourier-transform infrared spectra**

Using a Shimadzu 8400 FTIR spectrophotometer, FTIR spectra of free and vacuum dried encapsulated phage in CS containing samples were recorded. FTIR analysis was done at Agilent Technology to determine the various bands and compounds present in the chitosan molecule. Twenty scan per spectra were recorded between 4000 to 400 cm⁻¹.

**Accelerated stability studies**

The stability of chitosan microparticle encapsulating *Acinetobacter baumannii* phage cocktails (ɸAB140 & ɸAB150) in hydrogel matrix (Formulation C1 and C2) was determined by resuspension of the formulation in sterile distilled water at the concentration of 20% w/w using a modified method of Pirnay et al. The hydrogel was incubated at 25°C ± 2°C and analysed for their size different time intervals (0, 10, 15, 30, 60 and 90 days) using scanning electron microscopy as previously described. Accelerated stability testing of the chitosan microparticle encapsulating *Acinetobacter baumannii* phage cocktails (ɸAB140 & ɸAB150) in hydrogel matrix was then evaluated using the ICH
guidelines (40 ºC/75 %RH). Formulations C1 and C2 were stored in amber coloured vials and kept in a stability chamber with set temperature and relative humidity. The formulations were subjected to accelerated stability testing at both room temperature and at 40ºC and parameters were recorded on day 0, 10, 15, 30, 90. The formulations were evaluated for pH, homogeneity, appearance, and viability of the phage within the formulation using earlier described methodology.

**Biological Characterization**

*Antimicrobial determination of Acinetobacter baumannii encapsulated phage hydrogel in vitro*

To investigate the lytic activity of *A. baumannii* phage cocktail (ϕAB140 and ϕAB150) hydrogel against multi drug resistant *A. baumannii* isolates, spot test was performed. Bacterial lawn was prepared using double layer agar plate method with Luria Bertani agar. The lawn was allowed to set and four microparticles of formulation C1 and C2 were spotted on the bacterial lawns, the plates were left uncovered within aseptic zone for 5 min to allow the spots to dry and incubated (37 ºC for 24 h) and lytic activity observed. Draize tests was performed to ensure non reactivity of the formulated hydrogels on intact skin.16

**In-vitro release study**

ϕAB140 & ϕAB150 release from chitosan microparticle encapsulating *Acinetobacter baumannii* phage cocktails in hydrogel matrix was determined 25 mg microparticles were transferred to a tube with 5 mL 50mM phosphate buffered saline maintained at pH 7 and 37 ºC ± 1 ºC. At predetermined time intervals (2, 4, 6, 8, 10, 12 and 24 h), 2 mL of sample was removed after centrifugation at 21,700 × g for 30 min, and 2 mL of the supernatant removed was replaced with fresh 50mM phosphate buffered saline. The sample from each time interval was analysed by BCA assay to determine concentration of the released protein.

**Cell toxicity assessment**

Using method of Ilomuanya *et al.*,17 double enzymatic digestion, isolation of keratinocytes obtained from human foreskin (a waste product of male circumcision) was carried out. The formulation was carried using aseptic technique and hence it was not necessary to carry out sterilization. The chitosan based microparticles were also cultured to ensure that no bacterial contamination occurred in line with good quality control measures.17 MTT assay was carried out using chitosan-based microparticle encapsulated *A. baumannii* phage at concentrations of (0, 0.05, 0.1, 0.25, 0.5, 0.75 and 1 mg/mL). Cell cultured in wells devoid of phage was taken as control.

**In vivo wound healing**

Sixty thirteen-week-old rats breed specifically for research without exposure to medications and weighing between 150 and 165 g were used for the study after a seven-day acclimatization period. The animals were housed at a steady thermostatically controlled condition (12hr night/day cycle; 29 ± 2 ºC; 45 ± 10% RH). Standard feeding conditions was provided in line with international best practices, as stated within the study ethical approval (CMUL/HREC/09/19/676).

Experimental timelines (Fig. 1) show that the wound healing study was carried out within 14-day period. Diabetes was induced using 150 mg/kg body weight alloxan monohydrate at in a 2- day time lapse in the fasted rodents.18 After the alloxan injection was administered rats having fasting blood glucose levels above 190.5 ± 8.1 mg/dL with the random blood glucose levels of above 350 mg/dL were considered severely diabetic and selected for infection studies.18
**Wound Model:** Overnight culture of MDR *A. baumannii* isolate corresponding to $1 \times 10^9$ CFU/mL was prepared. Diabetic rats were taken and distributed in four different groups of six animals each randomly distributed. Epilation was carried and the skin cleaned with rubbing alcohol prior to wounding through the skin to the *panniculus carnosus* using a 5mm diameter punch. The wounds in Group 1, 2, 3 and 4 were left open and inoculated with 0.1 mL of *A. baumannii* ($1.5 \times 10^8$ CFU/mL) suspension. The animals were given ibuprofen suspension (5mg/kg bwt) to reduce pain. The infection was allowed to take hold for 24 hours before the treatment protocol commenced as follows.

Group 1: The infected rats that received no medication.
Group 2: The infected rats were treated with gentamicin ointment twice a day.
Group 3: Formulation C1 was applied on infected wounds, twice a day.
Group 4: Formulation C2 was applied on infected wounds, twice a day.

Wound size was determined using a digital camera with image calibration capacity and calculated using Relative wound size reduction measurements was calculated based difference in wound diameter $A_o$ and $A_t$; on at treatment time in days i.e. initial wound diameter and diameter at predetermined time intervals respectively (Eq. 3).

$$\text{Relative wound size reduction} \% = \frac{A_o - A_t \times 100}{A_o}$$  \hspace{1cm} \text{Eq. 3}

**Bacterial load in wounds:** MDR *Acinetobacter baumannii* on the surface of the wound, samples were collected from the wound surface using sterile swab stick for three times on day 7, 10, and 14. The samples were processed via nutrient broth inoculation and a 12-hour incubation at 37°C prior to identification and evaluation of the presence of MDR *Acinetobacter baumannii*. 18, 19

**Histology:** Fourteen days post treatment of the infected wound, skin sections fixed in 10% formaldehyde were stained using Hematoxylin/eosin and Masson’s trichome and histological profiles recorded using a Microsystems microscope (Mannheim, Germany). 2

**Histomorphometry**
DM1-300 Camera (South Korea) was used as the Image analyser was used to evaluate the histological skin samples. Percentage re-epithelialization was calculated as in Eq. 4.

$$\% \text{ Re-epithelialization} = \frac{[\text{Total wound length (mm)} - \text{Desquamated epithelium region (mm)}] \times 100}{\text{Total wound length (mm)}}$$  \hspace{1cm} \text{Eq. 4}

**Statistical analysis**
Graph pad prism software Ver. 5 was utilized for statistical analysis using data obtained in triplicate. All reported date are expressed as mean ± SD of experimental values for each variable.

**RESULTS**
**Phage isolation and Purification**
MRD *A. baumannii* was used in the study. Five *A. baumannii* specific phages were isolated among ten *A. baumannii* strains used for phage isolation. However, two unique phage (φAB140 and φAB150) were selected for the study amongst the five-phage library based on high lytic activity, broad host range and the plaque size formation. (Plate 1A and B). The encapsulated phage was freely released and highly sensitive to host bacteria (10⁸ CFU/mL, 1x10⁹CFU/mL) when tested using spot method on double agar layer technique (Plate 1C).

**Physical evaluation and pH determination of the gels**

The encapsulated chitosan microparticle hydrogel exhibited pH of 5.77 ±0.05, however after encapsulation of *A. Baumannii* phage (φAB140 and φAB150) the pH of the formulation increased to 6.54 ±0.03. This pH is still in tandem with the range of formulation pH required for management of chronic wounds. The viscosity of the C2 (1099 ± 2.15 MPa) was slightly lower than that of C1 (1120 ± 1.09 MPa). The viscosity of the formulations promotes ease of application as well as deters run-off of the formulation from site of application.

**Morphological Characterization**

SEM analysis showed that chitosan-based microcapsule presented as spheres of 30-40 µm in size. The shape was uniform throughout the sample and the microparticles existed as discrete particles. As shown in Figure 3C. Chitosan-based microparticle encapsulated *A. baumannii* phage presented as spherical shape approximately 40-60 µm in size (Figure 3A). The microparticles were suspended in hydrogel matrix to obtain formulation C1 and C2 and the SEM shows homogeneity of the microparticles within the hydrogel matrix (Figure 3B and D).

**Microparticle entrapment and yield determination.**

The microparticle yield of chitosan microparticles and chitosan-based microparticle encapsulated *A. baumannii* phage was 87.12 ± 2.93% and 80.99 ± 3.66% respectively. Entrapment efficiency of *A. baumannii* phage into chitosan microparticles was found to be 60.72 ± 3.09%.

**Fourier-transform infrared spectroscopy (FTIR)**

The development of chitosan-based microparticle encapsulated *A. baumannii* phage using plain chitosan microparticles as reference was analysed evaluated using FTIR. Sample C1 contained plain chitosan microparticles showed C-H stretches at 2875 cm⁻¹ with corresponding stretch vibration at 3283 cm⁻¹ reflecting-NH hydroxyl linkages (Fig 4A and B) The presence of the encapsulated *A. baumannii* phage caused a slight shift form 2128 cm⁻¹ to 2105 cm⁻¹ and shows eminent microparticle protein entrapment. The various bands seen include 1066 and 1021 cm⁻¹ which represents a C-O bending vibration accompanied with a peak, 1215 cm⁻¹ a representation of stretching vibration of P=O with a peak being observed, the bending of C-H formation is given within the range of 1457-1483 cm⁻¹, gives-H bending deformation, the carbonyl group of the amide bond is given as 1632 cm⁻¹ with a trough, C=O, the amine bond present N-H is given as 1580 cm⁻¹, and the C-H vibration stretch at 2867 cm⁻¹. The broad band N-H from the spectrum in Fig 4A and B is represented majorly as 3290 cm⁻¹ and 3253 cm⁻¹.

**Accelerated stability studies**

Formulations C1 and C2 were seen to be stable after resuspension and storage at different time intervals at 25°C ± 2°C. There was no change in morphology of the microparticle. C1 and C2 were stable when challenged with accelerated stability testing. There was no statistically significant variation in pH, lytic activity of the formulation and viscosity when exposed to ICH guidelines of 40 °C/75 %RH (Table 1)
In vitro antimicrobial determination of encapsulated Acinetobacter baumannii phage in hydrogel matrix

Bacterial load in wounds
The Acinetobacter baumannii infection was observed after 2, 7, 10 and 14 days on the wound surface in the no treatment (NT), control treatment (CT) and chitosan microparticle suspended in hydrogel group C1 and C2. A. baumannii contamination infection was not detected on days 7, 10, and 14 in the groups receiving the microparticle encapsulated A. baumannii phage hydrogel as in Table 2.

In vitro release study
The cumulative release of φAB from chitosan microparticle encapsulating Acinetobacter baumannii phage cocktails in hydrogel matrix was found to be 72.9 ± 7.09 % after 24 h of incubation. An initial release 32.76 at 2 hours followed by burst release of 72.24 which was sustained from 6 hours to 24 hours as shown in Figure 5.

Cell toxicity assessment
Microparticle encapsulated Acinetobacter baumannii phage (φAB140 and φAB150) were nontoxic to the cells at all concentration utilized. At the highest concentration of 1mg/mL, C1 and C2 showed similar percentage (117% ± 8.01% and 121% ± 2.83% respectively, after 24 hours following 183.03 ± 3.77 and 215% ± 7.89% after 120 hours) of cell proliferation with control. With respect to % cell proliferation, both hydrogels exhibited statistically significant difference at 120 hours compared to the value at t = 24hr. The optimized concentration of C2 adequately supported cell growth with a proliferation rate of 215% ± 7.89% compared to control (107.32% ± 4.55%) showing a lack of cell toxicity.

In vivo wound healing
The cumulative release of φAB from chitosan microparticle encapsulating Acinetobacter baumannii phage cocktails in hydrogel matrix was found to be 72.9 ± 7.09 % after 24 h of incubation. An initial release 32.76 at 2 hours followed by burst release of 72.24 which was sustained from 6 hours to 24 hours as shown in Figure 5.

Histology and histomorphometry
There was absence of haemorrhaging seen on the infected wounds in all groups including control post product application on the infected wound surface. Wound size reduction was most pronounced in formulation C2 which showed statistically significant wound seizure reduction at day 4 and day 7, 56.79% ± 2.02 and 62.15% ± 5.11, respectively. C1 containing plain hydrogel as well as control treatment showed a wound size reduction of 31.766% ± 3.07% and 31.28% ± 2.63% respectively. At day 14 only C2 showed completed wound closure with wound size reduction of 100% (Figure 6). Histologic section of tissue of animals in the no treatment group showed skin with dermis containing oil glands, as well as presence of clusters of inflammatory red cells (Figure 6A). Histologic section of tissue of animals treated with C2 reflected an absence of abnormalities after at Day14. The desquamated epithelial region for C1 measured at 1.55 mm which was statistically lower than control C1 and conventional drug measured at 2.91 mm ± 0.01 and 3.9 mm ± 0.03 mm respectively. No treatment group had desquamated epithelial region measured at 6.7
mm ± 0.10 mm due to increased inflammation in the dermis (Figure 7). Thickness of epidermis to the dermis in C2 was significantly higher than C1 (Figure 7B). The thickness of the central region in C1 (2.45 mm ± 0.09) was a clear indication of facilitated re-epithelization pathway that was not impaired by drug resistant *A. baumannii* infection. Re-epithelization rates was highest for C2 at 83.44% ±2.17 compared to 32.78% ± 0.11, 23.68% ± 1.01, and 11.99% ± 0.97 for C1, conventional drug and no treatment group respectively (Figure 7C).

**DISCUSSION**

This study developed encapsulated *A. baumannii* phage cocktails (ϕAB140 and ϕAB150) using chitosan and resuspended the microparticles in hydrogel to treat chronic wounds. *A. baumannii* phages were encapsulated using chitosan and then suspended in a hydrogel matrix. *A. baumannii* phages exhibited bactericidal activity in vitro. More so, in vivo lytic activity of the Chitosan-based microparticle encapsulated *Acinetobacter baumannii* phage cocktails in hydrogel matrix against Methicillin-resistant *A. baumannii* was studied in animal model. In the in-vivo animal wound experiment, we demonstrated that the phage cocktail in hydrogel was not toxic in the rodent model used in this study. Phage application in wound infections effectively reduced the number of bacteria isolates from the treated animal and all visible infection symptoms (red, swollen-purulent wound) disappeared. MDR *A. baumannii* is a rapidly emerging pathogen in the health care setting, it causes bacteraemia, pneumonia, meningitis, urinary tract, and wound infection. 20-21 The organism's ability to survive under a wide range of environmental conditions and to persist for extended periods of time on surfaces make it a frequent cause of outbreaks of infection. It also promotes its endemicity hence making it a health care–associated pathogen. 10 The developed microparticle phage formulation containing bacteriophages were stable and exhibited good organoleptic properties suitable for use as a wound healing formulation. There was also absence of irritation displayed on intact skin when tested prior to the wound healing study. Encapsulation of the phages followed by resuspension in a biomimetic hydrogel base ensured phage preservation within the drug delivery platform whilst aiding ease of usage in the clinical setting. In addition, the use of chitosan based microparticle encapsulated phage cocktail in hydrogel matrix sufficiently solved the problem of dropping of phage titre and inactivation of phage by immune cells around wound sites. Similarly, Colom *et al.,* 22 encapsulated *Salmonella spp* phages which were stable showed excellent bioavailability in the intestine thus increasing its therapeutic effect.

The animals treated with C2 containing bacteriophage exhibited bacterial clearance as there was absence of colonization drug resistant *Acinetobacter baumannii* on the wound surface. Kim *et al.,* 23 evaluated safety tests of phage lysin reflecting a success in systemic infection clearance of *A. baumannii*. This study however showed that hastened lysis of *A. baumannii* resulting in septic shock when 500 μg of phage was utilized. Encapsulation of the phage that was carried out in this study ensured that bacterial clearance occurred via sustained release of the phage through the microparticles.

The microparticle phage formulation C2 was able to facilitate complete wound closure by day 14. Similarly, Vinod-Kumar *et al.* 24 *S. aureus* clearance from wounds in immune compromised rats eight days post treatment with phage. Presence of drug resistant *A. baumannii* in the wound bed preclude the control drug and C1 treatment form achieving wound closure due to inability of the formulation achieve bacterial clearance due to lack of sensitivity and specificity for *A. baumannii* clearance. Cell proliferation and collagen deposition facilitates angiogenesis in development of granulation tissue. The animals treated with formulation C2, displayed an increase in granulation tissue with evidenced fewer inflammatory cells and increased collagen deposition. The % of
granulation tissue which was collagen occupied was increased in C2 when compared to C1 with increased fibroblast agglomeration seen in the tissue. Enhanced collagen deposition seen in animal treated with C2 was associated with well-organized collagen bands found within thin barrier blood vessels largely distributed throughout the granulation tissue with C2 exhibiting 50 microvessels within the granulation tissue. Weak scaffolds of collagen fibers accompanied by low number of microvessels at the tissue surface was observed for C1 and conventional drug treatment groups. C1 contained chitosan hence its ability to stimulated wound bed re-epithelization comparable with that seen in the convention treatment. Utilization of the hydrogel matrix as well has been seen to facilitate wound healing in contrast untreated wounds exhibited incomplete maturation of epidermal layer few fibroblasts, reduced number of microvessels and irregularly arranged collagen fibers which appeared coarse and lacking a compact structure.

Cytotoxicity of the microparticles is crucial for their in vivous use in clinical setting. Chitosan-based microparticle encapsulated A. baumannii phage cocktail was studied for their in-vitro cytotoxicity on human keratinocyte cells and showed high cell viability (1 mg/mL) of microparticles. Present study corroborated that C2 was non toxin on human keratinocyte cells. The use of phage cocktail provides effective solution which readily delivered the wounded animals without any delay, making cocktail phage therapy potentially more effective clinically than mono-phage therapy. Similarly, Chhibber et al., 25 demonstrated that, fast reduction in bacterial concentration and hastened wound healing occur in animal model treated with phage cocktail therapy.

CONCLUSION

Micro particle carrier technology was utilized to show the lytic activity against multi drug resistant A. baumannii phage. In vivo results showed significant wound size reduction which was most pronounced in formulation C2 at day 4. Complete clearance of A. baumannii isolates was detected on days 7, 10, and 14 in all the groups which received the microparticle encapsulated A. baumannii phage hydrogel as treatment. The optimized concentration of C2 is safe, nontoxic and effect in complete clearance of multidrug resistant A. baumannii infection from the wound bed.

References


<table>
<thead>
<tr>
<th>Time</th>
<th>Formulation</th>
<th>pH</th>
<th>Lytic activity</th>
<th>Viscosity (m Pas at 40 rpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>C1</td>
<td>5.77 ±0.05</td>
<td>Absence of lytic activity</td>
<td>1120 ± 1.09</td>
</tr>
<tr>
<td></td>
<td>C2</td>
<td>6.54 ±0.03</td>
<td>Presence of lytic activity</td>
<td>1099 ± 2.15</td>
</tr>
<tr>
<td>Day 10</td>
<td>C1</td>
<td>5.79 ±0.10</td>
<td>Absence of lytic activity</td>
<td>1120 ± 2.05</td>
</tr>
<tr>
<td></td>
<td>C2</td>
<td>6.53 ±0.04</td>
<td>Presence of lytic activity</td>
<td>1098 ± 2.31</td>
</tr>
<tr>
<td>Day 15</td>
<td>C1</td>
<td>5.79 ±0.09</td>
<td>Absence of lytic activity</td>
<td>1119 ± 1.72</td>
</tr>
<tr>
<td></td>
<td>C2</td>
<td>6.53 ±0.10</td>
<td>Presence of lytic activity</td>
<td>1101 ± 2.00</td>
</tr>
<tr>
<td>Day 30</td>
<td>C1</td>
<td>5.78 ±0.07</td>
<td>Absence of lytic activity</td>
<td>1121 ± 1.18</td>
</tr>
<tr>
<td></td>
<td>C2</td>
<td>6.54 ±0.11</td>
<td>Presence of lytic activity</td>
<td>1100 ± 1.45</td>
</tr>
<tr>
<td>Day 60</td>
<td>C1</td>
<td>5.77 ±0.07</td>
<td>Absence of lytic activity</td>
<td>1119 ± 1.11</td>
</tr>
<tr>
<td></td>
<td>C2</td>
<td>6.55 ±0.05</td>
<td>Presence of lytic activity</td>
<td>1097 ± 1.33</td>
</tr>
<tr>
<td>Day 90</td>
<td>C1</td>
<td>5.79 ±0.02</td>
<td>Absence of lytic activity</td>
<td>1121 ± 0.98</td>
</tr>
<tr>
<td></td>
<td>C2</td>
<td>6.55 ±0.04</td>
<td>Presence of lytic activity</td>
<td>1100 ± 1.32</td>
</tr>
</tbody>
</table>

Table 1 Accelerated stability testing on the microparticle encapsulated *Acinetobacter baumannii* phage cocktail in hydrogel (p ≤ 0.05).

Table 2 The assessment of the colonization of drug resistant *A. baumannii* on the wound surface
<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Day 2</th>
<th>Day 7</th>
<th>Day 10</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NT</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>2</td>
<td>CT</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>3</td>
<td>C1</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>4</td>
<td>C2</td>
<td>Positive</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
</tbody>
</table>

**Figure 1** Experimental timelines
Figure 2: Plate A and B showed the lytic activity and the titer of *A. baumannii* phage (φAB140 and φAB150) on double agar layer spot test. Plate C showed lytic activity of chitosan-based microparticle encapsulated *A. baumannii* phage cocktails (φAB140 and φAB150) on host strains.
Figure 3 SEM images of Chitosan-based microparticle encapsulated *Acinetobacter baumannii* phage cocktail (ϕAB140 and ϕAB150) (A) in hydrogel C2 (B), encapsulated chitosan microparticles (C) in hydrogel C1 (D).
Figure 4 FTIR spectra encapsulated phage in chitosan containing samples (A) C1 and (B) C2 containing *Acinetobacter baumannii* phage cocktail (ϕAB140 and ϕAB150)
Figure 5 Release profile of $\phi$AB from Chitosan-based microparticle encapsulated *Acinetobacter baumannii* phage ($\phi$AB140 & $\phi$AB150) cocktail (C2) at pH 7 at different time intervals.
Figure 6 (A) Representative sections stained with hematoxylin and eosin (H&E) after post treatment. (B) Wound size/ biometric analysis (mm) of wound area of Acinetobacter baumannii. (1.5 × 10^8 CFU/mL) infected animals (n = 6, each group) following no treatment (NT), conventional treatment (CT) with gentamicin ointment, C1 and C2. Wound area was measured on various days post-infection. Error bars represent the standard deviation (SD) from three independent values. *P < 0.05 indicate statistically significant differences between infected control and treated groups.
Figure 7 Histomorphometrical values showing (A) microvessels in granulation tissue (vessels/mm² of field), (B) thickness of central region (mm from epidermis to dermis) (C) re-epithelisation rates (%), (D) number of inflammatory cells (cell/mm² of field), (E) percentage occupied by collagen in granulation tissue (%/mm² of field), (F) desquamated epithelial region (mm) Treatments were compared with control and conventional drug (hydrogel without bacteriophage) *p < 0.05. The composition of hydrogels C1 and C2 is provided in Table 1