BIOPOLYMER MICROPARTICLES AS A POTENTIAL SURROGATE FOR *LEGIONELLA PNEUMOPHILA*

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ABSTRACT (500 WORDS MAXIMUM)

Legionella pneumophila is an opportunistic pathogen in engineered water systems (EWS) that has led to numerous legionellosis outbreaks worldwide. They are often associated with pre-existing EWS biofilms as secondary colonizers, and their release to the bulk water phase upon biofilm maturation results in further contamination. Despite this known risk, the attachment and mobility of *L. pneumophila* in EWS biofilms has not been investigated in detail due to the biohazard and cost of detection. Improvement of legionellosis risk management in EWS requires routine investigations of *L. pneumophila* persistence in EWS using safe, representative, cost-effective, and easy-to-detect surrogates. The commonly used faecal indicator surrogate *E. coli* is a poor model for *L. pneumophila* due to their different properties. In this study, we developed a novel biopolymer surrogate with size, shape, surface charge, and hydrophobicity similar to stationary phase *L. pneumophila* using polyelectrolyte-layered, DNA-encapsulated, alginate-calcium carbonate microparticles. We validated the surrogate's ability to mimic *L. pneumophila* biofilm attachment/detachment kinetics using *Pseudomonas fluorescens* biofilms established on the surfaces of stainless-steel plumbing material in a laboratory-scale bioreactor under a continuous flow regime, in the absence and presence of chlorine.

The results of our preliminary validation studies showed that the surrogate produced similar attachment/detachment kinetics and magnitudes to/from biofilms as that of *L. pneumophila* in the bioreactor. The relative concentrations and peak attachment values for both entities were within the same orders of magnitude and their attachment processes were slower than detachment. Biofilm attachment of both the bacteria and surrogate were reduced in the presence of chlorine. In addition, *P. fluorescens* biofilm concentrations were not affected by exposure to the surrogate due to its biopolymer composition. These results show that with further, more robust validations under more EWS representative conditions, this novel surrogate may provide new insight into understanding *L. pneumophila* mobility and persistence in water systems.

KEYWORDS: *L. pneumophila*, biofilm attachment/detachment, surrogate, biopolymer

PRESENTER PROFILE

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INTRODUCTION

Engineered water systems (EWS) such as cooling towers, air conditioning systems, potable water distribution systems, and recreational pools can be contaminated with *L. pneumophila* which poses a risk of causing legionellosis in the community. In EWS, *L. pneumophila* is frequently found associated with biofilms formed by other species or internalised within free-living amoebae (FLA). Maturation of biofilms and replication of *L. pneumophila* within FLA can release *L. pneumophila* into the bulk water phase, resulting in their dissemination throughout the EWS. Subsequent inhalation or ingestion of water droplets from EWS containing *L. pneumophila* can lead to legionellosis in susceptible individuals (Atlas, 1999).

Despite the global occurrence of legionellosis, the mobility and persistence of *L. pneumophila* in EWS remains poorly understood, mainly due to a lack of a representative surrogate. Although the faecal indicator *E. coli* has been used as a surrogate for *L. pneumophila*, differences in cell surface characteristics of the two bacteria resulted in different transportation behaviours (McBurnett *et al*., 2018, Mondal *et al*., 2020). As such, *E. coli* is an inadequate model for *L. pneumophila.* Use of non-specific, poorly representative surrogates may lead to over or underrepresentation of pathogen mobility, attenuation, and persistence in the environment (Sinclair *et al.,* 2012). Therefore, improved management of *L. pneumophila* risk in EWS requires a better, more suitable, detection-sensitive surrogate that closely mimics the surface properties of the bacteria.

Recently, research groups, including ours, have demonstrated the ability of surface-modified synthetic surrogates to closely mimic the transportation kinetics of important waterborne pathogens such as rotavirus (Pang *et al*., 2014) and *Cryptosporidium parvum* (Pang *et al.*, 2012, Liu *et al.*, 2019). However, the non-biodegradability of these surrogates limits their applications in real-world operational systems and eco-sensitive environments. Therefore, surrogates with biocompatible, biodegradable properties are sought. In this study, we report the development and preliminary validation of a novel, DNA-loaded, biocompatible, and biodegradable alginate-calcium carbonate ($CaCO_3$) surrogate with similar cell surface charge, hydrophobicity, size, and shape to *L. pneumophila*.

METHODS

SURROGATE SYNTHESIS AND MODIFICATION

Size-controlled, rod-shaped surrogate microparticles were produced by co-precipitating equimolar solutions of calcium chloride ($CaCl_2$) and sodium carbonate (Na_2CO_3) in the presence of low viscosity alginate under vigorous homogenization. For DNA loading, an aqueous suspension of alginate-$CaCO_3$ surrogate was incubated overnight with a DNA tracer previously produced in the lab (Pang *et al*., 2020) under slow magnetic stirring. DNA-loaded alginate-$CaCO_3$ surrogate microparticles were recovered by centrifugation. The surface charge and hydrophobicity of the DNA loaded surrogate was modified by assembling positively and negatively charged polyelectrolytes (poly-L-lysine and poly-L-glutamic acid,

respectively) using layer-by-layer method. Surface charge and hydrophobicity of the surrogate were measured before and after polyelectrolyte assembly (using microbial adhesion to hydrocarbon assay and zeta potential, respectively) to confirm successful surface modification. Detailed methods for surrogate biosynthesis and modification are given in Ariyadasa et al. (2021b).

SURROGATE VALIDATION

The surface-modified, DNA-loaded alginate-$CaCO_3$ surrogate microparticles were validated for their ability to mimic *L. pneumophila* attachment/detachment kinetics in flow-through bioreactors under continuous flow at 30 °C, 200 rpm. For bioreactor studies, *P. fluorescens* biofilms were developed on coupons of stainless-steel, a commonly used plumbing material. After the biofilms were developed on the coupon surfaces for 3 days under batch phase, the bioreactors were inoculated with freshly prepared surrogate or stationary phase *L. pneumophila* with known concentrations. The bioreactors were maintained in the continuous flow phase and the coupons were sampled in triplicate every 2-3 days over a period of 12 days. Coupons were washed in Butterfield buffer to remove loosely bound biofilm and the *L. pneumophila* or surrogate-associated biofilms were harvested by sonication and vortexing. Surrogate and *L. pneumophila* DNA were extracted using a commercial DNA extraction kit. Concentrations of biofilm associated surrogate and bacterial DNA were quantified using quantitative polymerase chain reaction.

RESULTS AND DISCUSSION

Scanning electron microscopy observations (Figure 1 A and B) confirmed that the rod-shaped alginate-$CaCO_3$ surrogate microparticles were ~1.3 µm in length and ~1 µm in width. As such, the surrogate closely matched the length of stationary phase *L. pneumophila*. However, it was ~3 times larger in width compared to the bacteria. Our previous studies have shown that the stationary phase *L. pneumophila* is 1.43 ± 0.34 µm long and 0.32 ± 0.03 µm wide (Ariyadasa et al., 2021a). Microbial adhesion to hydrocarbon assay showed that the surface modification using polyelectrolytes increased the surrogate surface hydrophobicity from 6.19 (±1.68) % (unmodified surrogate) to 37.88 (±0.46) %. Zeta potential of the surface modified surrogate was −21.70 (± 0.88) mV. These values closely represented the cell surface hydrophobicity and charge of stationary phase *L. pneumophila* (44.69 ± 1.20 % and -27.16 ± 0.01 mV, respectively).

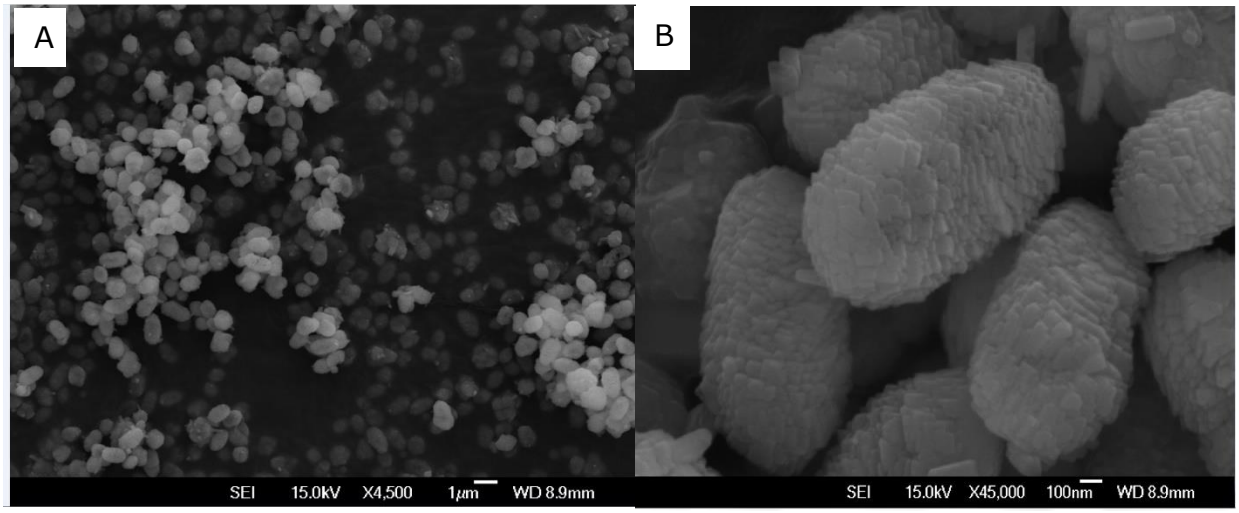

Figure 1. Scanning electron microscopy images of alginate-calcium carbonate surrogate microparticles at low (A) and high (B) magnifications. (2021, American Chemical Society)

The size, shape, and surface properties of the surrogate were similar in batches of surrogate microparticles produced in different dates, suggesting that they were reproducible. The surrogate also showed high DNA loading efficiency, with an average DNA loading efficiency of 94.73 (±5.99) % reported from qPCR of 6 independent batches. This may be due to the nanoporous structure and the high surface area of the surrogate microparticles, as indicated by the Brunauer-Emmett-Teller (BET) and Barrett-Joyner-Halenda (BJH) analyses. DNA leaching of the surrogate was very low in tap water over a period of 17 days suggesting that the DNA marker was effectively retained by the surrogate, possibly due to the surface modification.

Bioreactor experiments showed that the surrogate and *L. pneumophila* had similar attachment/detachment patterns (Figure 2). Both surrogate and *L. pneumophila* were detected in the biofilms on coupon surfaces from day 3, suggesting their attachment. The concentration of biofilm-associated surrogate and *L. pneumophila* continued to increase until day 10-14 in surrogate experiments and day 12 in *L. pneumophila* experiments. The relative concentrations of biofilm-attached surrogate and *L. pneumophila* were in the same orders of magnitude during peak attachment.

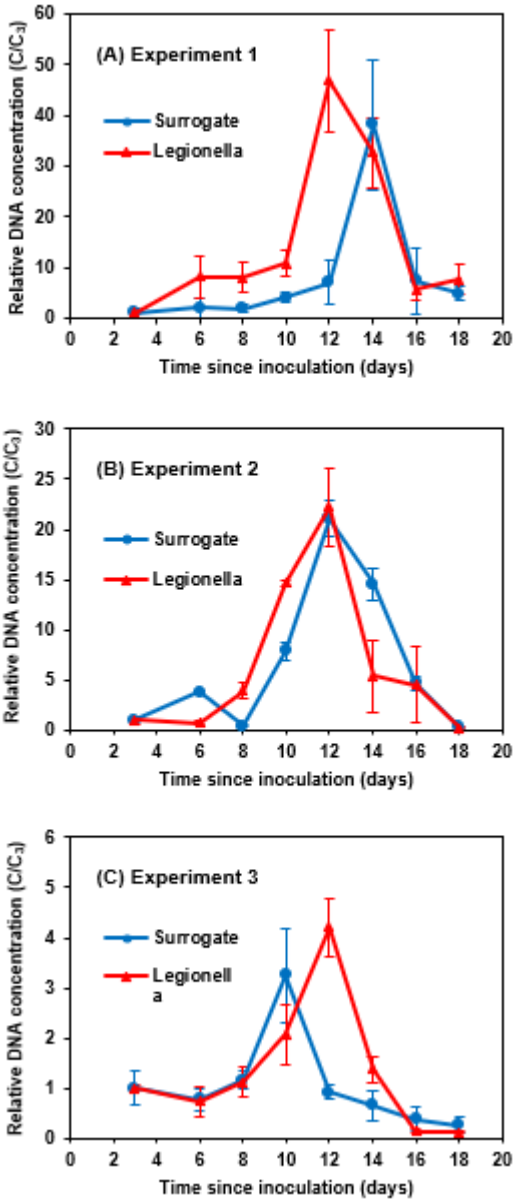

Figure 2. Normalised relative concentrations of biofilm-associated surrogate and *L. pneumophila* DNA harvested from the bioreactor experiments conducted in the absence (A and B) and presence (C) of chlorine (2021, American Chemical Society)

A decrease in biofilm-associated surrogate and *L. pneumophila* concentration were observed after the peak attachment point indicating the onset of the detachment process. Detachment processes of both entities were much faster than the attachment processes. The relative concentrations of biofilm-associated surrogate and *L. pneumophila* were lower in the experiments conducted in the presence of chlorine compared to those conducted in the absence, suggesting a reduction in biofilm attachment of both the surrogate and *L. pneumophila* in the presence of the disinfectant. A temporal moment analysis showed that the surrogate to *L. pneumophila* ratios at the zeroth, normalised first, and the normalised second moments were close to 1, indicating similarity in attachment/detachment kinetics of the surrogate and *L. pneumophila.*

CONCLUSIONS

Better prediction and control of legionellosis risk in EWS systems requires the use of a representative surrogate that can be applied in a wide variety of experimental and environmental settings. In this study, we have developed a novel new biopolymer surrogate for *L. pneumophila*. The DNA marker encapsulated in the surrogate enabled their easy detection using qPCR. The biosynthesis process resulted in a high and reproducible microparticle yield. Bioreactor studies showed that the biopolymer surrogate could mimic *L. pneumophila* attachment/detachment kinetics to and from *P. fluorescens* biofilms established on stainless steel coupon surfaces under continuous flow conditions, both in the absence and presence of chlorine. This suggests the potential of the newly developed surrogate to mimic mobility and persistence of *L. pneumophila* in EWS*.* However, future validation studies using more realistic EWS conditions such as multispecies biofilms, low nutrient conditions, different pluming materials, and temperatures are required to further understand the potential of the surrogate to mimic *L. pneumophila*. With future investigations, this surrogate could provide an effective tool to predict *L. pneumophila* risk more accurately and effectively than existing tools.

Figure 1. Scanning electron microscopy images of alginate-calcium carbonate surrogate microparticles at low (A) and high (B) magnifications. (2021, American Chemical Society)

Figure 2. Normalised relative concentrations of biofilm-associated surrogate and *L. pneumophila* DNA harvested from the bioreactor experiments conducted in the absence (A and B) and presence (C) of chlorine (2021, American Chemical Society)

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REFERENCES

ARIYADASA, S., ABEYSEKERA, G., BILLINGTON, C., FEE, C. & PANG, L. 2021a. Growth phase-dependent surface properties of Legionella pneumophila and their role in adhesion to stainless steel coated QCM-D sensors. *Letters in Applied Microbiology,* 73**,** 257-267.

ARIYADASA, S., ABEYSEKERA, G., PREMARATNE, A., ROBSON, B., BILLINGTON, C., FEE, C. J. & PANG, L. 2021b. Surface-Modified Biopolymer Microparticles: A Potential Surrogate for Studying Legionella pneumophila Attachment to Biofilms in Engineered Water Systems. *ACS ES&T Water,* 1**,** 2057-2066.

ATLAS, R. M. 1999. Legionella: from environmental habitats to disease pathology, detection and control. *Environmental Microbiology,* 1**,** 283-293.

LIU, L., WANG, Y., NARAIN, R. & LIU, Y. 2019. Functionalized polystyrene microspheres as Cryptosporidium surrogates. *Colloids and Surfaces B: Biointerfaces,* 175**,** 680-687.

MCBURNETT, L. R., HOLT, N. T., ALUM, A. & ABBASZADEGAN, M. 2018. Legionella-A threat to groundwater: Pathogen transport in recharge basin. *Science of the Total Environment,* 621**,** 1485-1490.

MONDAL, I., ACOSTA, J., ALUM, A., MAYER, B. K., DAHLEN, P. & ABBASZADEGAN, M. 2020. Comparative Transport of Legionella and E. coli through Saturated Porous Media in a Two-Dimensional Tank. *Water,* 12**,** 3170.

PANG, L., ABEYSEKERA, G., HANNING, K., PREMARATNE, A., ROBSON, B., ABRAHAM, P., SUTTON, R., HANSON, C., HADFIELD, J. & HEILIGENTHAL, L. 2020. Water tracking in surface water, groundwater and soils using free and alginate-chitosan encapsulated synthetic DNA tracers. *Water Research,* 184**,** 116192.

PANG, L., FARKAS, K., BENNETT, G., VARSANI, A., EASINGWOOD, R., TILLEY, R., NOWOSTAWSKA, U. & LIN, S. 2014. Mimicking filtration and transport of rotavirus and adenovirus in sand media using DNA-labeled, protein-coated silica nanoparticles. *Water research,* 62**,** 167-179.

PANG, L., NOWOSTAWSKA, U., WEAVER, L., HOFFMAN, G., KARMACHARYA, A., SKINNER, A. & KARKI, N. 2012. Biotin-and glycoprotein-coated microspheres: potential surrogates for studying filtration of Cryptosporidium parvum in porous media. *Environmental science & technology,* 46**,** 11779-11787.

SINCLAIR, R. G., ROSE, J. B., HASHSHAM, S. A., GERBA, C. P. & HAAS, C. N. 2012. Criteria for selection of surrogates used to study the fate and control of pathogens in the environment. *Applied and environmental microbiology,* 78**,** 1969-1977.