



# Chitosan-Based Microparticle Encapsulated *Acinetobacter baumannii* Phage Cocktail in Hydrogel Matrix for the Management of Multidrug Resistant Chronic Wound Infection

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## ABSTRACT

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

**Materials and Methods:** Two isolates of *A. baumannii*-specific lytic phases  $\phi$ AB140 and  $\phi$ AB150 alone, in combination (cocktail) encapsulated within a chitosan (CS) microparticle was suspended in CS hydrogel and evaluated for their therapeutic efficacy to ensure bacterial clearance in *A. baumannii* induced diabetic wound infection. Microencapsulation of the phage was carried out using ionic gelation techniques. Biological characterization *via* cell cytotoxicity, *in vivo* wound healing, histology and histomorphometry was carried out.

**Results:** Two characterized *A. baumannii* phages ( $\phi$ AB140 and  $\phi$ AB150), specific to twenty *A. baumannii* isolates, were isolated. The encapsulated CS microparticle hydrogel exhibited a pH of  $5.77 \pm 0.05$ . The wound size reduction was most pronounced in formulation C2, which showed statistically significant wound size reduction on days 4 and 7,  $56.79 \pm 2.02\%$  and  $62.15 \pm 5.11\%$ , respectively. The optimized concentration of C2 was not toxic to the cells as it adequately supported cell growth with a proliferation rate of  $215 \pm 7.89\%$  compared to control ( $107.32 \pm 4.55\%$ ).

**Conclusion:** Microparticle carrier technology was used to show the lytic activity against multi drug-resistant *A. baumannii*. *In vivo* results showed significant wound size reduction that was most pronounced in formulation C2 on day 4.

**Key words:**  $\phi$ AB140 and  $\phi$ AB150 phage, microparticle hydrogel, chronic wound, cytotoxicity

## INTRODUCTION

Wound healing requires collagen synthesis, cell migration, angiogenesis, blood clotting, extracellular matrix adhesion, and immune/inflammatory response,<sup>1</sup> amongst other complex biological processes to ensure the complete restructure of the skin in the area where the wound occurred.<sup>2</sup> Diabetic wound healing is however mitigated by both systemic and local factors. Systemic factors include the use of steroids, antineoplastic agents and non-steroidal anti-inflammatory drugs, deficiencies of vitamins A, C and E, magnesium, zinc and

copper; nutritional status, associated illnesses/immunity and patient age.<sup>3</sup> 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.<sup>3</sup> Diabetic wounds are also promoted by the presence of microorganisms antimicrobial-resistant.

Infection of diabetic wounds impairs the inflammatory phase of natural wound healing.<sup>3</sup> This is due to pathogenic microbes competing with macrophages and fibroblasts for limited

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resources, hence impairing neutrophil function, chemotaxis, phagocytosis and decreased T-cell response, leading to necrosis in the wound bed; sepsis and perhaps death.<sup>4</sup> In a bid to combat antimicrobial resistance, encapsulation of bacteriophage has been used to tackle systemic infections caused by *Staphylococcus* sp. and *Mycobacterium* sp.<sup>4,5</sup> 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.<sup>5</sup>

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 patients with diabetes as against 50% in non-diabetics.<sup>6-9</sup> Multidrug-resistant organisms (MDRO) were more prevalent in the population with diabetes and co-infections were also higher. In a more recent study by Lee et al.<sup>7</sup>, high levels of antibiotic resistance and MDRO were observed to limit the treatment options of individual with diabetic wound ulcers.<sup>10</sup> Chitosan (CS)-based hydrogels have been used as carriers for other therapeutic molecules in enhancing diabetic wound healing.<sup>11</sup> They are ideal to promote wound healing as they serve as both wound dressings and drug delivery systems.<sup>12</sup> Different polymeric and oil-based drug delivery platforms have been examined for delivery of bacteriophages, however, these drug delivery platforms for bacteriophage delivery of  $\phi$ AB140 and  $\phi$ AB150 have not been documented.<sup>8-11</sup> A simplified stepwise system for drug delivery involving bacteriophages for translation to bedside use in the management of chronic life threatening and debilitating infections is currently unavailable. This study presents a novel attempt to encapsulate  $\phi$ AB140 and  $\phi$ AB150. This study developed an approach to tackle MDR *Acinetobacter baumannii* infection in a chronic wound model through *A. baumannii* phage ( $\phi$ AB140 and  $\phi$ AB150 phage) encapsulation resuspension in hydrogel.

## MATERIALS AND METHODS

### Chemicals and reagents

CS (MW 80,000 g/mol, degree of deacetylation 80%), polyether sulfone, sodium chloride, polyethylene glycol, 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,  $\text{KH}_2\text{PO}_4$ , sodium hydroxide, pancreatin, and BCA solution were obtained from (Macklin Biochemical Co. Ltd, Pudong China). Ketamine and xylazine were obtained from Tocris Bioscience Bio-Techne Corporation MN, USA. High performance liquid chromatography 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. Ethical approval was obtained from the Health Research Ethics Committee, College

of Medicine, University of Lagos (approval number: CMUL/HREC/09/19/676).

### Bacterial strains

Fifty wound samples were collected from Medical Microbiology Laboratory of Teaching Hospital in Lagos University, Nigeria. The samples were subcultured on varying media including MacConkey and blood agar at 37°C with time equals 24 hrs. Bacteria strain was identified by conventional methods and confirmed using microbact 12A and B system.<sup>6,13</sup> The identified *A. baumannii* isolates were confirmed using microbact 12A and B system.

### Antibiotic susceptibility test

Kirby-Bauer method was used 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.<sup>13</sup>

### Phage isolation and purification

Ten MDR *A. baumannii* strains were selected for the 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 the canal water sample was collected from the canal behind Teaching Hospital of Lagos University. The phage was isolated using enrichment protocol as previously described.<sup>14</sup>

Two clonal differences in the *A. baumannii* isolates ( $\phi$ AB140 and  $\phi$ AB150) that showed very distinct and clear plaques were picked for further purification tests. The phage was purified through optimization and biokinetic measuring process as previously described.<sup>8</sup> The phage was amplified against their specific host bacterial stains and titer quantified; this was followed by storage at 4°C.

### Chitosan microparticle encapsulating *Acinetobacter baumannii* phage cocktails ( $\phi$ AB140 and $\phi$ AB150) in hydrogel matrix

CS (0.1% w/w) was dissolved in 10 mL acetic acid (4% v/v). Fifty microliter of *A. baumannii* phage cocktails ( $1 \times 10^{11}$  PFU/mL) was added to the CS solution under magnetic stirring and continuous sonication to obtain a homogenized fluid at  $25 \pm 0.5^\circ\text{C}$ . Using a gauge 25 needle, 20 mL of the phage-CS 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.<sup>15</sup>

To prepare the plain gel to be used as the control, CS (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  $\mu$ L of the phage cocktail ( $1 \times 10^{11}$  PFU/mL) was mixed with 20 mL of the gel and homogenized at 100 rpm for 30 min and then stored in a glass vial at  $25 \pm 0.5^\circ\text{C}$ .

Formulation C1 contained 20 mL hydrogel alone whilst formulation C2 contained 20 mL of hydrogel and 50  $\mu$ L of bacteriophage component comprising *A. 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  $25^\circ\text{C}$ , 20 rpm using spindle 04. Scanning electron microscopy (SEM) using JEOL JSM-6360\L instrument was used to determine approximate shape, size, and uniformity of the microparticles.

#### *Microparticle entrapment and yield determination*

Microparticle suspension (10 mL) was centrifuged ( $20,000 \times g/30$  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 CS, TPP. The weight of the phage particles was excluded from the microparticle yield calculation (equation 1).

$$\text{Microparticle yield (\%)} = \frac{\text{microparticle weight}}{\text{Total solids (CS + TPP - 1) weight}} \times 100$$

Equation 1

*A. baumannii* phage microparticle suspension was evaluated for entrapment efficiency. Under centrifugal force of  $21700 \times g$  for 15 min. Aided the concentration in the supernatant was determined by bicinchoninic assay where the supernatant from CS nanoparticles is used as the blank sample. Using bicinchoninic assay the phage entrapment efficiency was determined using equation 2.

$$\% \text{ Entrapment efficiency} = \frac{(\text{Total phage} - 1) - (\text{free phage} - 1)}{\text{total phage} - 1} \times 100$$

Equation 2

#### *Fourier-transform infrared spectra (FTIR)*

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 in the CS molecule. Twenty scans *per* spectra were recorded between 4000 and  $400 \text{ cm}^{-1}$ .

#### *Accelerated stability studies*

The stability of CS microparticle encapsulating *A. baumannii* phage cocktails ( $\phi$ AB140 and  $\phi$ AB150) in hydrogel matrix (formulation C1 and C2) was determined by the resuspension

of the formulation in sterile distilled water at the concentration of 20% w/w using a modified method of Pirnay et al.<sup>14</sup> The hydrogel was incubated at  $25^\circ\text{C} \pm 2^\circ\text{C}$  and analysed for their size different time intervals (0, 10, 15, 30, 60, and 90 days) using SEM as previously described.

Accelerated stability testing of the CS microparticle encapsulating *A. baumannii* phage cocktails ( $\phi$ AB140 and  $\phi$ AB150) in the hydrogel matrix was then evaluated using the ICH guidelines ( $40^\circ\text{C}/75\% \text{ RH}$ ). Formulations C1 and C2 were stored in amber colored 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  $40^\circ\text{C}$  and parameters were recorded on days 0, 10, 15, 30, and 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 A. baumannii encapsulated phage hydrogel in vitro*

To investigate the lytic activity of *A. baumannii* phage cocktail ( $\phi$ AB140 and  $\phi$ AB150) hydrogel against multi drug-resistant *A. baumannii* isolates, a 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 ( $37^\circ\text{C}$  for 24 h) and lytic activity observed. Draize tests were performed to ensure non-reactivity of the developed hydrogels on intact skin.<sup>16</sup>

##### *In vitro release study*

$\phi$ AB140 and  $\phi$ AB150 release from CS microparticle encapsulating *A. baumannii* phage cocktails in the hydrogel matrix was determined 25 mg microparticles were transferred to a tube with 5 mL 50 mM phosphate buffered saline maintained at pH 7 and  $37^\circ\text{C} \pm 1^\circ\text{C}$ . At predetermined time intervals (2, 4, 6, 8, 10, 12, and 24 h), 2 mL sample was removed after centrifugation at  $21,700 \times g$  for 30 min, and 2 mL of the supernatant removed was replaced with fresh 50 mM phosphate buffered saline. The sample from each time interval was analysed by bicinchoninic acid (BCA) assay to determine the concentration of the released protein.

##### *Cell toxicity assessment*

Using the method of Iloмуanya et al.<sup>17</sup>, double enzymatic digestion, isolation of keratinocytes obtained from human (a waste product of male circumcision) was carried out. The formulation was carried using aseptic technique and, hence, it was not necessary to conduct sterilization. The CS-based microparticles were also cultured to ensure that no bacterial contamination occurred in line with good quality control measures.<sup>17</sup> 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was carried out using CS-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 (12 hr night/day cycle;  $29 \pm 2^\circ\text{C}$ ;  $45 \pm 10\%$  RH). Standard feeding conditions were provided in line with international best practices, as stated within the study ethical approval (CMUL/HREC/09/19/676).

Experimental timelines (Figure 1) show that the wound healing study was conducted within a 14 day period. Diabetes was induced using 150 mg/kg body weight alloxan monohydrate in 2 day time lapse in the fasted rodents.<sup>18</sup> After the alloxan injection was administered rats having fasting blood glucose levels above  $190.5 \pm 8.1$  mg/dL with the random blood glucose levels of above 350 mg/dL were considered severely diabetic and selected for infection studies.<sup>18</sup>

### *Wound model*

Overnight culture of MDR *A. baumannii* isolates 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 before wounding through the skin to the panniculus carnosus using a 5 mm 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 (5 mg/kg bwt) to reduce pain. The infection was allowed to take hold for 24 h before the treatment protocol started 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 an image calibration capacity and calculated using relative wound size reduction measurements was calculated based difference in wound diameter  $A_o$  and  $A_t$  on treatment time in days *i.e.* initial wound diameter and diameter at predetermined time intervals respectively (equation 3).

$$\text{Relative wound size reduction \%} = \frac{A_o - A_t}{A_o} \times 100 \quad \text{Equation 3}$$

### *Bacterial load in wounds*

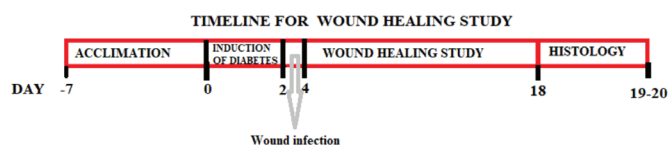


Figure 1. Experimental timelines

MDR *A. baumannii* on the surface of the wound, samples were collected from the wound surface using a 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  $37^\circ\text{C}$  before identification and evaluation of the presence of MDR *A. baumannii*.<sup>18,19</sup>

### *Histology*

Fourteen days post-treatment of the infected wound, skin sections fixed in 10% formaldehyde was stained using hematoxylin/eosin and Masson's trichome and histological profiles recorded using a microsystems microscope (Mannheim, Germany).<sup>2</sup>

### *Histomorphometry*

DML-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 equation 4.

$$\text{Re-epithelialization \%} = \frac{[\text{Total wound length (mm)} - \text{Desquamated epithelium region (mm)}]}{\text{Total wound length (mm)}} \times 100 \quad \text{Equation 4}$$

### *Statistical analysis*

Graph pad prism software ver. 5 was used for statistical analysis using data obtained in triplicate. All reported date is expressed as mean  $\pm$  standard deviation 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 phages ( $\phi\text{AB140}$  and  $\phi\text{AB150}$ ) were selected for the study amongst the five-phage library based on high lytic activity, broad host range and the plaque size formation (Figure 2A, B). The encapsulated phage was freely released and highly sensitive to host bacteria ( $10^6$  CFU/mL,  $1 \times 10^9$  CFU/mL) when tested using spot method on double agar layer technique (Figure 2C).

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

The encapsulated CS microparticle hydrogel exhibited pH of  $5.77 \pm 0.05$ , however after encapsulation of *A. baumannii* phage ( $\phi\text{AB140}$  and  $\phi\text{AB150}$ ) the pH of the formulation increased

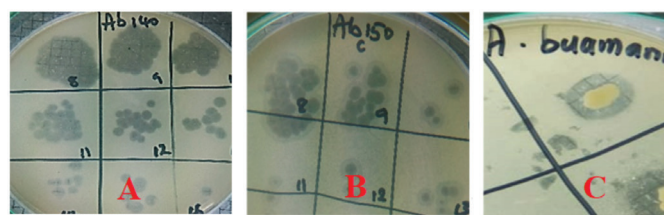


Figure 2. Plate A and B showed the lytic activity and the titer of *Acinetobacter baumannii* phage ( $\phi\text{AB140}$  and  $\phi\text{AB150}$ ) on double agar layer spot test. Plate C showed lytic activity of chitosan-based microparticle encapsulated *A. baumannii* phage cocktails ( $\phi\text{AB140}$  and  $\phi\text{AB150}$ ) on host strains



to  $6.54 \pm 0.03$ . This pH is still in tandem with the range of formulation pH required for the management of chronic wounds. The viscosity of the C2 ( $1099 \pm 2.15$  MPa) was slightly lower than that of C1 ( $1120 \pm 1.09$  MPa). The viscosity of the formulations promotes ease of application as well as deters run-off of the formulation from the site of application.

#### Morphological characterization

SEM analysis showed that CS-based microcapsule presented as spheres of 30–40  $\mu\text{m}$  in size. The shape was uniform throughout the sample and microparticles existed as discrete particles. As shown in Figure 3C, CS-based microparticle encapsulated *A. baumannii* phage presented as spherical shape approximately 40–60  $\mu\text{m}$  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 (Figures 3B and D).

#### Microparticle entrapment and yield determination

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

#### Fourier-transform infrared spectroscopy

The development of CS-based microparticle-encapsulated *A. baumannii* phage using plain CS microparticles as reference was analysed evaluated using FTIR. Sample C1 contained plain CS microparticles showed C-H stretches at  $2875\text{ cm}^{-1}$  with corresponding stretch vibration at  $3283\text{ cm}^{-1}$  reflecting-NH hydroxyl linkages (Figures 4A and B). The presence of the encapsulated *A. baumannii* phage caused a slight shift from  $2128\text{ cm}^{-1}$  to  $2105\text{ cm}^{-1}$  and shows eminent microparticle protein entrapment. The various bands seen include  $1066$  and  $1021\text{ cm}^{-1}$ , which represent a C-O bending vibration accompanied by a peak,  $1215\text{ cm}^{-1}$  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  $1457$ – $1483\text{ cm}^{-1}$ , gives-H bending deformation, the carbonyl group of the amide bond is given as  $1632\text{ cm}^{-1}$  with a trough, C=O, the amine bond present N-H is given as  $1580\text{ cm}^{-1}$ , and the C-H vibration stretch at  $2867\text{ cm}^{-1}$ . The broad band N-H from the spectrum in Figure 4A, B is represented majorly as  $3290\text{ cm}^{-1}$  and  $3253\text{ cm}^{-1}$ .

#### Accelerated stability studies

Formulations C1 and C2 were seen to be stabile after resuspension and storage at different time intervals at  $25^\circ\text{C} \pm 2^\circ\text{C}$ . There was no change in the 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  $40^\circ\text{C}/75\% \text{RH}$  (Table 1).

#### In vitro antimicrobial determination of encapsulated *A. baumannii* phage in hydrogel matrix

##### Bacterial load in wounds

The *A. baumannii* infection was observed after 2, 7, 10, and 14<sup>th</sup> days on the wound surface in the no treatment (NT), control

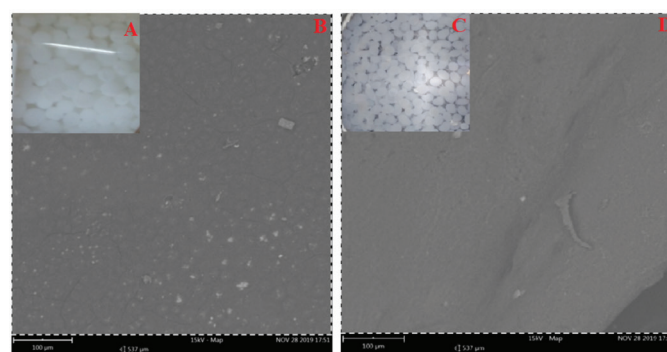
treatment (CT) and CS microparticle suspended in hydrogel group C1 and C2. *A. baumannii* contamination infection was not detected on days 7, 10, and 14 in the groups receiving the microparticle encapsulated *A. baumannii* phage hydrogel as in Table 2.

#### In vitro release study

The cumulative release of  $\phi\text{AB}$  from CS microparticle encapsulating *A. baumannii* phage cocktails in the hydrogel matrix was found to be  $72.9 \pm 7.09\%$  after 24 h of incubation. An initial release  $32.76$  at 2 h followed by burst release of  $72.24$  which was sustained from 6 h to 24 h as shown in Figure 5.

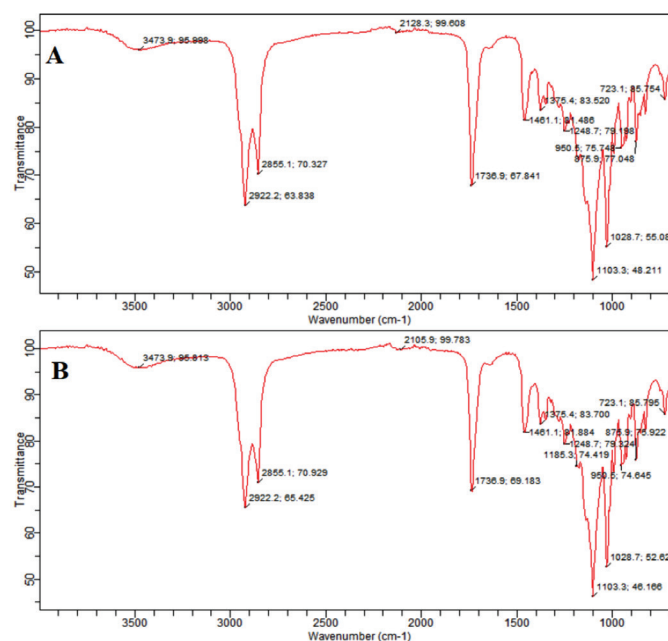
#### Cell toxicity assessment

Microparticle encapsulated *A. baumannii* phage ( $\phi\text{AB140}$  and  $\phi\text{AB150}$ ) were non-toxic to the cells at all concentration utilized.



**Figure 3.** SEM images of chitosan-based microparticle encapsulated *Acinetobacter baumannii* phage cocktail ( $\phi\text{AB140}$  and  $\phi\text{AB150}$ ) (A) in hydrogel C2 (B), encapsulated chitosan microparticles (C) in hydrogel C1 (D)

SEM: Scanning electron microscopy



**Figure 4.** FTIR spectra encapsulated phage in chitosan containing samples (A) C1 and (B) C2 containing *Acinetobacter baumannii* phage cocktail ( $\phi\text{AB140}$  and  $\phi\text{AB150}$ )

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

Time (duration)	Formulation	pH	Lytic activity of formulation	Viscosity (mPas at 40 rpm)
Day 0	C1	$5.77 \pm 0.05$	Absence of lytic activity	$1120 \pm 1.09$
	C2	$6.54 \pm 0.03$	Presence of lytic activity	$1099 \pm 2.15$
Day 10	C1	$5.79 \pm 0.10$	Absence of lytic activity	$1120 \pm 2.05$
	C2	$6.53 \pm 0.04$	Presence of lytic activity	$1098 \pm 2.31$
Day 15	C1	$5.79 \pm 0.09$	Absence of lytic activity	$1119 \pm 1.72$
	C2	$6.53 \pm 0.10$	Presence of lytic activity	$1101 \pm 2.00$
Day 30	C1	$5.78 \pm 0.07$	Absence of lytic activity	$1121 \pm 1.18$
	C2	$6.5 \pm 0.11$	Presence of lytic activity	$1100 \pm 1.45$
Day 60	C1	$5.77 \pm 0.07$	Absence of lytic activity	$1119 \pm 1.11$
	C2	$6.55 \pm 0.05$	Presence of lytic activity	$1097 \pm 1.33$
Day 90	C1	$5.79 \pm 0.02$	Absence of lytic activity	$1121 \pm 0.98$
	C2	$6.55 \pm 0.04$	Presence of lytic activity	$1100 \pm 1.32$

**Table 2. The assessment of the colonization of drug resistant *Acinetobacter baumannii* on the wound surface**

Group	Treatment protocol	Chemotherapy utilized	Presence of multi drug resistant <i>A. baumannii</i> at			
			Day 2	Day 7	Day 10	Day 14
1	NT	No treatment	Positive	Positive	Positive	Positive
2	CT	Gentamicin ointment	Positive	Positive	Positive	Positive
3	C1	Chitosan microparticle suspended in hydrogel	Positive	Positive	Positive	Positive
4	C2	Microparticle encapsulated <i>A. baumannii</i> phage cocktail in hydrogel	Positive	Negative	Negative	Negative

NT: No treatment, CT: Conventional treatment

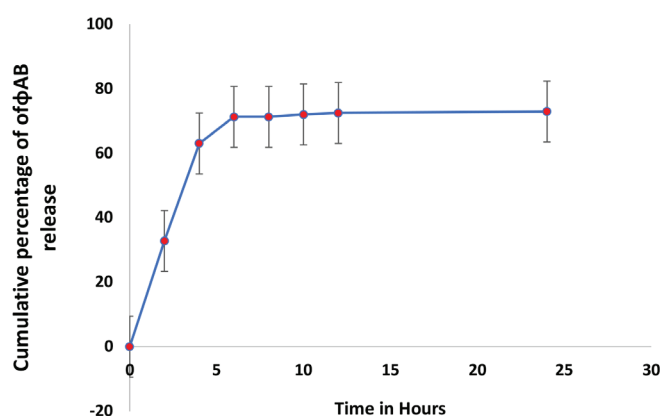
At the highest concentration of 1 mg/mL, C1 and C2 showed similar percentage ( $117 \pm 8.01\%$  and  $121 \pm 2.83\%$  respectively, after 24 hours following  $183.03 \pm 3.77\%$  and  $215 \pm 7.89\%$  after 120 hrs) of cell proliferation with control. With respect to % cell proliferation, both hydrogels exhibited statistically significant difference at 120 hours compared to the value at  $t=24$  hr. The optimized concentration of C2 adequately supported cell growth with a proliferation rate of  $215 \pm 7.89\%$  compared to control ( $107.32 \pm 4.55\%$ ) showing a lack of cell toxicity.

#### *In vivo wound healing*

The cumulative release of  $\phi$ AB from CS microparticle encapsulating *A. baumannii* phage cocktails in hydrogel matrix was found to be  $72.9 \pm 7.09\%$  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 an absence of hemorrhaging seen on the infected wounds in all groups including the control post product application on the infected wound surface. The wound size reduction was most pronounced in formulation C2 that showed statistically significant wound size reduction on days 4 and



**Figure 5.** Release profile of  $\phi$ AB from chitosan-based microparticle encapsulated *Acinetobacter baumannii* phage ( $\phi$ AB140 and  $\phi$ AB150) cocktail (C2) at pH 7 at different time intervals

7,  $56.79 \pm 2.02$  and  $62.15 \pm 5.11\%$ , respectively. C1 containing plain hydrogel and CT showed a wound size reduction of  $31.766 \pm 3.07\%$  and  $31.28 \pm 2.63\%$  respectively. On day 14<sup>th</sup>, only C2 showed completed wound closure with wound size reduction of 100% (Figure 6). Histologic section of tissue of animals in

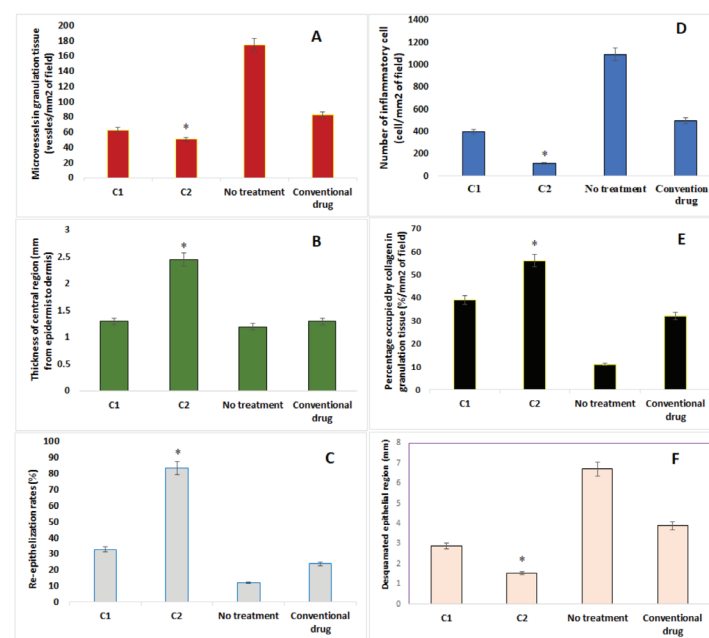
the NT group showed skin with dermis containing oil glands, as well as presence of clusters of inflammatory red cells (Figure 6A). Histologic section of tissue of animals treated with C2 reflected an absence of abnormalities after at day 14<sup>th</sup>. The desquamated epithelial region for C1 measured at 1.55 mm that was statistically lower than control C1 and conventional drug measured at  $2.91 \pm 0.01$  mm and  $3.9 \pm 0.03$  mm, respectively. NT group had desquamated epithelial region measured at  $6.7 \pm 0.10$  mm due to increased inflammation in the dermis (Figure 7). The thickness of epidermis to the dermis in C2 was significantly higher than C1 (Figure 7B). The thickness of the central region in C1 ( $2.45 \pm 0.09$ ) was a clear indication of facilitated re-epithelization pathway that was not impaired by drug-resistant *A. baumannii* infection. Re-epithelization rates were highest for C2 at  $83.44 \pm 2.17\%$  compared to  $32.78 \pm 0.11\%$ ,  $23.68 \pm 1.01\%$ , and  $11.99 \pm 0.97\%$  for C1, conventional drug and NT groups, respectively (Figure 7C).

## DISCUSSION

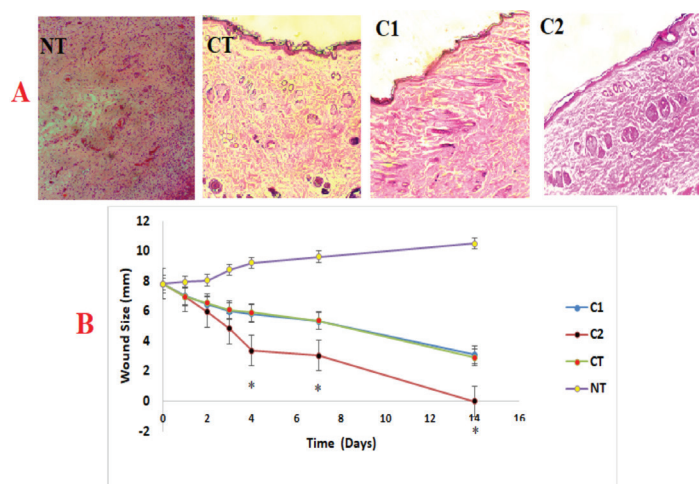
This study developed encapsulated *A. baumannii* phage cocktails ( $\phi$ AB140 and  $\phi$ AB150) using chitosan and resuspended the microparticles in hydrogel to treat chronic wounds. *A. baumannii* phages were encapsulated using CS and then suspended in a hydrogel matrix. *A. baumannii* phages exhibited bactericidal activity *in vitro*. More so, *in vivo* lytic activity of the CS-based microparticle-encapsulated *A. baumannii* phage cocktails in hydrogel matrix against methicillin-resistant *A. baumannii* was studied in an 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 healthcare setting, it causes bacteremia, pneumonia, meningitis, urinary tract, and wound infection.<sup>20,21</sup> The organism's ability to survive under various environmental conditions and to persist for extended time on surfaces make it a frequent cause of outbreaks of infection. It also promotes its endemicity hence making it a healthcare-associated pathogen.<sup>10</sup> 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 an absence of irritation displayed on intact skin when tested before the wound healing study. The 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. Additionally, the use of CS based microparticle-encapsulated phage cocktail in hydrogel matrix sufficiently solved the problem of dropping of phage titer and inactivation of phage by immune cells around wound sites. Similarly, Colom et al.<sup>22</sup> encapsulated *Salmonella* spp. phages that 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 an absence of colonization drug-resistant *A. baumannii* on the wound surface. Kim et al.<sup>23</sup> evaluated safety tests of phage lysis reflecting success in systemic infection clearance of *A. baumannii*. This study,



**Figure 7.** Histomorphometrical values showing (A) microvessels in granulation tissue (vessels/mm<sup>2</sup> of field), (B) thickness of central region (mm from epidermis to dermis) (C) re-epithelisation rates (%), (D) number of inflammatory cells (cell/mm<sup>2</sup> of field), (E) percentage occupied by collagen in granulation tissue (%/mm<sup>2</sup> of field), (F) desquamated epithelial region (mm). Treatments were compared with control and conventional drug (hydrogel without bacteriophage) \**p* < 0.05. The compositions of hydrogels C1 and C2 are provided in Table 1



**Figure 6.** (A) Representative sections stained with hematoxylin and eosin after post treatment. (B) Wound size/biometric analysis (mm) of wound area of *Acinetobacter baumannii* ( $1.5 \times 10^8$  CFU/mL). Infected animals (*n* = 6, each group) following NT, CT with gentamicin ointment, C1 and C2. Wound area was measured on various days post-infection. Error bars represent the standard deviation from three independent values. \**p* < 0.05 indicate statistically significant differences between infected control and treated groups

NT: No treatment, CT: Conventional treatment

however, showed that hastened lysis of *A. baumannii* resulting in septic shock when 500 µg of phage was used. The 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 could facilitate complete wound closure by day 14. Similarly, Vinod Kumar et al.<sup>24</sup> *Staphylococcus aureus* clearance from wounds in immune compromised rats eight days post-treatment with phage. The presence of drug-resistant *A. baumannii* in the wound bed preclude the control drug and C1 treatment from achieving wound closure due to the inability of the formulation achieve bacterial clearance due to lack of sensitivity and specificity for *A. baumannii* clearance. Cell proliferation and collagen deposition facilitate angiogenesis in the 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 percentage of granulation tissue which was collagen occupied was increased in C2 compared to C1 with increased fibroblast agglomeration seen in the tissue. The 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 a low number of microvessels at the tissue surface was observed for C1 and conventional drug treatment groups. C1 contained CS hence its ability to stimulate wound bed re-epithelization comparable with that seen in the convention treatment. The 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 that appeared coarse and lacking a compact structure.

The cytotoxicity of the microparticles is crucial for their *in vivo* use in clinical setting. CS-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. The present study corroborated that C2 was non-toxin on human keratinocyte cells. The use of phage cocktail provides an 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.<sup>25</sup> demonstrated that fast reduction in bacterial concentration and hastened wound healing occur in an animal model treated with phage cocktail therapy.

## CONCLUSION

Microparticle carrier technology was used to show the lytic activity against multi drug-resistant *A. baumannii* phage. *In vivo* results showed significant wound size reduction that was most pronounced in formulation C2 on day 4. Complete clearance of *A. baumannii* isolates was detected on days 7, 10, and 14

in all groups that received the microparticle encapsulated *A. baumannii* phage hydrogel as treatment. The optimized concentration of C2 is safe, non-toxic and effect in complete clearance of MDR *A. baumannii* infection from the wound bed.

## Ethics

**Ethics Committee Approval:** Ethical approval was obtained from the Health Research Ethics Committee, College of Medicine University of Lagos approval number: CMUL/HREC/09/19/676.

**Informed Consent:** This was not required.

**Peer-review:** Externally peer-reviewed.

## Authorship Contributions

Concept: M.O.I., Design: M.O.I., N.V.E., Data Collection or Processing: E.A., A.F., A.A., Analysis or Interpretation: M.O.I., N.V.E., E.A., A.F., C.I.I., Literature Search: M.O.I., A.F., E.A., Writing: M.O.I., N.V.E., E.A.

**Conflict of Interest:** No conflict of interest was declared by the authors.

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