Composition, Storage, and Ultraviolet Sterilization Effect Bacteriophage Elution from Biodegradable Microspheres

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INTRODUCTION: The current standard of care for orthopaedic device-related infections (ODRIs) includes IV antibiotics, and multiple surgical procedures to irrigate and debride the infection site and remove and replace the infected device.[1] *Staphylococcus aureus* is responsible for 33-43% of ODRIs[2,3] and is increasingly challenging to treat due to antimicrobial resistance (AMR) and biofilm formation.[4,5] Bacteriophage (phage) therapy (PT) is now being considered as a treatment option for AMR ODRIs.[4] Phage lyse targeted bacterial cells while leaving human cells intact and self-replicate in the presence of host bacteria allowing for amplification at the site of infection, in contrast to antibiotics whose concentration decreases over time.[6] Previously, we demonstrated successful incorporation of phage K into poly(lactic-co-glycolic acid) (PLGA) microspheres to allow local phage delivery. Here, we aimed to optimize our PLGA particle formulation and determine how size, phage encapsulation, and elution profile varied across the formulations, to identify manufacturing protocols that maximize phage elution over time and minimize the particle size average and range. Furthermore, we began preliminary experiments to evaluate the shelf life of the phage-containing microspheres and to examine how ultraviolet (UV) irradiation affected phage stability.

METHODS: PLGA microspheres were synthesized using a water-oil-water protocol as described previously.[7] The effect of the polymer molecular weight (MW), percentage of polyethylene glycol (PEG), and volume (125 µL) of the phage K solution (1.5E11 phage/mL) was evaluated using five different formulations of microspheres: low MW (7,000-17,000 g/mol) PLGA (Sigma Aldrich 719897) with 10% PEG by weight, medium molecular weight (45,000 g/mol) PLGA (Sigma Aldrich 805726) with 0%, 5% or 10% PEG by weight, and medium MW PLGA with double volume of phage solution added. Formulations were evaluated via elution assay using 25 mg of lyophilized microspheres added to 1 mL of synthetic interstitial fluid (SIF), incubated at 37°C for 24 hr, 4 days, and 11 days. Plaque forming units (PFUs) of the eluents were quantitated after spotting 10 µL of serially diluted eluents in triplicate onto a lawn of *S. aureus* UAMS-1 in 0.5% nutrient agar and incubating the plates overnight at 37°C. Total phage eluted during the 11-day study and size were monitored for each batch. Microparticle size was evaluated using scanning electron microscopy (SEM). Shelf life of medium MW PLGA with 10% PEG was evaluated using the standard elution protocol after 0, 2, and 3 weeks of storage at 4°C. The effect of UV irradiation was evaluated after various exposure times (0, 0.5, 1.0, 1.5, 2.0, 3.0 hr) at 60 cm from a 30W germicidal UV bulb. Following UV irradiation, phage titers at 24 hr of elution were performed to determine the phage activity following sterilization.

RESULTS: The MW of the PLGA polymer had a significant effect on phage elution, whereas the percentage of PEG had little effect (**Fig. 1A)**. The microspheres made from low MW PLGA with 10% PEG had significantly lower phage elution at 24 hr compared to microspheres made from medium MW PLGA with 0% PEG (p=0.018). At 24 hr and 4 days, microspheres made with doubled phage volume (p=0.005) had the highest phage elution, which was significantly higher than the elution from low MW microspheres (p=0.005 and p=0.001). Interestingly, the microspheres made from lower percentage of PEG had higher elution at 11 days, but the difference was not significant. None of these formulation alterations resulted in significant changes in microsphere size (**Fig 2**). The preliminary shelf-life study indicated that there may be a detrimental effect of storage at 4°C after 2 weeks (**Fig. 3**). UV irradiation resulted in a larger reduction of phage activity exposure times greater than 1 hr (**Fig. 1B**).

DISCUSSION: In this *in vitro* study, we evaluated different formulations of microspheres to determine the effect phage activity and particle size. Additionally, we conducted preliminary experiments on the effects of storage time and UV sterilization. The medium MW PLGA used to synthesize our particles is ester terminated, making it more hydrophobic. Although the greater attraction between monomers yielded larger particles, they were on average smaller than the low MW PLGA polymer, which is acid terminated. Deviation from the expected size distribution can partially be attributed to batch variability and particles measured. Decreases in PEG percentage resulted in increasing particle size, which is consistent with current literature. The phage eluting capacity appears to be diminished after 2 weeks of 4°C storage. To clinically translate phage-loaded microspheres into use, the particles must be stable during storage and sterilized without significantly reducing phage activity. We evaluated the effect of UV irradiation on phage eluting ability of medium MW PLGA with 10% PEG microspheres and noted a 10^2 reduction in active phage ability after 1.5 hr. Future experiments will evaluate radiation exposure times less than 1 hr and the capacity of UV irradiation to eliminate contamination. These results indicate additional studies are warranted to gauge the effectiveness of the microspheres against *S. aureus* in *in vitro* biofilms and in an *in vivo* animal model.

SIGNIFICANCE: This study evaluated different formulations of bacteriophage containing PLGA microspheres to assess the effect of formulation, storage, and sterilization on active bacteriophage delivery over 11-days to develop a superior alternative to current treatments of ODRIs caused by AMR *S. aureus*.

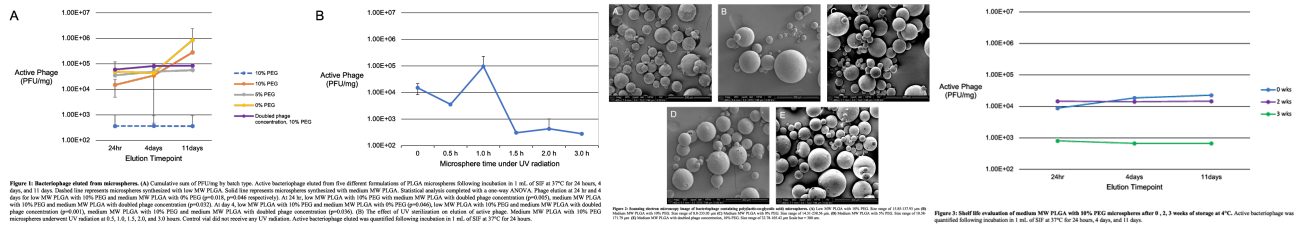

Figure 1: Bacteriophage eluted from microspheres. (**A**) Cumulative sum of PFUmg by batch type. Active bacteriophage eluted from five different formulations of PLGA microspheres following incubation in 1 mL of SIF at 37°C for 24 hours, 4 days, and 11 days. Dashed line represents microspheres synthesized with low MW PLGA. Solid line represents microspheres synthesized with medium MW PLGA. Statistical analysis completed with a one-way ANOVA. Phage elution at 24 hr and 4 days for low MW PLGA with 10% PEG and medium MW PLGA with 0% PEG (p=0.018, p=0.046 respectively). At 24 hr, low MW PLGA with 10% PEG with medium MW PLGA with doubled phage concentration (p=0.005), medium MW PLGA with 10% PEG and medium MW PLGA with doubled phage concentration (p=0.032). At day 4, low MW PLGA with 10% PEG and medium MW PLGA with 0% PEG (p=0.046), low MW PLGA with 10% PEG and medium MW PLGA with doubled phage concentration (p=0.001), medium MW PLGA with 10% PEG and medium MW PLGA with doubled phage concentration (p=0.036). (**B**) The effect of UV sterilization on elution of active phage. Medium MW PLGA with 10% PEG microspheres underwent UV radiation at 0.5, 1.0, 1.5, 2.0, and 3.0 hours. Control vial did not receive any UV radiation. Active bacteriophage eluted was quantified following incubation in 1 mL of SIF at 37°C for 24 hours.

Figure 2: Scanning electron microscopy image of bacteriophage containing polylactic-co-glycolic acid) microspheres. (**A**) Low MW PLGA with 10% PEG. Size range of 13.65-137.93 µm (**B**) Medium MW PLGA with 10% PEG. Size range of 8.62-221.05 µm (**C**) Medium MW PLGA with 0% PEG. Size range of 14.31-238.36 µm. (**D**) Medium MW PLGA with 5% PEG. Size range of 19.26-171.79 µm. All Medium MW PLGA with doubled phage concentration, 10% PEG. Size range of 53.78-181.42 µm Scale bar = 100 µm.

Figure 3: Shelf life evaluation of medium MW PLGA with 10% PEG microspheres after 0 , 2, 3 weeks of storage at 4°C. Active bacteriophage was quantified following incubation in 1 mL of SIF at 37°C for 24 hours, 4 days, and 11 days.

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