Polyvalent Phages Conjugated to Magnetic Nanospheres for Mixed Biofilm Treatment

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Targeting ARB in Activated Sludge Communities

Biofilms are formed by complex communities of attached microbes associated with eutrophic aquatic systems in nature. They also form on structures and processing components found in Waste Water Treatment Plants (**WWTP**).

Fig. 1
Biofilms form on hard surfaces and are complex microbial communities

A common method of treating municipal waste water is by transferring microbially-rich sludge (activated sludge) from further along the WWTP process back into the aerobic digesters near the beginning.

Newer methods employing membrane bioreactors (**MBR**) have increased efficiencies by reducing WWTP steps, but have inherent challenges.

- Activated sludge method has limitations
- Biofilms foul MBRs
- Flux decreases of 60% are not uncommon
- The community of microorganisms are mostly beneficial and required for aerobic decomposition of biosolids

Further complicating the MBR process are antibiotic resistant bacteria (**ARB**).

- ARB are not uncommon within biofilms in WWTP facilities
- Pose special risks within MBRs fouled by ARB
- MBRs may prove the best site to control ABR with our process

Where treatment options are limited, non-conventional solutions may exploit nanotechnology and genetic resources, including bacteriophages (phages), to target ARB.

Unique Approach to Overcome Biofilms

Control of target ARB may be accomplished with polyvalent bacteriophages. These phages lyse multiple bacterial hosts and may offer broad spectrum control. Especially important are effective, non-chemical controls for *Pseudomonas aeruginosa* (PAO-1) and *Enterobacteriaceae* (*E. coli* 15597). Our polyvalent phage nanosphere process demonstrates better control of target ARB.

- Biofilms' extracellular polymeric substances (EPS) effectively shelter target bacteria. Our conjugation technique combines magnetic nanospheres, polyvalent phages and the biofilm treatment.
- By conjugating phages to specific magnetic nanospheres, we
  - Overcame the biofilm barrier by introducing phages though biofilms—*penetrating* them
  - Increased phage efficacy
- Our process targets specific bacteria (*E. coli* 15597 and PAO-1) without affecting beneficial community members.

Selecting for Polyvalency is Key

Native phages were collected from campus soil and activated sludge media from City of Houston WWTP. Phages were isolated and selected for polyvalent action against specific enterobacteria via a sequential approach (Chart 1).

Our polyvalent phage selection proved to have high plaque forming units (PFU) across a spectrum of enterobacteria, including *Pseudomonas aeruginosa* (PAO-1) and *Enterobacteriaceae* (*E. coli* 15597). The selection process involves laboratory-scale protocols including ...

- Isolating, purifying and culturing phages
- Maintaining strong multi-ARB infectibility as a key objective
- *E. coli* 15597 & PAO-1 effectiveness at 1:1 ratio

Our polyvalent phage PEL01 was successfully isolated and achieved the above objectives with PFU of $6*10^6$

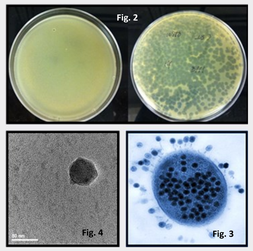
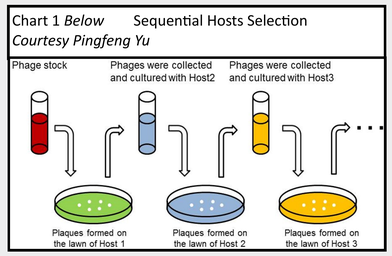


Figure 2 *Left top*
Assay of control (L) and phage (R)

Figure 3 *Left bottom*
Phage PEL01 *Courtesy of Lingli Li*

Figure 4 *Right bottom*
Bacterium under phage attack
Figures 2 and 4 courtesy Pingfeng Yu



Conjugation of Polyvalent Phages to Magnetic Nanospheres

Nanospheres were synthesized as these conjugate candidate materials, designated as :

- **FN**—An amination synthesis (Fe_3O_4@SiO_2-NH_2)
- **FS**—A synthesized silicate coating (Fe_3O_4@SiO_2)
- **FC**—A carboxylation synthesis (Fe_3O_4@SiO_2-COOH)

Prepared, washed nanospheres were agitated, incubated with EDC and NHS, and again washed.
Our Phage PEL01 was introduced to the three synthesized materials, FN, FS and FC, by overnight agitation.
Any additional conjugation sites were blocked.

Zeta potential affects biofilm penetrability, potentially allowing phage-conjugated nanospheres to be manipulated though the biofilms that foul MBRs, and targeting ARB.
Our unique nanospheres' zeta potential properties were confirmed in a laboratory setting.

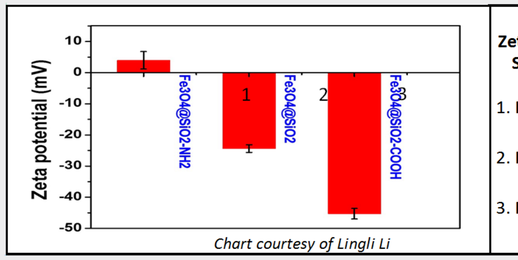


Chart courtesy of Lingli Li

Chart 2
Zeta Potential (mV) of Synthesized Nanospheres
1. **FN** Amination **+4 mV**
2. **FS** Silicate Coating **-25mV**
3. **FC** Carboxylation **-44mV**

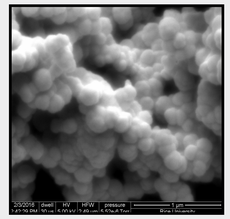
Confirmation and Efficacy of Conjugated Nanospheres

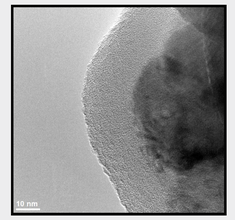
Transmission and Scanning Electron Microscope images confirmed conjugation to the synthesized nanospheres.

Figure 5 *Near right,*
TEM image of **FS**

Figure 6 *far right*
SEM image of **FS**

Images courtesy of Lingli Li





PFU density on a lawn of the mixed targets show evidence of strong infection and the effectiveness of our processes with our three synthesized materials. Further research will focus on the polyvalent phages' environmental resiliency, such as, *in situ* ranges for pH and temperature.

Our successful polyvalent, conjugated nanospheres that are can be manipulated magnetically may prove to be a new method to combat ARB, especially within biofilms on MBRs.

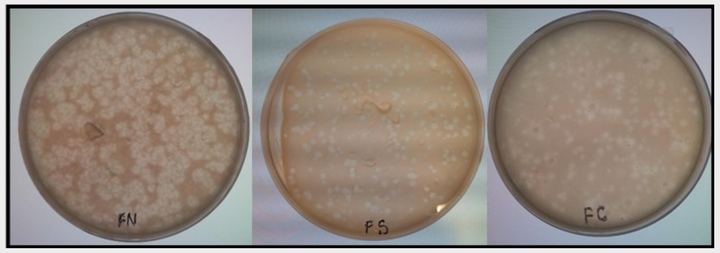


Figure 7 Cultivation of Polyvalent Phages
FN (left), **FS** (center), & **FC** (right) showing plaques among a lawn of mixed *E. coli* 15597 & PAO-1

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