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Bacteriophage-nanocomposites: An easy and reproducible method for the construction, handling, storage and transport of conjugates for deployment of bacteriophages active against *Pseudomonas aeruginosa*

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ABSTRACT

The purpose of this work was proof of concept to develop a novel, cost effective protocol for the binding of bacteriophages to a surface without loss of function, after storage in various media. The technology platform involved covalently bonding bacteriophage 13 (a *Pseudomonas aeruginosa* bacteriophage) to two magnetised multiwalled carbon nanotube scaffolds using a series of buffers; bacteriophage–nanotube (B–N) conjugates were efficacious after storage at 20 °C for six weeks. B–N conjugates were added to human cell culture *in vitro* for 9 days without causing necrosis and apoptosis. B–N conjugates were frozen (−20 °C) in cell culture media for several weeks, after which recovery from the human cell culture medium was possible using a simple magnetic separation technique. The retention of viral infective potential was demonstrated by subsequent spread plating onto lawns of susceptible *P. aeruginosa*. Analysis of the human cell culture medium revealed the production of interleukins by the human fibroblasts upon exposure to the bacteriophage. One day after exposure, IL-8 levels transitorily increased between 60 and 100 pg/mL, but this level was not found on any subsequent days, suggesting an initial but not long lasting response. This paper outlines the development of a method to deliver antimicrobial activity to a surface that is small enough to be combined with other materials. To our knowledge at time of publication, this is the first report of magnetically coupled bacteriophages specific to human pathogens which can be recovered from test systems, and could represent a novel means to conditionally deploy antibacterial agents into living eukaryotic systems without the risks of some antibiotic therapies.

1. Introduction

1.1. Pseudomonas aeruginosa and bacterial resistance to antibiotics

Antibacterial agents have been in public use since the early twentieth century, and continue to act as the main route of curative therapy in Western medicine. Since the mass promulgation of the benefits of penicillin, a range of novel drug classes have been developed to target an increasingly resistant range of bacterial human pathogens. Of key importance to global healthcare scientists is the emergence of multi-drug resistant (MDR) organisms, with species such as *Acinetobacter baumanii*, *Staphylococcus aureus* and *P. aeruginosa* being of particular concern (Hanlon, 2007). *P. aeruginosa* is a ubiquitous organism found in environmental matrices such as soil, and is heavily implicated in incidents of wound, respiratory and burn associated infections (Karatuna and Yagci, 2010). The bacterium has developed MDR status to many of the antibiotics commonly used to treat incidents of human disease, including ciprofloxacin, imipenem and gentamicin (Lambert, 2002). This means that the ability of healthcare workers to treat infections associated with this bacterium is rapidly decreasing. Master et al. (2011) reported that whilst resistance rates for *P. aeruginosa* either showed a small decrease or remained steady in the USA, between 1997 and 2009, high cross resistance rates for imipenem, aztreonam, ciprofloxacin and gentamicin amongst *P. aeruginosa* isolates were observed. Resistance patterns are not confined to one country, and in 2010 Dogru et al. demonstrated 29% ciprofloxacin resistance amongst *P. aeruginosa* isolates recovered from device-associated infections (Dogru et al., 2010). More recently, in 2013 Kataoka et al. reported that amongst *P. aeruginosa* isolates associated with urinary, blood, respiratory and other infections, resistance rates ranged from: 100% resistant to ceftazidime, imipenem, meropenem, ciprofloxacin & tobramycin; 53.4% resistant to piperacillin; 57% to aztreonam; 89.4% to gentamicin; 83% to amikacin; 87.2% to arbekacin; and 6.4% to colistin (Kataoka et al., 2013). This level of MDR leads to the conclusion that *P. aeruginosa* has the potential to develop pan resistance against a host of antibiotic actives, and underlines

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the urgent need for tighter antibiotic stewardship and healthcare governance strategies, as well as the need for new avenues of antibacterial research to be explored.

1.2. Bacteriophages as bio-control agents and delivery strategies

Bacteriophages have long been used to treat bacterial infections and could represent a viable alternative treatment strategy for MDR bacterial infections (Hanlon, 2007). They are highly specific to a single bacterial species, making them ideal agents to target specific infections, with a reduced potential to interfere with the constitution of the normal human microbiota. For example, bacteriophages demonstrating high efficacy against planktonic cultures of *P. aeruginosa* have been used to treat biofilm associated infections. Pires et al. (2011) demonstrated that one species of bacteriophage achieved a three-log reduction of *P. aeruginosa* PAO1 biofilm cell numbers, with continued efficacy demonstrated 24 h after inoculation. The ability to successfully treat biofilms without the use of systemic or broad spectrum antibiotics is of particular use in the treatment of non-healing wounds, ulcers and high body percentage burn injuries (Mulcahy et al., 2014).

Given the specificity of bacteriophages for the target bacterium, they have been used against pathogenic bacteria in the gastrointestinal tract, where, in theory, they should not disrupt the hosts' normal flora, and are nonpathogenic to the host. This has led to the formulation of several bacteriophage based technologies currently on the market, most notable in animal husbandry and food production. Pertinent examples include EcoShield® and ListShield® produced by Intralytix Ltd (USA), which are intended to protect against contamination by *Escherichia coli* and *Listeria monocytogenes* in the food chain (Mai et al., 2010).

Some technologies require the application of bacteriophage cultures directly to the skin (Hooton et al., 2011); others include their adhesion to a biodegradable film which can be applied directly to treat ulcers (Markoishvili et al., 2002). However, many of the original trials conducted targeting the human gastrointestinal tract were subject to less rigorous regulation, meaning that the data has not been scrutinised and validated under accepted, Westernised standards (Verbeken et al., 2012). Questions concerning phage ability to survive passage through the gastrointestinal tract and of persistence in the body remain, with the effects of peristaltic movement, splenic clearance and the removal of the host pathogen all shortening phage availability. It has been suggested, however, that these problems can be overcome to facilitate persistence within the body by the selection of sequestration resistant mutants (Alisky et al., 1998). The delivery of bacteriophages into the human body has been reported to have important consequences for the patient, such as an enhanced antibody based immune response (Bastien et al., 1997), which must be properly investigated. This work did show, however, that immune response can be ameliorated by exposure to a modified bacteriophage, conferring a degree of protection against other viral particles. These works suggest that it is indeed possible to formulate bacteriophage preparations to safely treat internal bacterial human infections.

Research demonstrates that it is the protein coat of the bacteriophage which holds the key to the development of an immune response upon the introduction of bacteriophage to humans and animals. Through biological manipulation, small immunogenic peptides can be attached to the capsid, which act as a novel delivery system for these peptides to key areas, or for the inclusion or delivery of DNA vaccines (Clark and March, 2004). The presence of bacteriophages in long term blood circulation has caused concern about an ongoing immune response after the target bacterium has been eradicated. However, Merril et al. (1996) successfully demonstrated that by modifying the bacterial protein coat, the virus could remain in circulation within animal models to avoid entrapment by the reticuloendothelium system. This suggests that it might be possible to use bacteriophages as long term preventative agents, as well as curative ones, potentially with single dose therapies being developed.

However, the notion of live, circulating virus particles within patients has also caused concern, and needs to be addressed in order to make the procedure viable for large scale clinical use, as it represents a source of considerable public angst. Indeed, there is a pressure for scientists to be sure that the use of live viruses is safe to the patient, can be removed at the end of the treatment process, and will cause no additional harm to the patient during the window of exposure. It should be noted that bacteriophages have been shown to modulate bacterial resistance in some instances (Wagner and Waldor, 2002). Hence it would be prudent for any further studies to investigate gene transmission and enhanced bacterial virulence.

Gram negative infections of large burns during reconstructive surgery is a major factor in morbidity and mortality, systemic antibiotics are often the only way to treat these infections (Azzopardi et al., 2014). The addition of *Pseudomonas* bacteriophage to a wound dressing, for the conditional treatment of infected burns, would represent a significant step forward in burn care. Attachment of the phage to the wound dressing would be advantageous in many respects; the phage would remain separate from the wound, only being released in the event of an infection. To achieve this goal we considered the attachment of phage to carbon nanotubes. Carbon nanotubes are nanoscale and so can be incorporated into many dressing types, they are also strong, so may increase the robustness of many modern hydrogel burn dressings which are often quite fragile.

1.3. Bio–nano technology development

Nanomaterials possess at least a single dimension less than 100 nm, which leads to the dominance of surface atom properties over its bulk form constituents and can lead to changes, or significant enhancement, of performance. Moreover, the reduction of size of a material also increases its surface area thus improving the density of packing and the ability to construct multifunctional devices with a small cross sectional area. Control over their surface chemistry facilitates bottom-up assembly with precise control over the desired properties or functionality, which is exceptionally useful for the development of advanced therapeutics.

The effect of nanomaterials on biological systems can be diverse and related to a dominance or combination of fundamental properties, including solvation rate, geometry, aspect ratio, zeta potential and surface chemistry. Though carbon nanotubes have been found in certain formulations and depending on the cell or tissue of choice to possess toxic responses, not all physiological responses to nanomaterials are malignant. Carbon nanotubes, for example, can positively impact enzymatic catalysis (Caseli and Sigueira, 2012), increase proliferation of neuronal outgrowths (Matsumoto et al., 2007), increase stimulation of biological (and mechanical) performance in hydroxyapatite coatings (Hahn et al., 2009), and, when the chemistry is controlled, provides target therapy of cancer cells (Hong et al., 2010). Therefore, careful consideration and design can lead to useful carbon nanotube-based technologies at the bio–nano interface.

The surface interaction of carbon nanotubes with biomolecules, such as proteins, peptides and nucleotides (Katz and Willner, 2004), also extends to bacteriophages as viral sensors (Mandal et al., 2012) or self-assembly of carbon nanotube-based photovoltaic devices (Dang et al., 2011). However, non-covalent attachment comprises “weaker” forces that exist in equilibrium with its surroundings, which may leave such couplings prone to detachment. Covalent binding of bacteriophages to glass substrates has been conducted to show improved biosensor properties (Handa et al., 2008), but the binding to nanomaterials or nano-structured surfaces for improved performance has not yet been reported.

The surface chemistry of carbon nanomaterials has been well-reported, though for covalent modification, acid-oxidation is an attractive approach for many applications (Banerjee et al., 2005). The *in situ* generation and immobilisation of fulvic acid analogues need to be verified and