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Award Number: W81XWH-10-1-0756

TITLE: Noninvasive Thermal Ablation of Osteomyelitis-Causing Bacteria Using Functionalized Nanoparticles

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REPORT DATE: March 2012

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-03-2012	Final	1 SEP 2010 - 29 FEB 2012

4. TITLE AND SUBTITLE

Noninvasive Thermal Ablation of Osteomyelitis-Causing Bacteria Using Functionalized Nanoparticles

5a. CONTRACT NUMBER

5b. GRANT NUMBER

W81XWH-10-1-0756

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Maryland
College Park, MD 20742

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

During the performance period, we successfully constructed an N-terminal serine to cysteine mutant of PlyCB. This mutant was expressed, purified, reacted with gold nanoparticles and the resulting PlyCB/gold nanoparticle complex was then purified. A fraction of the complex was further conjugated to a fluorescent dye and specific binding of the complex to streptococcal cells, but not non-target cells, was observed by both bright field and fluorescent microscopy. Next, the nanoparticles were exposed to various power settings on an infrared spectrophotometer and showed a measured response to input power. In parallel we tested the viability of streptococcal cells at different temperatures and at different exposure times. Finally, we made a fusion of PlyCB with a pneumococcal binding domain and demonstrated that we could can change or expand specificity of the nanoparticle complex through biological engineering.

15. SUBJECT TERMS

Gold, Nanoparticle, Infrared Radiation, Infrared Spectroscopy, Thermal Ablation, Bacteriophage

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	15	**19b. TELEPHONE NUMBER** *(include area code)*

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INTRODUCTION:

Due to the changing bacteriology and multi-drug resistant nature of osteomyelitis (bone infections) associated with war wounds, choosing appropriate antimicrobial therapy is a challenging task (Calhoun, 2008; Johnaon, 2007; Murray, 2006; Murray, 2008; Yun, 2008). One alternative approach to antibiotics includes the use of near infrared (IR) radiation to thermally kill pathogenic organisms (Kam, 2005; Zharov, 2006). Whereas near IR wavelengths pass harmlessly through the human body, they are known to heat gold nanoshells to high temperatures (>70°C), which in principle will thermally ablate any cell (bacterial or eukaryotic) in close proximity to these particles. Due to the rapid dissipation of thermal energy over very short distances, it is speculated that only cells in direct contact with gold particles will be killed and surrounding tissues will be unaffected (Hu, 2006). The challenge to this approach is to develop a system to bind the gold nanoparticles to the surface of targeted cells. Our aim is to use a scaffold protein, PlyCB, which can be functionalized to bind gold nanoparticles as well as bacterial-specific targeting domains. PlyCB is stable up to 100°C, resistant to proteolysis, high ionic strength, extreme pH conditions, and most detergents, making it a perfect platform for engineering. The "top" surface of PlyCB will be modified to contain cysteine residues to covalently bind gold nanoparticles (Fig 1). The "bottom" surface of PlyCB inherently binds streptococcal cells with nanomolar affinity, which will be used for proof-of-principle thermal ablation studies. We further propose to engineer the "bottom" surface to contain an array of binding domains isolated from tail fibers of bacteriophage that are specific for osteomyelitis causing pathogens.

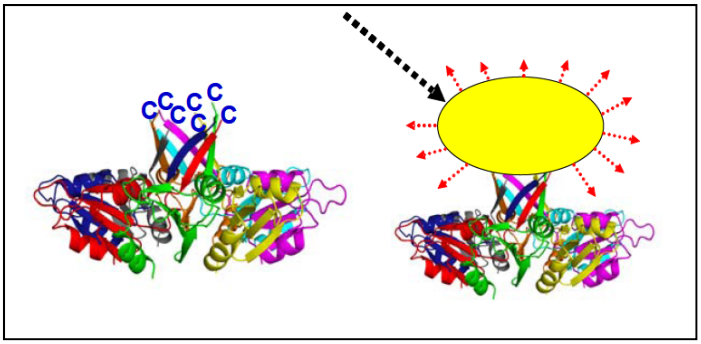


Fig. 1. Left, PlyCB with a Ser to Cys mutation at the N-terminal amino acid. Right, gold particles will bind PlyCB via the cysteine residues, making the PlyCB/gold nanoparticle complex that can be used for thermal ablation studies.

BODY:

<u>Aim 1. Make PlyCB/Gold nanocomplexes.</u>

Task 1. Obtain reagents. (month 1)

All reagents have been obtained. The gold nanoparticles causes us the most trouble. Initially, we were using 100 nm gold nanoparticles (round balls). However, after fine tuning our infrared spectrophotometer and receiving more technical advice from the vendor, we found that a nanorod, not a sphere, produced better surface plasmon resonance for our applications. We now have normal nanorods as well as neutravidin-coated rods.

Task 2. Make PlyCB mutations (change serine at position 1 to cysteine (S1C mutant). (month 1-3)

The single point mutant (Ser at position 1 to cysteine) was easily accomplished by a QuickChange Mutagenesis kit. The S1C mutant was expressed, purified, and confirmed to contain the mutation by both mass spectral analysis and N-terminal sequence analysis (data not shown). Additionally, we confirmed by analytical gel filtration that the mutant had no adverse affect on formation of the PlyCB octamer. We were able to generate ~50 mg of pure S1C mutant from a 6L fermentation.

Task 3. Make PlyCB/gold nanoparticle complex. (month 4-9)

Initially, 1 mg of the S1C mutant of PlyC was reacted with ~$1x10^{10}$ gold nanoparticles (nps)/ml and purified by size exclusion chromatography (Fig. 2). The chemistry involved relied on free, reduced sulfhydral moieties of the cysteine residues to interact directly with the gold. This approach was very successful at forming the complex. However, the complexes were "sticky" and would often bind to the container they were stored in or they would aggregate and fall out of solution. To solve this problem, we switched to using gold nanoparticles that were coated with a neutravidin shell. In order to bind to the neutravidin, we biotinylated the cysteine residues of the S1C mutant. Although this was a setback, we did achieve formation of the complex, were able to purify it, and successfully store it without aggregation issues (data not shown).

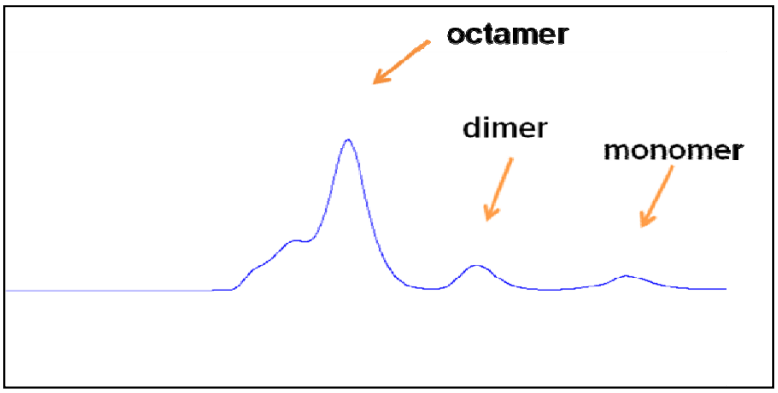


Fig. 2. Analytical gel filtration showing formation of the PlyCB octamer complexed to the gold nanoparticles.

Another issue we encountered (later in Aim 2), caused us to return to this task. Namely, the 100 nm spherical gold nanoparticles we were using were not optimized for thermal ablation by near infrared spectroscopy. Through trial and error, we determined a gold nanorod, roughly 10 nm x 30 nm, gave the best surface plasmon resonance for thermal ablation studies by infrared radiation. Thankfully, the vendor made neutravidin rods in the size/length we required. We now have a stock of PlyCB/gold nanorod complexes for future studies.

Next, we chemically crosslinked the complex with a fluorophore (AlexaFluor 488), mixed it with streptococcal cells, washed, and viewed under bright field and fluorescent microscopy. As can be seen in Fig. 3, gold nanoparticles adhered to the surface of the streptococci. We wanted to determine if this binding event was specific for the streptococcal surface or simply an artifact of a "sticky" complex. Therefore, we repeated the experiment in the presence of both streptococci and *E. coli*, a non-target host for PlyCB. As can be seen in Fig. 4, the PlyCB/gold nanoparticle complex specifically binds streptococci.

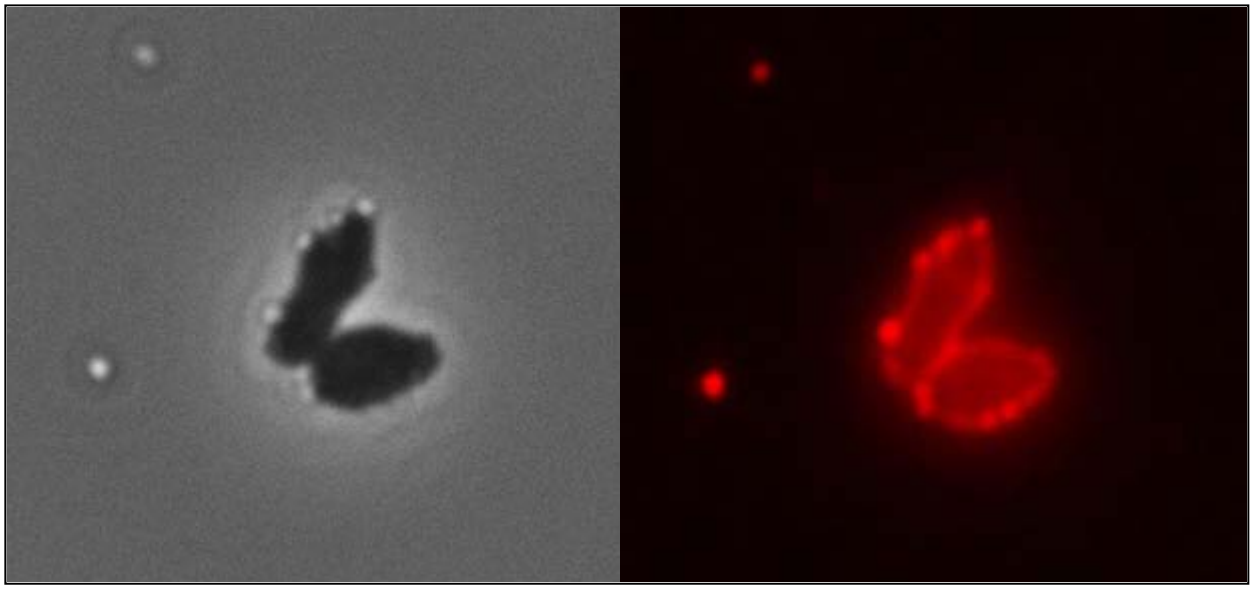
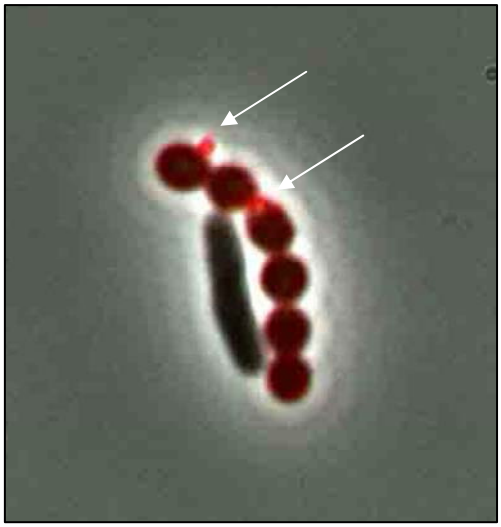


Fig. 3. Left, bright field image of PlyCB/gold nanoparticle complexes binding streptococcal cells. The 100 nm gold spheres coated with PlyCB can be seen as white dots on the streptococcal cell. Right, fluorescent image. PlyCB was labeled with a fluorophore before being complexed to the gold nanoparticles. As such, the particles can be seen as bright red spheres.

Fig. 4. The same experiment as in Figure 3, but this time, E. coli was mixed with streptococci. The PlyCB/gold complexes specifically bind streptococci (white arrows) but not the E. coli.



Aim 2. Proof-of-principle thermal ablation experiments.

Task 1. Irradiation and power tests. (month 9-10)

Before we can perform irradiation and power test on our nanocomplexes, we needed to do some basic studies on how much heat is needed to kill streptococci and at what duration. Therefore,

we heated streptococci to various temperatures for different amounts of time. As shown in Fig. 5, all streptococci (i.e. 10^7) were killed when exposed to 50°C for 10 minutes. However, when the temperature was raised to 60°C, only 10 seconds was needed to produce a 4 log drop in viability and 65°C sterilized the culture. Given that thermal ablation can produce very high temperatures, we may only need a fraction of a second of contact time.

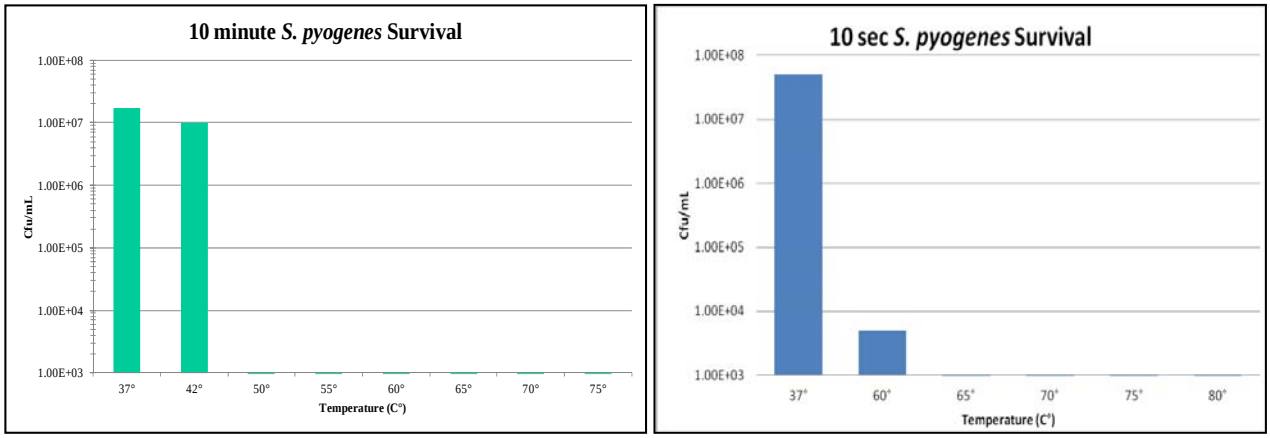


Fig. 5. Left, thermal killing after 10 min exposure. Right, thermal killing after 10 second exposure.

Finally, we were ready for power testing. As can be seen in Fig. 6, just 5 or 10 milijules of laser power was sufficient to produce a strong resonant energy. We are may be sufficient for thermal ablation studies.

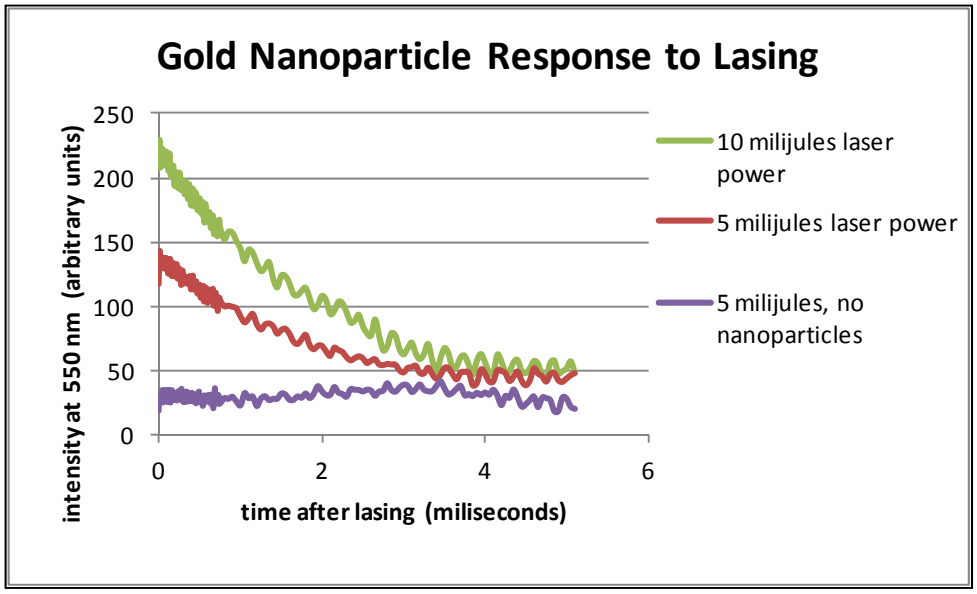


Fig. 6. Resonance energy response of PlyCB/gold nanoparticle complex to near infrared lasing at 550 nm.

Task 2. Bacterial ablation testing. (month 11-13)

In our initial bacterial ablation experiments, it appeared as if thermal ablation of streptococci worked at all power settings (data not shown). However, we noticed that controls where infrared lasing did not occur also produced a significant drop in viability. Therefore, we had immediate concerns that the gold nanoparticles themselves were toxic to streptococci. Indeed, as seen in Fig. 7, bare gold nanoparticles were somewhat toxic to the bacteria in the absence of near infrared lasing. However, the neutravidin-coated particles were found to be non-toxic. The neutravidin-coated particles bound to the biotinylated S1C mutant of PlyCB were therefore used for all future experiments. Significantly, we were able to show thermal ablation of streptococcal strain D471 by neutravidin gold nanoparticles both alone and complexed with PlyCB (Fig. 8).

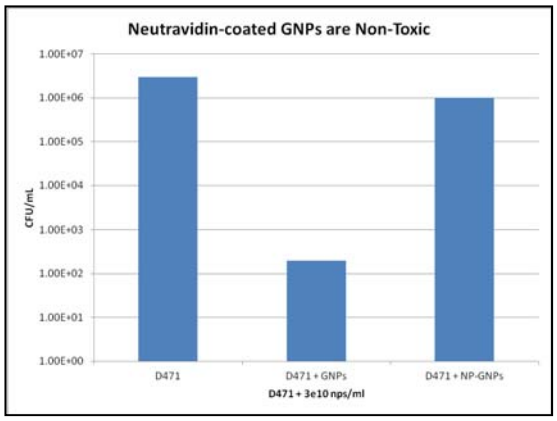


Fig. 7. "Bare" gold nanoparticles was toxic to streptococcal strain D471, but when the same particles were coated with neutravidin, toxicity was avoided.

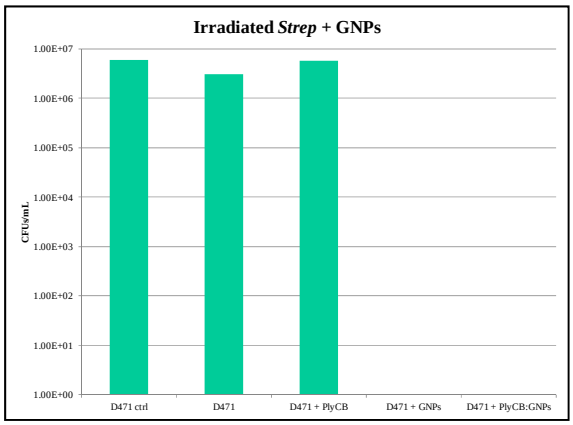


Fig. 8. $6x10^6$ colony forming units/ml of streptococcal strain D471 was mixed with $6x10^{10}$ nanoparticles/ml and irradiated with 50 pulses of a 5 mJ laser at 569 nm. Only wells containing gold nanoparticles were sterilized.

Task 3. Determine depth of IR radiation penetration and range of thermal damage. (months 11-15)

For this task, we cultured a monolayer of Hep-2 epithelial cells and seeded one corner with streptococcal cells pre-coated in PlyCB/gold nanocomplexes. The entire well was then exposed to 10 milijules lasing. Trypan blue was then added to the media to identify lysed epithelial cells. No cell lysis was noted, which suggest the eukaryotic cells there not thermally damaged.

We next tried to recover streptococcal cells from the co-culture environment by differential centrifugation in order to measure streptococcal ablation. Unfortunately, due to the adherent properties of the bacteria toward the eukaryotic cells, we were not able to obtain reproducible results in our experimental or control wells.

Aim 3. Engineer bacteriophage tail fiber domains.

Task 1. Obtain and characterized bacteriophage. (month 1-6)

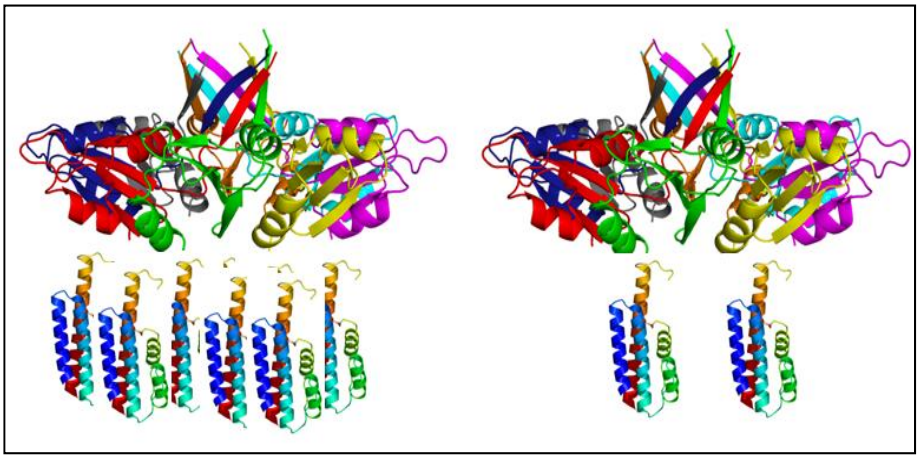
We have obtained several phage through depositories or international collaborators. Sequencing has been an issue and even when sequencing is available, there is a bit of a bioinformatic bottleneck trying to elucidate which domains on a tail fiber are responsible for binding to the bacterial surface and which domains are structural. Nonetheless, we have identified a binding domain on a pneumococcal phage that we believe is responsible for binding to the surface. This domain, known as a choline binding domain (CBD), will serve us for our proof of concept studies.

Task 2. Make PlyCB/gold nanoparticle constructs with selected phage tail fiber or lysin binding domains. (month 7-12)

As stated above, we have identified a pneumococcal binding domain for proof of principle experiments, synthesized this gene, and made a fusion of this gene to the C-terminal domain of PlyCB. Unfortunately, when we expressed the fusion protein, insoluble inclusion bodies were formed. We speculate the reason was due to structural hindrance attributable to the size of the fusion protein. Significantly, each PlyCB monomer is 8 kDa, making the octamer 64 kDa and we attempted to add a ~10 kDa CBD to each PlyCB monomer, making the overall structure ~150 kDa. We always knew this would be a potential pitfall, but we nonetheless attempted to make the fusion.

In order to address the misfolding of our fusion protein, we decided to attempt co-expressing WT PlyCB along with the PlyCB-CBD fusion in hopes that the resulting octamers would only incorporate a few CBD domains instead of all eight. Under this scenario, there would be a Gaussian distribution ranging from 8 of 8 monomers being WT to 8 of 8 monomers being PlyCB-CBD, with the majority of the octamers containing 3, 4, or 5 monomers of WT and 3, 4, or 5 monomers of PlyCB-CBD (see Fig. 9). Those that contained a majority of PlyCB-CBD would be insoluble and those that contained only WT could be selected against by using two chromatography steps; one specific for PlyCB and one specific for CBD.

Fig. 9. When all PlyCB monomers are fused to CBD (left), insoluble inclusion bodies are formed due to mis-folding. Our co-expression scheme was designed to incorporate just a few fusion monomers in an otherwise WT octamer (right).



Expression of a soluble, recombinant, hetero-octamer protein was successful. Fig. 10 below shows both the 8 kDa WT PlyCB and the ~18 kDa PlyCB-CBD fusion co-purified by double affinity chromatography (hydroxyapatite column to bind PlyCB and a choline column to bind CBD) and Fig. 11 confirms hetero-octamer formation by analytical gel filtration.

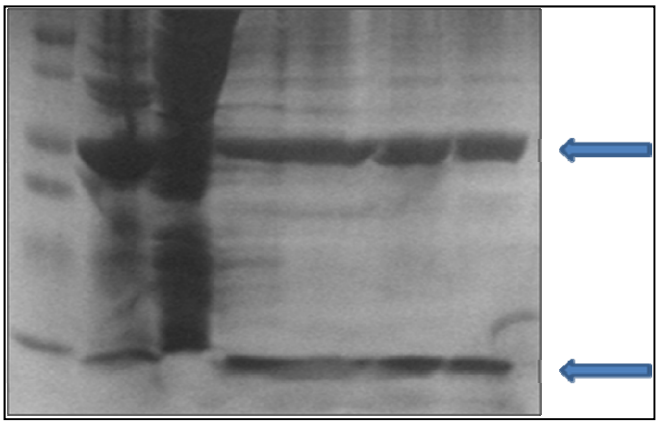


Fig. 10. Double affinity purified PlyCB x PlyCB/CBD fusions. The 18 kDa PlyCB/CBD fusion is show by the top arrow and the 8 kDa WT PlyCB is shown by the bottom arrow.

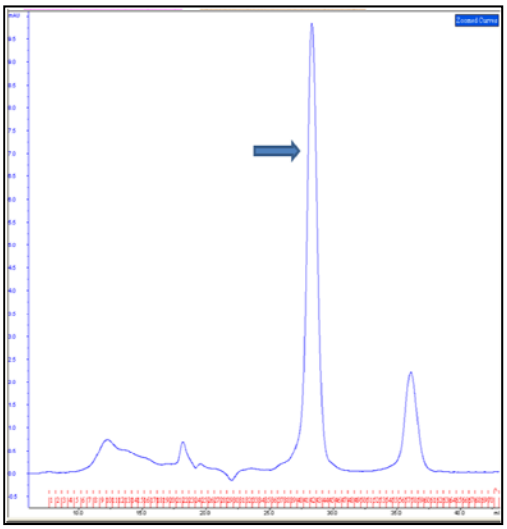


Fig. 11. Analytical gel filtration showing the purified hetero-octamer (arrow).

Next, we labeled the PlyCB-PlyCB/CBD hetero-octamers with an AlexaFluor dye in order to determine if the complex could not bind pneumococcal cells. As can be seen in Fig. 12, the complex has not only acquired the ability to bind pneumococcal cells (left image), but it retains full binding to the parental streptococcal cells (middle image). As a control, it does not bind non-target *E. coli* cells (right image). Thus, through protein engineering, we have expanded the host range of our PlyCB platform, which was the overall goal of Aim 3.

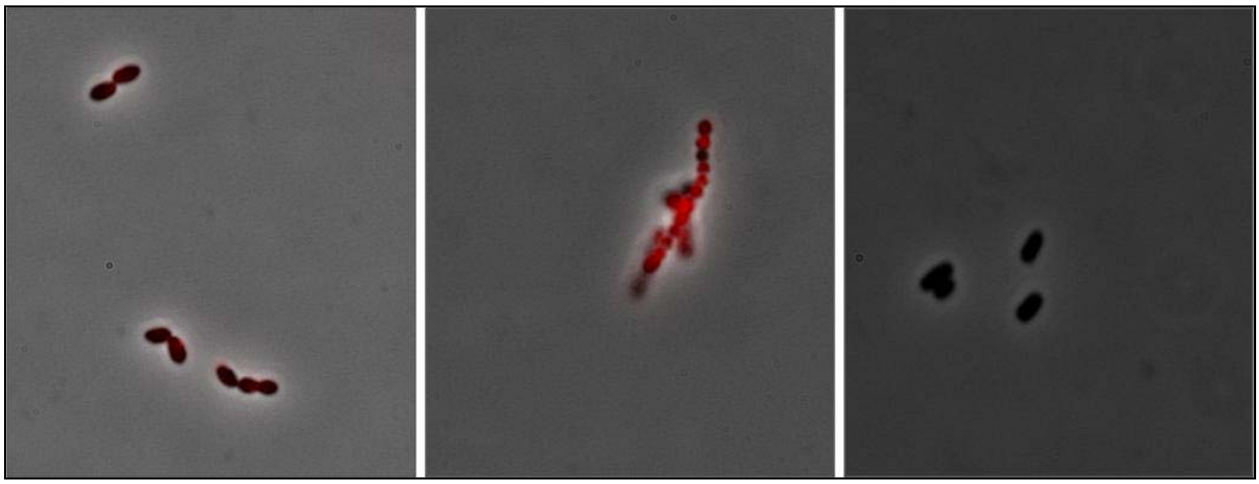


Fig. 12. Merged brightfield and fluorescent images of labeled PlyCB-PlyCB/CBD complexes with pneumococci (left), streptococci (middle), and *E. coli* (right).

Task 3. Thermal ablation experiments on osteomyelitis-causing pathogens. (months 13-18)

As stated above, this project was much more intricate than anticipated and in order to keep pace with the stated aims, we fused a pneumococcal CBD domain to PlyCB for proof of principle experiments demonstrating that the specificity of PlyCB could be changed. Task 2, which consisted of making the PlyCB/CBD complex took almost 8 months due to the solubility issues when expressing fusions of PlyCB. Nonetheless, we did form stable PlyCB/CBD complexes as outlined above. For the current task, we biotinylated the PlyCB/CBD complex and reacted it to neutravidin conjugated gold particles. We were able to purify the large complex by gel filtration. However, whereas streptococcal cells were stable with neutravidin gold particles in the absence of lasing (Fig. 7), pneumococcal cells, which were tested in this instance, underwent autolysis in the presence of neutravidin coated gold, so we could not fully evaluate the effects of lasing.

While PlyCB/CBD did allow us to demonstrate the platform utility of PlyCB (i.e. we could change specificity while still using PlyCB as a scaffold to bind gold nanoparticles), the instability of pneumococcal cells in the presence of gold nanoparticles was unanticipated. Looking toward the future, a variety of different cell wall binding domains could be evaluated in this system now that we have worked out the methodology to selectively express just a few fusion proteins in the scaffold octamer.

KEY RESEARCH ACCOMPLISHMENTS:

- Successfully created S1C mutation of PlyCB
- Made PlyCB/gold nanoparticle complexes
- Demonstrated that PlyCB/gold nanoparticle complexes retain specific binding to streptococcal cells
- Overcame hurdles with respect to size/shape of gold nanoparticle and toxicity of uncoated gold nanoparticles
- Demonstrated resonance energy output from nanoparticles lased with near infrared radiation
- Demonstrated thermal ablation of streptococci with PlyCB/gold nanoparticle complexes
- Created a chimeric fusion of PlyCB with a pneumococcal binding domain (CBD)
- Overcame hurdles with misfolding and developed a system to selectively express just a few fusion monomers in the octamer
- Demonstrated that the fusion octamers acquired a new species specificity while retaining the parental specificity
- Made PlyCB-PlyCB/CBD-gold nanoparticle complexes

REPORTABLE OUTCOMES:

Some aspects of our preliminary data and/or overall strategy have been presented at the following meetings/symposia during the past year:

- Nineteenth Evergreen International Phage Biology Meeting. Olympia, WA. Poster presentation titled "Thermal Ablation of Streptococci using an Endolysin Binding Domain as a Functionalized Nanoparticle"
- Georgia Institute of Technology, Atlanta, GA. Seminar speaker.
- Catholic University, Washington, D.C. Seminar speaker.
- Rockefeller University, New York, NY. Seminar speaker.
- National Institute for Standards and Technology. Seminar speaker.

In addition, this project has now become the subject of collaboration with scientists from the National Institute for Standards and Technology who are interested in using PlyCB/gold nanocomplexes to calibrate their infrared lasers. See the joint poster in the appendix.

CONCLUSION:

We had several technical hurdles with the size of the gold nanoparticle and the toxicity of bare nanoparticles, but we have overcome all of these issues. More importantly, we successfully made the S1C mutation, formed PlyCB/gold nanoparticle complexes, and demonstrated these complexes retained species-specific binding properties of wild-type PlyCB and were able to thermally ablate streptococci in the presence of lasing. The later goals involved demonstration of PlyCB as a platform technology whereby different binding domains could be fused to the PlyCB octamer in order to change bacterial specificity of the nano-complex. Toward this end, we successfully created PlyCB/CBD complexes that bound both streptococcal and pneumococcal cells with and without gold particles attached. Although we were not able to evaluate thermal ablation of pneumococcal cells (due to gold toxicity of pneumococci), we nonetheless worked out the methodology to display fusion proteins on PlyCB without causing misfolding. It is anticipated that this technique can be extended to other binding domains in future research for evaluation on a wide range of pathogens.

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APPENDICES:

The following abstract was presented as a poster at the Nineteenth Evergreen International Phage Biology Meeting in August, 2011 in Olympia, Washington.

Thermal Ablation of Streptococci using an Endolysin Binding Domain as a Functionalized Nanoparticle

Michael S. Mitchell[1], Joseph Kotarek[2], Curtis Meuse[2], and Daniel C. Nelson[1,3]

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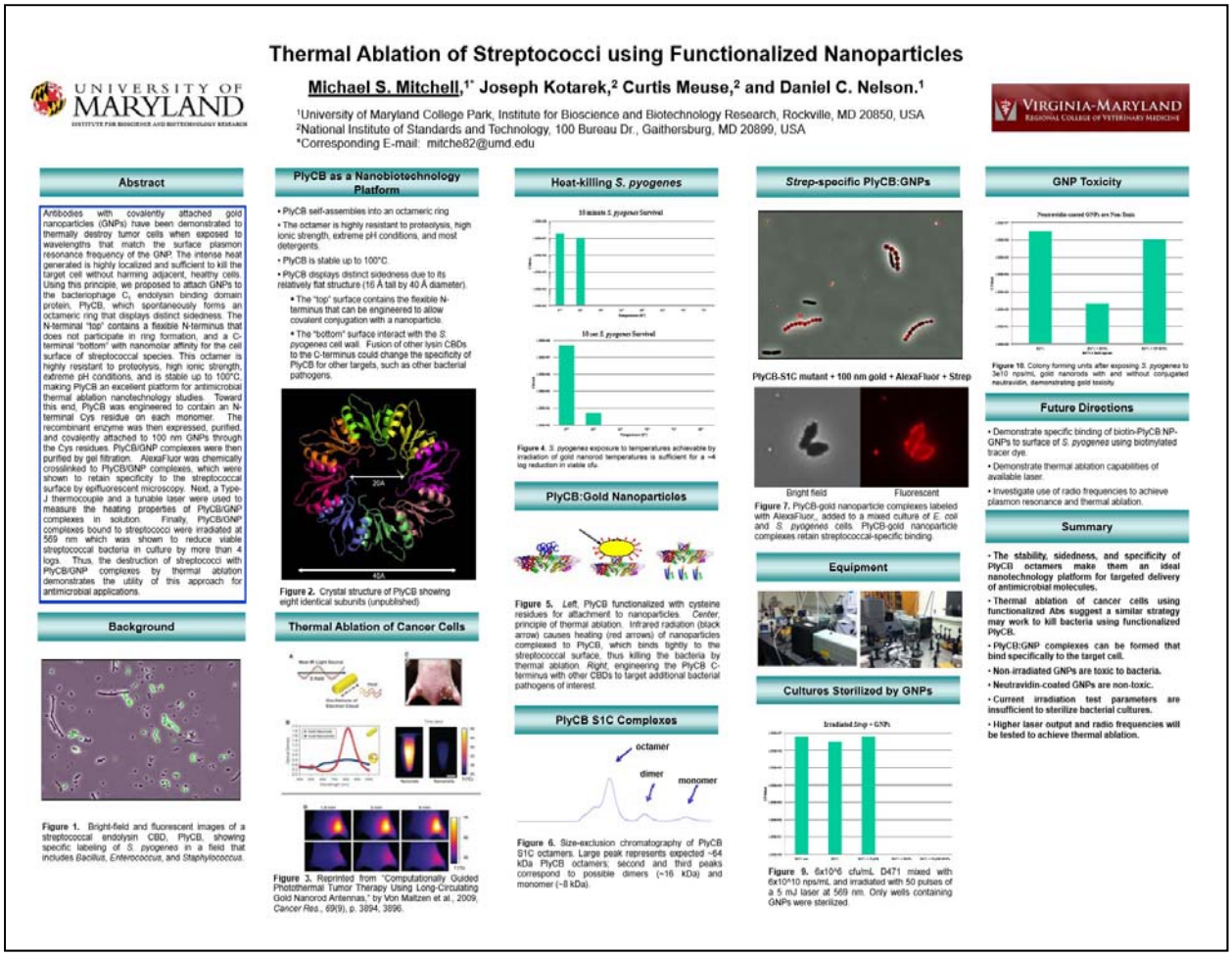
[3]Department of Veterinary Medicine, Virginia-Maryland Regional College of Veterinary Medicine, College Park, MD, USA.

Antibodies with covalently attached gold nanoparticles (GNPs) have been demonstrated to thermally destroy tumor cells when exposed to wavelengths that match the surface plasmon resonance frequency of the GNP. The intense heat generated is highly localized and sufficient to kill the target cell without harming adjacent, healthy cells. Using this principle, we proposed to attach GNPs to the bacteriophage C_1 endolysin binding domain protein, PlyCB, which spontaneously forms an octameric ring that displays distinct sidedness. The N-terminal "top" contains a flexible N-terminus that does not participate in ring formation, and a C-terminal "bottom" with nanomolar affinity for the cell surface of streptococcal species. This octamer is highly resistant to proteolysis, high ionic strength, extreme pH conditions, and is stable up to 100°C, making PlyCB an excellent platform for antimicrobial thermal ablation nanotechnology studies. Toward this end, PlyCB was engineered to contain an N-terminal Cys residue on each monomer. The recombinant enzyme was then expressed, purified, and covalently attached to 100 nm GNPs through the Cys residues. PlyCB/GNP complexes were then purified by gel filtration. AlexaFluor was chemically crosslinked to PlyCB/GNP complexes, which were shown to retain specificity to the streptococcal surface by epifluorescent microscopy. Next, a Type-J thermocouple and a tunable laser were used to measure the heating properties of PlyCB/GNP complexes in solution. Finally, PlyCB/GNP complexes bound to streptococci were irradiated at 569 nm which was shown to reduce viable streptococcal bacteria in culture by more than 4 logs. Thus, the destruction of streptococci with PlyCB/GNP complexes by thermal ablation demonstrates the utility of this approach for antimicrobial applications.

A copy of the poster is follows:



Thermal Ablation of Streptococci using Functionalized Nanoparticles

Michael S. Mitchell,[1*] Joseph Kotarek,[2] Curtis Meuse,[2] and Daniel C. Nelson.[1]

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Abstract

Antibodies with covalently attached gold nanoparticles (GNPs) have been demonstrated to thermally destroy tumor cells when exposed to wavelengths that match the surface plasmon resonance frequency of the GNP. The intense heat generated is highly localized and sufficient to kill the target cell without harming adjacent, healthy cells. Using this principle, we proposed to attach GNPs to the bacteriophage C_1 endolysin binding domain protein, PlyCB, which spontaneously forms an octameric ring that displays distinct sidedness. The N-terminal "top" contains a flexible N-terminus that does not participate in ring formation, and a C-terminal "bottom" with nanomolar affinity for the cell surface of streptococcal species. This octamer is highly resistant to proteolysis, high ionic strength, extreme pH conditions, and is stable up to 100°C, making PlyCB an excellent platform for antimicrobial thermal ablation nanobiotechnology studies. Toward this end, PlyCB was engineered to contain an N-terminal Cys residue on each monomer. The recombinant enzyme was then expressed, purified, and covalently attached to 100 nm GNPs through the Cys residues. PlyCB/GNP complexes were then purified by gel filtration. AlexaFluor was chemically crosslinked to PlyCB/GNP complexes, which were shown to retain specificity to the streptococcal surface by epifluorescent microscopy. Next, a Type-J thermocouple and a tunable laser were used to measure the heating properties of PlyCB/GNP complexes in solution. Finally, PlyCB/GNP complexes bound to streptococci were irradiated at 569 nm which was shown to reduce viable streptococcal bacteria in culture by more than 4 logs. Thus, the destruction of streptococci with PlyCB/GNP complexes by thermal ablation demonstrates the utility of this approach for antimicrobial applications.

Background

Figure 1. Bright-field and fluorescent images of a streptococcal endolysin CBD, PlyCB, showing specific labeling of *S. pyogenes* in a field that includes *Bacillus*, *Enterococcus*, and *Staphylococcus*.

PlyCB as a Nanobiotechnology Platform

- PlyCB self-assembles into an octameric ring
- The octamer is highly resistant to proteolysis, high ionic strength, extreme pH conditions, and most detergents.
- PlyCB is stable up to 100°C.
- PlyCB displays distinct sidedness due to its relatively flat structure (16 Å tall by 40 Å diameter).
  - The "top" surface contains the flexible N-terminus that can be engineered to allow covalent conjugation with a nanoparticle.
  - The "bottom" surface interact with the *S. pyogenes* cell wall. Fusion of other lysin CBDs to the C-terminus could change the specificity of PlyCB for other targets, such as other bacterial pathogens.

Figure 2. Crystal structure of PlyCB showing eight identical subunits (unpublished)

Thermal Ablation of Cancer Cells

Figure 3. Reprinted from "Computationally Guided Photothermal Tumor Therapy Using Long-Circulating Gold Nanorod Antennas," by Von Maltzen et al., 2009, *Cancer Res.*, 69(9), p. 3894, 3896.

Heat-killing *S. pyogenes*

Figure 4. *S. pyogenes* exposure to temperatures achievable by irradiation of gold nanorod temperatures is sufficient for a >4 log reduction in viable cfu.

PlyCB:Gold Nanoparticles

Figure 5. *Left*, PlyCB functionalized with cysteine residues for attachment to nanoparticles. *Center*, principle of thermal ablation. Infrared radiation (black arrow) causes heating (red arrows) of nanoparticles complexed to PlyCB, which binds tightly to the streptococcal surface, thus killing the bacteria by thermal ablation. *Right*, engineering the PlyCB C-terminus with other CBDs to target additional bacterial pathogens of interest.

PlyCB S1C Complexes

Figure 6. Size-exclusion chromatography of PlyCB S1C octamers. Large peak represents expected ~64 kDa PlyCB octamers; second and third peaks correspond to possible dimers (~16 kDa) and monomer (~8 kDa).

Strep-specific PlyCB:GNPs

PlyCB-S1C mutant + 100 nm gold + AlexaFluor + Strep

Bright field Fluorescent

Figure 7. PlyCB-gold nanoparticle complexes labeled with AlexaFluor, added to a mixed culture of *E. coli* and *S. pyogenes* cells. PlyCB-gold nanoparticle complexes retain streptococcal-specific binding.

Equipment

Cultures Sterilized by GNPs

Figure 9. 6x10^6 cfu/mL D471 mixed with 6x10^10 nps/mL and irradiated with 50 pulses of a 5 mJ laser at 569 nm. Only wells containing GNPs were sterilized.

GNP Toxicity

Figure 10. Colony forming units after exposing *S. pyogenes* to 3e10 nps/mL gold nanorods with and without conjugated neutravidin, demonstrating gold toxicity.

Future Directions

- Demonstrate specific binding of biotin-PlyCB:NP-GNPs to surface of *S. pyogenes* using biotinylated tracer dye.
- Demonstrate thermal ablation capabilities of available laser.
- Investigate use of radio frequencies to achieve plasmon resonance and thermal ablation.

Summary

- **The stability, sidedness, and specificity of PlyCB octamers make them an ideal nanotechnology platform for targeted delivery of antimicrobial molecules.**
- **Thermal ablation of cancer cells using functionalized Abs suggest a similar strategy may work to kill bacteria using functionalized PlyCB.**
- **PlyCB:GNP complexes can be formed that bind specifically to the target cell.**
- **Non-irradiated GNPs are toxic to bacteria.**
- **Neutravidin-coated GNPs are non-toxic.**
- **Current irradiation test parameters are insufficient to sterilize bacterial cultures.**
- **Higher laser output and radio frequencies will be tested to achieve thermal ablation.**

SUPPORTING DATA:

Figures and figure legends are contained within the body of the text above.