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Thermally triggered release of the bacteriophage endolysin $CHAP_K$ and the bacteriocin lysostaphin for the control of methicillin resistant *Staphylococcus aureus* (MRSA)

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ABSTRACT

Staphylococcus aureus infections of the skin and soft tissue pose a major concern to public health, largely owing to the steadily increasing prevalence of drug resistant isolates. As an alternative mode of treatment both bacteriophage endolysins and bacteriocins have been shown to possess antimicrobial efficacy against multiple species of bacteria including otherwise drug resistant strains. Despite this, the administration and exposure of such antimicrobials should be restricted until required in order to discourage the continued evolution of bacterial resistance, whilst maintaining the activity and stability of such proteinaceous structures. Utilising the increase in skin temperature during infection, the truncated bacteriophage endolysin $CHAP_K$ and the staphylococcal bacteriocin lysostaphin have been co-administered in a thermally triggered manner from Poly(*N*-isopropylacrylamide) (PNIPAM) nanoparticles. The thermoresponsive nature of the PNIPAM polymer has been employed in order to achieve the controlled expulsion of a synergistic enzybiotic cocktail consisting of $CHAP_K$ and lysostaphin. The point at which this occurs is modifiable, in this case corresponding to the threshold temperature associated with an infected wound. Consequently, bacterial lysis was observed at 37 °C, whilst growth was maintained at the uninfected skin temperature of 32 °C.

1. Introduction

Staphylococcus aureus (*S. aureus*) is a frequent inhabitant of the human skin flora colonizing up to 30% of individuals at any given time, primarily through nasal carriage [1]. Requiring a suitable portal of entry into the body, the skin normally provides such a barrier to progressive infection. However a breach in the skin, often as a result of a scratch, cut or burn provides a suitable infection point for the opportunistic bacteria. *S. aureus* is the leading cause of skin and soft tissue infections (SSTI) across all continents, thus resulting in both delayed wound healing and further systemic infections, such as sepsis, osteomyelitis and endocarditis [2]. With the discovery of staphylococcal drug resistance and the subsequent global epidemic that methicillin resistant *S. aureus* (MRSA) has become, the need to source alternative treatment has become paramount. Hospital acquired MRSA (HA-MRSA) has a mortality rate twice that of its methicillin susceptible counterpart, and is more than twice as expensive to treat [3]. Furthermore, the isolation of these 'super-bugs' is not confined to the hospital setting. Indeed, community acquired MRSA (CA-MRSA) is proving an equal challenge to clinicians worldwide [4].

Bacteriophage (phage), (the naturally occurring parasitic viruses of bacteria, able to infect and destroy bacterial cells) were first utilised as a treatment to infection in the 1930s within the former Soviet Union. Despite the continued development of phage products throughout the Cold War, bacteriophage therapy was largely disregarded in the West from under the relative comfort of the antibiotic blanket. However, the alarming rise in multi-drug resistance (MDR) in recent times has regenerated interest in phage therapy [5]. One of the main disadvantages associated with the use of whole phage to treat infection is the viral nature of the phage itself. Containing a vast amount of genetic material, temperate phage have been known to increase the virulence of certain species of bacteria through transduction, an example of which includes the bacterial acquisition of the gene encoding the Panton Valentine Leucocidin toxin, causing 'scalded skin syndrome' [6]. Whilst this is selected against when sourcing phage for treatment, the regulation and control of suitable virulent phage for clinical use is often timely and uncertain.

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Bacteriophage-encoded endolysins (peptidoglycan hydrolases synthesised by phage infected bacterial cells) are utilised in the end stages of phage infection. Lysins are capable of destroying the bacterial cell wall through digestion of the peptidoglycan polymer, resulting in cell death through osmolysis [7]. The isolation of these hydrolases has the potential to overcome many issues surrounding the use of whole phage. As hydrolytic enzymes, they retain specificity without affecting commensal flora, are capable of rapid bacterial lysis, are unlikely to encounter resistance owed to the essential bacterial binding sites and they do not contain transducible genetic information [8]. The specific mechanism of action of these endolysins is discussed elsewhere [9]. Endolysins demonstrating activity towards both Gram-positive and Gram-negative bacteria have been isolated and characterised, including lysins active against *Acinetobacter baumanni*, *Bacillus anthracis*, *Streptococcus pyogenes* and in some cases active against both Gram-positive and –negative bacteria simultaneously [10–12]. The phage endolysin designated LysK isolated from the staphylococcal bacteriophage K has been shown to have potent antimicrobial activity against a range of staphylococci including MRSA [13]. LysK has been truncated to its single catalytic domain, a cysteine, histidine-dependent aminohydrolase/peptidase ($CHAP_K$). This single domain, 18.6 kDa antimicrobial enzyme has been fully characterised and has demonstrated retention of lytic activity in vitro, in vivo and against staphylococcal biofilms [14–16].

Another class of potential alternative antimicrobials are bacteriocins. Lysostaphin, a 26.8 kDa metalloendopeptidase is produced naturally by *Staphylococcus simulans* [17]. Consisting of a single lytic domain (glycylglycine M23 endopeptidase), lysostaphin demonstrates potent antistaphylococcal activity through cleavage of the pentaglycine crossbridges within the peptidoglycan of the bacterial cell wall. Active against a multitude of antibiotic susceptible, intermediate and resistant strains of bacteria, lysostaphin exhibits synergistic behaviour with multitude of antibiotics, phage lysins and antimicrobial peptides [18–20]. Successful application of lysostaphin has been demonstrated in cases of ocular infection, osteitis and endocarditis [21–23].

Despite the discovery and development of new potential antimicrobial candidates, the mode of delivery of any such pharmacologically active substance remains equally as important as the discovery itself. Ensuring activity, stability and dosage conditions are correct (especially when considering biological material), is crucial to successful administration in order to maximise the therapeutic benefit and to reduce any potential side effects. The triggered release of a therapeutic agent (small molecule, protein or virus) may rely on a variety of external stimuli in order to release the active cargo, including pH, temperature, ultrasound, magnetism or biomarker signals [24,25]. Utilising the difference between the healthy and the diseased state may provide certain conditions whereby treatment can be administered in a controlled fashion. Consequently, high local concentrations are achieved only in the specific location required. When considering treatment of bacterial infection, preventing the administration of unnecessary or sub-lethal concentrations of an antibiotic or antimicrobial agent is crucial in preventing the continued development of antibiotic resistance [26].

Poly(*N*-isopropylacrylamide) (PNIPAM) has been widely investigated as a triggered drug release vehicle [27]. As a thermoresponsive polymer, PNIPAM undergoes a reversible, entropically driven phase transition at a lower critical solution temperature (LCST), resulting in the expulsion of water and a subsequent change in polymer volume. The LCST of PNIPAM and its associated structures (nanoparticles, micelles, nanogels etc.) can be manipulated through control of polymer concentration, copolymers and surfactants. Through adjusting the LCST to that of a clinically relevant temperature, PNIPAM has been extensively investigated in a wide range of biomedical applications including cancer therapy, wound healing, bioscaffolding and cell cultivation [28–31].

In previous studies, PNIPAM nanoparticles were formulated with allylamine for the controlled release of Bacteriophage K, which demonstrated potent antistaphyloccal activity through the thermally controlled collapse of the nanoparticles [32]. However in this study, phage virions were replaced with a synergisitic enzybiotic cocktail. Allylamine was used in order to adjust the LCST to a biologically relevant temperature of 34 °C, which is indicative of an infected wound. A bacterial infection of a wound as been shown to present as an elevation in skin temperature of around 3.6 °C (demonstrated in infected leg ulcers), in comparison to the surface temperature of approximately 32 °C seen in healthy skin [33]. PNIPAM nanoparticles were anchored to non-woven polypropylene to simulate a wound dressing using plasma deposited maleic anhydride and free amine groups from allylamine. Plasma

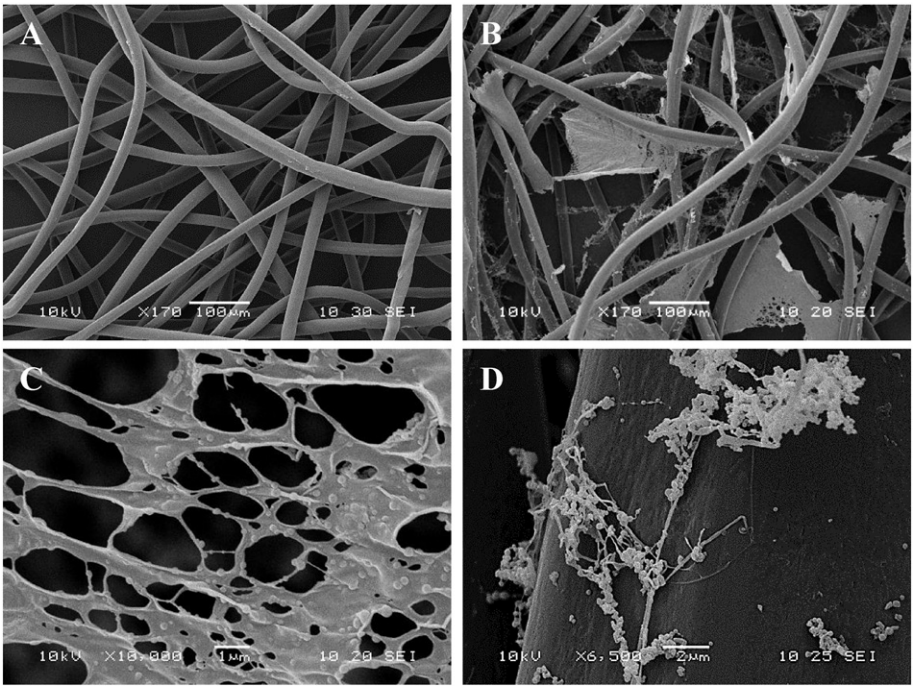

Fig. 1. SEM images of non-woven polypropylene fabric (A) Untreated (B) Following PNIPAM nanoparticle attachment (C) Polymeric matrix seen dispersed within the fibre network (D) Nanoparticles attached to the surface of a polypropylene fibre.