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DEVELOPMENT AND CHARACTERIZATION OF METHODS TO ENCAPSULATE NISIN FOR USE AS AN ANTIMICROBIAL AGENT

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14. ABSTRACT

Polyelectrolyte complexation is a useful technique to encapsulate sensitive molecules for increased stability and sustained, controlled delivery. It can be performed under mild conditions and is highly tailorable. Combinations of polyacrylic acid (PAA) and polyethyleneimine (PEI) were used to encapsulate the bacteriocin nisin. In its crude form, nisin has significant antimicrobial activity, but impurities made it difficult to encapsulate, so a purification protocol was developed using fast liquid protein chromatography (FPLC) methods. Once purified, the nisin was incorporated into optimized formulations (PAA:Nisin:PEI, PAA:PEI:Nisin, PAA:Nisin and a control PAA:PEI), and compared for loading efficiency and antimicrobial activity. Loading efficiencies were high for PAA:Nisin:PEI and PAA:Nisin, but dropped by more than half when the Nisin was incorporated as an outer coating (PAA:PEI:Nisin). In quantitative assays all nisin-containing particle types demonstrated antibacterial activity, even at reduced concentrations. The level of activity did vary somewhat, and none of the optimized formations performed as well as the purified nisin, nor did they confer any more stability on the nisin than in crude form. More studies are needed to be able to harness controlled release kinetics for long-term anti-microbial activity from a biological molecule.

15. SUBJECT TERMS

FPLC	PARTICLES	SEPARATION	ENCAPSULATION	SOLUTIONS(MIXTURES)
NISIN	DROP TESTS	ENTRAPMENT	STRAINS(BIOLOGY)	PATHOGENIC BACTERIA
YIELD	INFECTIONS	PREPARATION	CHARACTERIZATION	ANTIMICROBIAL AGENTS
PEPTIDES	INHIBITION	PURIFICATION	POLYELECTROLYTES	
TEXTILES	PROTOCOLS	BACTERIOCINS	ANTIMICROBIAL PEPTIDES	
ASSAYING	MOLECULES	ION EXCHANGE	STAPHYLOCOCCUS AUREUS	
BACTERIA	ANTIBIOTICS	ANTIMICROBIAL	BCA(BICINCHONINIC ACID)	
DILUTION	PROTECTION	NANOPARTICLES	FAST LIQUID PROTEIN CHROMATOGRAPHY	

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DEVELOPMENT AND CHARACTERIZATION OF METHODS TO ENCAPSULATE NISIN FOR USE AS AN ANTIMICROBIAL AGENT

1 Introduction

This report presents results and findings from research conducted at the U.S. Army Natick Research, Development and Engineering Center (NSRDEC) to encapsulate a bacteriocin in a polyelectrolyte complex, in support of a Bootstrap effort entitled: Nisin Protection from Degradation and Controlled Release via Polyacrylic Acid Encapsulation. The work was performed from March 3, 2015 – March 3, 2017 by a National Research Council (NRC) postdoctoral research fellow.

Combat wounds are often incurred in remote locations far from conventional treatment facilities, where the possibility of infection from pathogenic bacteria is significantly increased. Currently, broad spectrum antibiotics are used to prevent and/or treat such infections. However, these are active against both harmful and beneficial bacteria, which can disrupt the native ecosystem residing on the skin, and can allow opportunistic species to proliferate, leading to secondary infections. To address this, there is a need for antibiotics with high specificity for the pathogenic species. Recently, compounds known as bacteriocins have been recognized for their potential as alternatives to traditional antibiotics. Bacteriocins are a class of antimicrobial peptides produced by many species of bacteria to kill or inhibit competing bacteria for access to space and nutrients [1]. These compounds are produced naturally and are typically targeted towards closely related species, making them highly specific [2]. Their narrow spectrum of activity makes them ideal candidates for therapeutic applications.

Nisin is a well-studied, commercially available cationic bacteriocin that is active against gram positive bacteria [3,4]. The mode of action, representative of this class of antimicrobial, starts with binding to the cell membrane, either nonspecifically through electrostatic interaction, or specifically to Lipid II. Once bound, nisin changes conformation, which causes cell wall synthesis inhibition and creation of a pore in the membrane, killing the bacterium [3,4]. It exhibits low toxicity towards humans and in over 40 years of study has not given rise to resistant strains of bacteria [5]. Nisin's antimicrobial properties make it an excellent prospect as a topical therapeutic to prevent wound infection. Wipes, bandages, or apparel impregnated with nisin would be useful for battlefield application and could be a lifesaving technology. Current industrial uses for this bacteriocin are confined to the food industry as a preservative, and it has been shown to exhibit activity loss under certain conditions [6]. There has been extensive research on encapsulation of nisin using biodegradable polymers for increased stability and controlled release in food systems. However, there has been little research on its use as a therapeutic, and it has not been studied in the textile literature.

Encapsulation is a useful technique to create a stabilizing microenvironment for sensitive compounds so that they may retain their activity in non-native environments. Liposomes made from phospholipid bilayers are commonly used for this. Were et al. investigated several different formulations of liposomes to encapsulate nisin and found that encapsulated nisin had higher

antimicrobial activity over free nisin [7]. There are also many accounts of encapsulation with proteins. Zein, a protein isolated from corn, was used by Xiao et al. and soy was used by Malheiros et al [8,9]. Some groups have used emulsions to sequester nisin. Ji et al. developed particles with controllable size and properties using a water/oil nisin/poly(lactic acid) emulsion [10]. Polyelectrolyte complexes (PECs) are created by mixing two oppositely charged polymers together in solution. Housseini et al. developed nisin-containing particles using PECs based on negatively charged alginate [11]. While these methods have been successful in the food industry, these techniques are not made to withstand the same sort of conditions a textile might experience. The work described here expands on the methods in the literature by employing more robust polymers that are not biodegradable, to develop an encapsulation technique better suited to applications in military textiles that can help protect the Soldier (Figure 1).

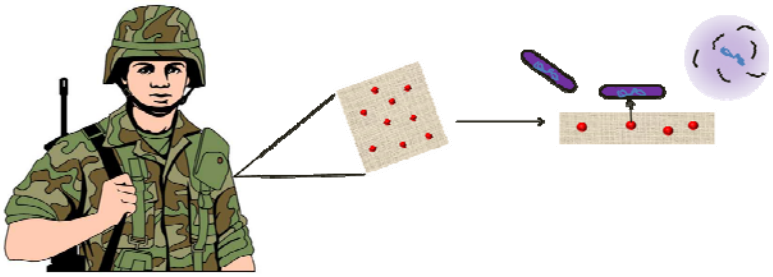

Figure 1: Schematic of active fabric used in a Soldier uniform to protect against bacteria.

Polyelectrolyte complexation was chosen due to its simplicity, mild conditions, and tunability. Particle properties are influenced by polymer size and concentration, mixing ratio and order, pH, and ionic strength [12]. Through these simple manipulations of the method, the resulting particles can be tailored to the desired specifications. Examples of architectures resulting from differences in polymer size, charge, and mixing ratio are illustrated in Figure 2.

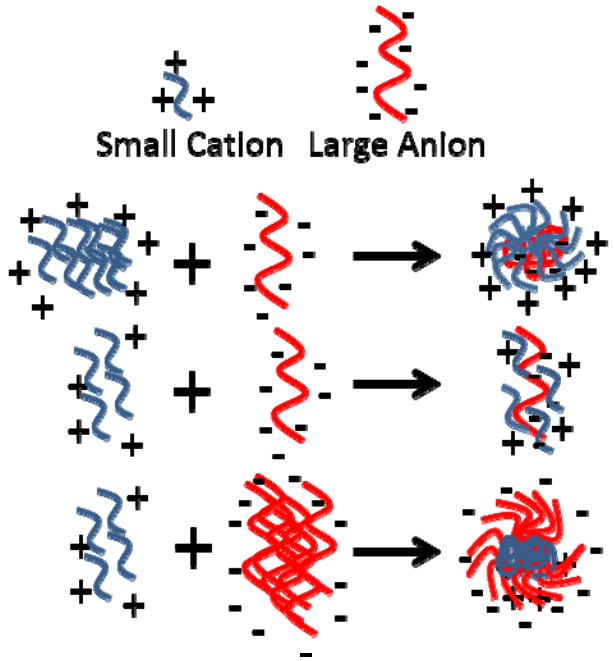

Figure 1: Examples of different PEC structures resulting from different polymer sizes, charge, and mixing ratio. The polymer in excess dictates the overall charge.

This method can be applied to any number of polymer combinations. The end goal is to apply these to a textile; thus, polymers that are either not biodegradable or can be crosslinked to improve their durability were chosen. Polyacrylic acid (PAA) was selected because it is anionic, water soluble, and contains carboxylic acids along its backbone that can be crosslinked or functionalized. Its anionic nature can be exploited to bind nisin or to electrostatically interact with a polycation such as Polyethyleneimine (PEI) to form a PEC with desirable properties. The ability to functionalize the PAA backbone is useful for crosslinking, immobilization, and controlling release kinetics of a cargo. Branched PEI was selected because it is cationic, water soluble, nonbiodegradable, its branched structure increases the surface area available for binding with the PAA, and it contains many amine functional groups. Similar to PAA, its polyionic nature is exploited to form PECs, and functional groups are useful for immobilization and any post-synthesis modifications. Both polymers need to be water soluble so that mild conditions, which will not denature the nisin, can be used for particle synthesis. The combination of a branched and unbranched polymer allows for control of particle size and structure, so that the PAA will be the primary component in the corona as long as it is in excess. Nisin is an ideal candidate for use as a therapeutic antimicrobial and its small size (3 kDa) and cationic nature should allow facile complexation and encapsulation with the proposed polymer system. The chemical structures are shown in Figure 3.

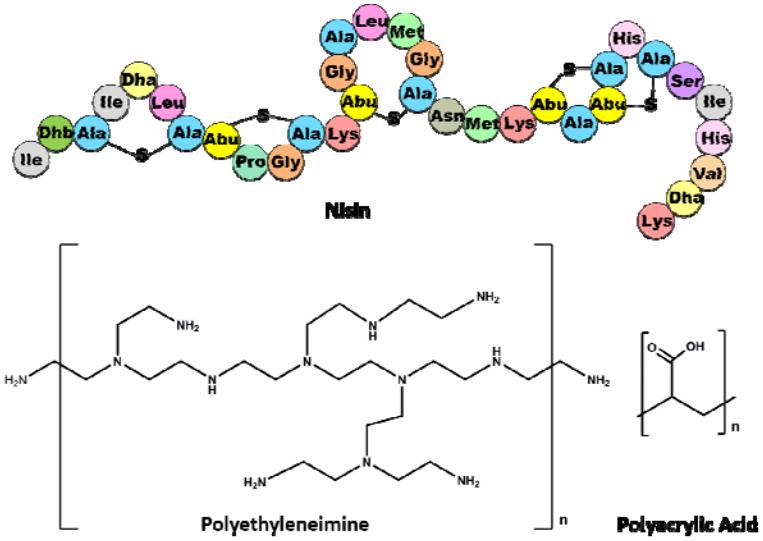

Figure 2: Chemical structures of particle components used in this study.

The work described herein optimizes several nisin-containing polyelectrolyte complex nanoparticle (PCN) formulations and tests them against *S. aureus*. Parameters that were studied include: polymer concentration, mixing ratio, polymer molecular weight, composition, and mixing order. It was determined that crude nisin contained too many contaminants to be encapsulated through electrostatic interactions. Fast liquid protein chromatography (FPLC) methods were explored and a protocol for nisin purification was established. The optimized particles were characterized for size, zeta potential, and loading efficiency. Interesting relationships between ion-pairing and resulting structures and properties were observed. These may have an effect on bioavailability or stability of the nisin.

2 Materials

Nisin (20% pure) was purchased from Chihonbio (Zhengzhou, Henan, P.R. China). PAA (450 kDa) was obtained from Polysciences (Warrington, PA). PAA molecular weight standards of different sizes (18 kDa, 35 kDa, 80 kDa, and 130 kDa), bought from American Polymer Standard (Mentor, OH), were investigated as well. PEI (750 kDa)), sodium phosphate dibasic, and sodium phosphate monobasic were purchased from Sigma Aldrich (St. Louis, MO). TRIS Base, TRIS HCl, agar, Alamar Blue, and Pierce™ Bicinchoninic (BCA) Protein Assay Kit were purchased from Thermo Fisher Scientific (Waltham, MA). *Staphylococcus aureus* (*S. aureus*) ATCC Strain #27217 (Manassas, VA) was used for antimicrobial testing. Nutrient Broth and Dev-Engley (DE) broth were purchased from Becton, Dickinson and Company (Franklin Lakes, NJ). Mannitol salt broth was purchased from Alpha Aesar (Ward Hill, MA). All solutions were prepared using milliQ deionized water (diH_2O, EMD Millipore, Billerca, MA). A Bio-Rad NGC^{TM} Chromatography System with a BioFrac fraction collector (Hercules, CA) was used to purify nisin and to calculate loading efficiency. Particles were characterized for size and zeta potential using a Malvern Instruments (Westborough, MA) Zetasizer Nano ZS. Biological activity was evaluated qualitatively using a Spiral Biotech (Norwood, MA) Color QCount and quantitatively with a bioMérieux TEMPO® enumerator and a BioTek (Winooski, VT) Eon plate reader running on Gen5 2.03 software.

3 Methods

3.1 Preparation of Particles

3.1.1 Nisin Preparation

The crude nisin powder was dissolved in TRIS buffer 25 mM pH 5 at 50 mg/mL, equivalent to 10 mg/mL nisin content, by vortexing for 10 min. The solution was then centrifuged to separate insoluble milk proteins at 3500 rpm for 15 min. The supernatant was collected and filtered through a hydrophilic, low protein binding, 0.22 µm membrane. At this point, the filtrate was either used or further purified using FPLC.

3.1.2 Nisin Purification

The filtrate from Section 3.1.1 was desalted using a Bio-Rad chromatography system equipped with a 10 mL Bio-Scale™ Mini Bio-Gel P6 Desalting Column and a fraction collector. The column was first equilibrated with TRIS buffer 25 mM pH 5. A 5 mL sample of the filtrate was then injected, followed by seven column volumes of buffer. During this time, eluate was collected if the absorbance at 255 nm was above 50 mAu. The fraction containing the majority of the salt was discarded and fractions containing primarily protein were retained and combined (Figure 5).

The nisin in the combined protein rich fractions was then purified using ion exchange chromatography. A GE Healthcare Life Sciences (Pittsburgh, PA) HiPrep CM FF sepharose column (20 mL) was attached to the chromatography system and equilibrated with TRIS buffer 25 mM pH 5. A 10 mL sample of the desalted protein solution was injected, followed by two column volumes of buffer to elute contaminating protein. A linear salt gradient 0 to 2.0 M over ten column volumes was run to induce elution of the cationic nisin. Eluate was collected if the absorbance at 255 nm was above 50 mAu. Fractions containing nisin were kept and others were discarded. The FPLC traces are displayed in Figure 6 to clarify the purification process. The samples collected here were used for any experiments labeled purified nisin.

3.1.3 Nisin Concentration

The amount of nisin recovered after purification was determined using a BCA protein assay kit. Protein standards using bovine serum albumin (BSA) were made ranging from 5 to 500 µg/mL. Nisin was serially diluted by half to give concentrations of 1, 0.5, 0.25, and 0.125 times the concentration collected from the FPLC. The working reagent was prepared according to the manufacturer's instructions. A volume of 75 µL of sample or standard was pipetted into a 96-well plate in triplicate, and 150 µL of working reagent was added. The plate was incubated at 60 °C for 30 min and then read at 562 nm. The results displayed are the mean absorbance for each sample. The corresponding FPLC traces for nisin purification at 255 nm were integrated using the Bio-Rad ChromLab™ version 3.3 for comparison.

3.1.4 Nisin Encapsulation

Polymer solutions were made by dissolving the specified polymer, either PAA or PEI, at the designated concentration (0.1 mg/mL, 0.5 mg/mL, or 1 mg/mL) in TRIS buffer (25 mM pH 7.5). PAA was dissolved at 60 °C overnight, while PEI solutions were prepared at room temperature and stirred for 1 h. Nisin was either used as is or diluted to a desired concentration, typically 100-200 µg/mL. Four formulations of PCNs were developed: PAA with only PEI (PAA:PEI), PAA complexed with nisin followed by PEI (PAA:Nisin:PEI), PAA complexed with PEI followed by

nisin (PAA:PEI:Nisin), and PAA complexed with only nisin (PAA:Nisin). For all formulations, 4 mL of PAA is added to a beaker first and vigorously stirred to create a cyclone. For PAA:PEI, 2 mL of TRIS buffer (25 mM, pH 7.5) was added, followed by 2 mL of PEI, and the solution is stirred for 30 min. After 30 min, the particle solution is set aside. For PAA:Nisin:PEI, 2 mL of nisin at the desired concentration is added and stirred for 30 min to allow complexation. Next, 2 mL of PEI is added and the solution is stirred for another 30 min. For PAA:PEI:Nisin, 2 mL of PEI is added and stirred for 30 min. At this time, 2 mL of nisin is added and the solution is stirred for another 30 min to allow the nisin to bind to the outside of the particle. For PAA:Nisin, 2 mL of TRIS buffer (25 mM, pH 7.5) is added, followed by 2 mL of nisin, and the solution is stirred for 30 min. PAA:Nisin:PEI, PAA:PEI:Nisin, and PAA:Nisin are then transferred into 15 mL centrifuge tubes and centrifuged at 3500 rpm for 5 min to remove unincorporated nisin. The supernatant is either collected for analysis or discarded. Finally, particles are resuspended in 8 mL of TRIS buffer (25 mM, pH 7.5).

3.2 Particle Characterization

3.2.1 Size and Zeta Potential

Particle size was determined using dynamic light scattering (DLS). Samples were diluted by half and 1 mL was transferred into a quartz cuvette. Measurements were taken on a Malvern Zetasizer Nano ZS in triplicate at 25 °C. The sizes reported are the mean effective hydrodynamic diameter and the mean polydispersity index for each formulation. Surface charge was determined using electrophoretic light scattering (ELS). The zeta universal dip cell was inserted into the quartz cuvette and placed in the Zetasizer for analysis. Each sample was measured five times at 25 °C. The value reported is the mean zeta potential ± the standard deviation of the mean.

3.2.2 Loading Efficiency

Nisin loading was measured using ion exchange chromatography on the Bio-Rad FPLC equipped with a GE HiTrap CMFF sepharose column (1 mL). When particles were centrifuged during nisin encapsulation, the supernatant was collected for analysis. During this step, the particles were separated from unincorporated nisin. Samples of the collected supernatant (1 mL) were injected into the column, followed by wash buffer, and finally eluted and collected using a salt gradient, as in Section 3.1.2. Lasers at 255 nm and 280 nm, and a conductivity probe, are used to monitor the eluent. Nisin presence was monitored at 255 nm. The amount of nisin calculated in the supernatant was compared to the amount originally used to make each formulation to determine loading efficiency.

3.3 Antimicrobial Testing

3.3.1 Drop Test

Particles and purified nisin were tested against *S. aureus* for antibacterial activity. Mannitol salt agar was prepared according to the manufacturer's instructions, poured into sterile petri dishes, and allowed to solidify. Mannitol salt soft agar was prepared similarly, using half the concentration of agar as the standard recipe. *S. aureus* was cultured in a suspension of nutrient broth for 6 hours, or until the optical density of the solution was 1 (when read at 600 nm), indicating 10^7 CFU/mL. Mannitol salt soft agar (7 mL) was melted, allowed to cool but not solidify, and then inoculated with 20 µL of *S. aureus*. This was poured over mannitol salt agar and allowed to set to create a lawn of bacteria. Samples of 6 µL of each particle type and purified nisin (at an equivalent concentration) were dropped on top of the bacterial lawn in triplicate.

These plates were cultured overnight at 37 °C. Images were taken and examined for resulting zones of clearing. Drop tests are a qualitative measure of antimicrobial activity. Here they are described as Clear (C), meaning no growth and complete clearing in the drop; Inhibitory (I), meaning some growth with blurred edges; or No Activity (N), meaning completely overgrown with no evident clearing.

3.3.2 Alamar Blue

Purified nisin and particles at various concentrations were tested against *S. aureus* in solution. A minimal inhibitory concentration study was done on purified nisin by serially diluting the solution collected by the FPLC to 10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}. Additionally, an activity study was done on particles and compared to equivalent amounts of nisin. These were diluted to contain 0.4, 2, 10, and 50 µg/mL of nisin based on the concentration of the solution obtained from FPLC as determined by BCA (Section 3.1.3). Particles were diluted based on the amount of nisin used for their formulation rather than based on their loading efficiencies. *S. aureus* was cultured in a suspension of nutrient broth for 6 hours, or until the optical density of the solution was 1, indicating 10^{7} CFU/mL. The cell suspension was then diluted to 10^{6} CFU/mL for inoculation. A volume of 4 mL of sample (particles or purified nisin at each concentration, or nutrient broth) was transferred into a 15 mL centrifuge tube and 50 µL of *S. aureus* was added to each. These were cultured at 37 °C overnight. After 16 h of culture, 100 µL of sample was transferred into a 96-well plate. Additionally, a well was filled with 100 µL of TRIS buffer that had not been cultured with *S. aureus*. Each sample was tested in triplicate. An additional 100 µL of nutrient broth was added to each well to promote growth of any surviving bacteria. Next, 20 µL of Alamar Blue was added to each well, and the plate was incubated for 3 h at 37 °C. The plate was then read at 570 nm. Values reported are the means ± the standard deviation of the means. Statistical analysis was performed by running an ANOVA with Tukey's multiple comparisons tests and Dunnett's method, $p<0.05$, on Minitab 17 (Minitab, Inc., State College, PA).

3.3.3 TEMPO® Enumerator

The 50 µg/mL nisin-containing particles and purified nisin from Section 3.3.2 were also tested for their antibacterial activity using the TEMPO® Enumerator. After 16 h of culture, 100 µL of each sample was pipetted into 5 mL of D/E Neutralizing broth and vortexed to stay antimicrobial activity. Serial dilutions from 10^{-1} to 10^{-4} were prepared and tested using the Staphylococcus (STA) cards and vials according to the TEMPO® Enumerator manufacturer's instructions. Results reported are the mean log reduction of *S. aureus* for each nisin-containing formulation compared to the mean of a nisin-free control ± the error. Minitab software was used to run an ANOVA with Tukey's multiple comparisons tests and Dunnett's method, $p<0.05$, to assess activity loss over storage time.

4 Results and Discussion

4.1 Particle Formulation Development Summary

Polyelectrolyte complexation is driven by the electrostatic interactions of oppositely charged polyionic species. Their size and stability are impacted by mixing ratio, molecular weight, concentration, pH, and ionic strength [12,13]. All of these factors were taken into consideration when developing particle formulations to encapsulate nisin. Attempted parameters and their result, No (N) or Promising (P), are displayed in Table 1. No (N) indicates that no complexation occurred, PCNs that did form could not be purified and recovered, or large aggregates formed and precipitated immediately. A designation of Promising (P) means that PCNs were formed, could be purified and recovered, but recovery may have been difficult, resulting in low yields and high polydispersity, or resulting PCNs were not active against *S. aureus*.

Table 1: Summary of particle formulation development.

Development Stage	PAA MW	Concentration of PAA and PEI	Composition	Ratios	Results
Stage 1 Concentrations and Charge Ratios	450 kDa	0.1 mg/mL	PAA:Nisin	2 to 1	N
	450 kDa	0.1 mg/mL	PAA:Nisin	4 to 1	N
	450 kDa	0.5 mg/mL	PAA:Nisin	2 to 1	N
	450 kDa	0.5 mg/mL	PAA:Nisin	4 to 1	N
	450 kDa	0.5 mg/mL	PAA:Nisin	6 to 1	N
	450 kDa	0.5 mg/mL	PAA:Nisin	10 to 1	N
	450 kDa	0.5 mg/mL	PAA:Nisin	20 to 1	N
	450 kDa	1 mg/mL	PAA:Nisin	6 to 1	N
Stage 2 Molecular Weights, Concentrations, and Charge Ratios	18 kDa	0.5 mg/mL	PAA:Nisin	4 to 1	N
	18 kDa	1 mg/mL	PAA:Nisin	2 to 1	N
	35 kDa	0.5 mg/mL	PAA:Nisin	2 to 1	N
	35 kDa	0.5 mg/mL	PAA:Nisin	4 to 1	N
	35 kDa	1 mg/mL	PAA:Nisin	2 to 1	P
	80 kDa	0.5 mg/mL	PAA:Nisin	4 to 1	N
	80 kDa	1 mg/mL	PAA:Nisin	2 to 1	P
	130 kDa	0.5 mg/mL	PAA:Nisin	4 to 1	N
	130 kDa	1 mg/mL	PAA:Nisin	4 to 1	P
Stage 3 Testing Charge Ratios with Ideal System, No Nisin	80 kDa	0.5 mg/mL	PAA:PEI	2 to 1	P
	80 kDa	0.5 mg/mL	PAA:PEI	4 to 1	P
	80 kDa	0.5 mg/mL	PAA:PEI	6 to 1	P
	80 kDa	0.5 mg/mL	PAA:PEI	8 to 1	P
	80 kDa	1 mg/mL	PAA:PEI	2 to 1	P
Stage 4 Addition of PEI for More Complete Ion-Pairing	80 kDa	1 mg/mL	PAA:Nisin:PEI	10 to 1 to 2	P
	130 kDa	1 mg/mL	PAA:Nisin:PEI	10 to 1 to 2	P
	450 kDa	1 mg/mL	PAA:PEI	2 to 1	N
	450 kDa	1 mg/mL	PAA:Nisin:PEI	10 to 1 to 5	P
	450 kDa	1 mg/mL	PAA:PEI:Nisin	2 to 1 to 1	P

The original strategy, illustrated in Figure 2, was to introduce the nisin (small cation) to an excess solution of PAA (large anion) and allow the PAA to self-assemble around the nisin, encapsulating it. This was attempted as outlined in Table 1: Stage 1 of PCN development at low concentration and at volume ratios of 2 to 1, 4 to 1, 6 to 1, 10 to 1, and 20 to 1 PAA to nisin. When charge ratios are close to 1, larger particles are produced and aggregation is probable. The initial conditions chosen were designed to achieve charge ratios large enough to avoid these issues. Additionally Boddohi et al. suggests that the dilute regime ideal for PCN formation is less than 1 mg/mL; thus, all formulations were kept below this concentration [12]. However, these attempts resulted in either large aggregates or particles that could not be separated through centrifugation. Separation is an important step for removing unencapsulated nisin so that functionality testing would not be skewed by excess nisin in solution.

Initial failure of proper PCN formation was at first attributed to the high molecular weight PAA being used. Because the PAA 450 kDa could not be filtered, contaminants, incomplete dissolution of PAA, or high PDI could cause the inconsistent results obtained. Therefore, as outlined in Table 1: Stage 2, molecular weight standards of PAA (at 18 kDa, 35 kDa, 80 kDa, and 130 kDa) with narrow distributions were obtained to confirm this theory. However, for most test cases, no viable particles could be isolated from solution. Indeed, only the higher polymer concentrations, at 1 mg/mL, showed evidence of complexation. The PCNs isolated (from 35 kDa, 80 kDa, and 130 kDa PAA) were not monodisperse according to DLS, and the yields were significantly lower than expected.

The use of narrow distribution molecular weight (MW) polymers did not solve the formulation issues, and it is known that ionic strength can influence the polyelectrolyte complexation process [14]. The crude nisin stock is only 20% nisin, with the remainder being a mixture of salts and proteins. It was assumed that the preparation steps described in Section 3.1.1 were sufficient to purify the nisin, but these did not account for excess salt. To eliminate factors caused by the presence of crude nisin, PCNs made from only PAA:PEI were studied (Table 1: Stage 3). At all concentrations and ratios attempted, the PAA:PEI system formed uniform, recoverable particles. Since the polymers, in the absence of nisin, behaved as expected, it was determined that the crude nisin solution was the main cause of complexation failure.

Nisin is weakly charged, and it was hypothesized that the excess salt interferes with the complexation. It was speculated that if nisin was first allowed to bind to the PAA, followed by the addition of PEI to complete the PCN formation, the nisin would be encapsulated in the center of the complex. Additionally, the polymer concentration was held at 1 mg/mL to provide a maximum amount of binding sites for the nisin while remaining in the dilute regime ideal for polyelectrolyte complexation. These formulations were evaluated in Stage 4 (Table 1) and produced promising particles, but DLS continued to suggest that they were not monodisperse.

Nisin-containing particles that showed promise in the formulation optimization studies were drop tested against *S. aureus* to investigate their antimicrobial activity. Unfortunately, the PAA:Nisin:PEI formulation did not exhibit antibacterial activity in drop tests (Figure 4), suggesting that the nisin was tightly encapsulated on the inside of the complex in the PAA:Nisin:PEI formulation and thus was not available. A new procedure was developed based on Place et al. [15] where nisin was bound to the outside of a PAA:PEI particle, dubbed

PAA:PEI:Nisin. This new formulation had an inhibitory effect against *S. aureus* in drop tests (Figure 4). Like all the other unoptimized formulations with promising results, this particle type had low yields and high polydispersity. Even with changes to the process, it was determined that the nisin solution was too heterogeneous with too many contaminants; thus, purification protocols were pursued.

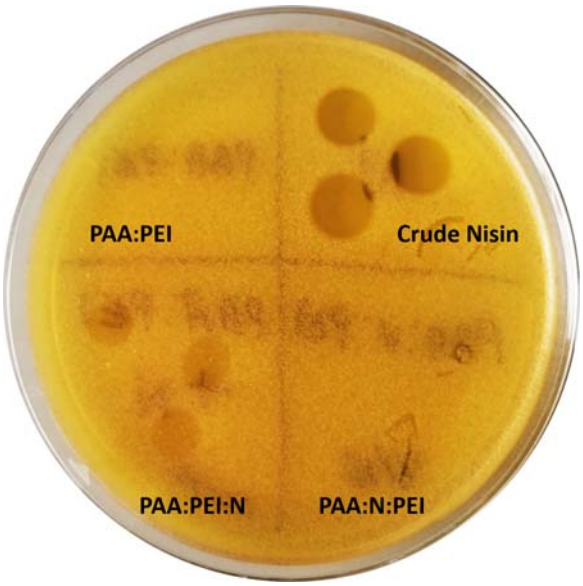

Figure 3: Drop test of particles, 0.1 mg/mL, PAA:PEI - 2 to 1, PAA:PEI:Nisin - 10 to 5 to 1, and PAA:Nisin:PEI 10 to 1 to 5. Crude nisin exhibits definitive zones of clearing, weaker zones are visible for PAA:PEI:Nisin, and no zones are observed for PAA:PEI or PAA:Nisin:PEI.

4.2 Nisin Purification

4.2.1 FPLC and BCA

To mitigate inconsistencies and low yields encountered during formulation development, nisin was purified using FPLC. Size exclusion chromatography (SEC) was first used to remove salts that seemed to interfere with complexation. The FPLC traces are displayed in Figure 5. The absorbance at 255 nm is shown in black and the conductivity in red. Absorbance is an indication of protein concentration and conductivity signifies the presence of salt. The boxes designate the protein-rich regions that were kept for further purification.

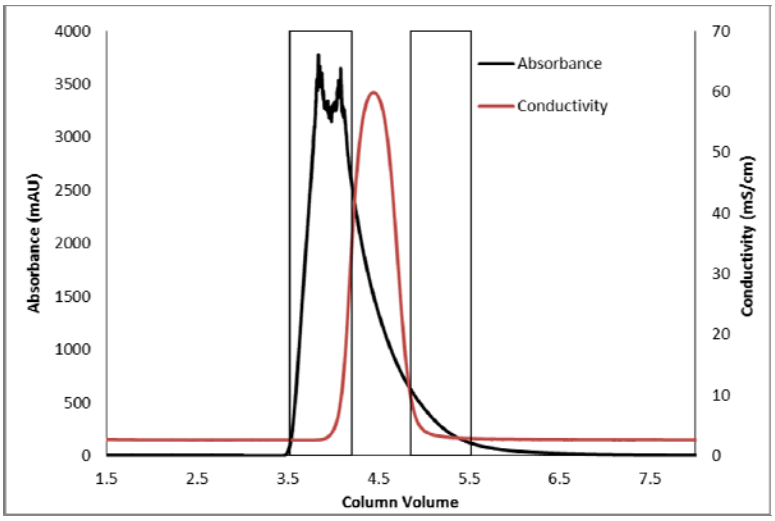

Figure 4: FPLC traces of SEC to remove salt. The boxed regions were collected and combined for further purification.

SEC separates molecules based on size. Columns are filled with porous beads that restrict entry. Large species are unable to enter the beads and flow quickly through the column, eluting first. Small molecules, conversely, travel into the pores, taking a tumultuous, longer path, and elute later. The bulk of the protein was in the first peak, but there remained a shoulder after the salt passed. It was a small amount, but according to the absorbance trace, protein was present, so it was collected to maximize recovery.

Once the salts were removed, ion exchange chromatography (IEX) was done to isolate nisin from the other proteins. In IEX the substrate inside the column is charged so that the molecule of interest will bind. Then counter ions are added and these exchange with the desired molecule causing it to elute. Nisin is a weak polyelectrolyte and thus eluted near the beginning of the salt gradient. It eluted within two column volumes of the beginning of the gradient, where the conductivity was between 10 and 30 mS/cm. The FPLC traces are shown in Figure 6. The boxed region highlights the location of the nisin peak. Due to the fact that nisin contains no aromatic amino acids [16], absorbance readings for this protein were monitored at 255 nm, rather than at 280 nm. In Figure 6, absorbance at 255 nm is drawn by the black trace and absorbance at 280 nm is drawn by the dashed gray trace. Both are exhibited in the first peak while only absorbance at 255 nm manifests in the second peak and absorbance at 280 nm remains at baseline. This indicates that the second peak is comprised of purified nisin.

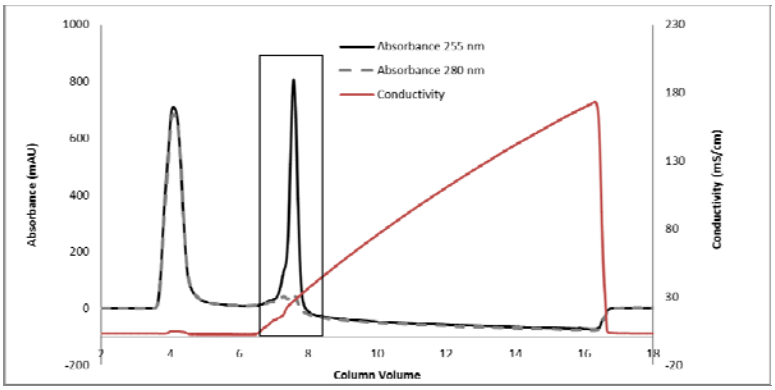

Figure 5: FPLC traces for IEX Absorbance at 255 nm is shown in black, at 280 nm in dashed gray, and conductivity is in red. The boxed region contains purified nisin and was collected for subsequent experiments and particle synthesis. Nisin does not absorb at 280 nm.

A BCA assay was used to determine the concentration of the recovered purified nisin. It was observed that there was variation in the BCA results between experiments ranging from 40 µg/mL to 400 µg/mL. Most experiments gave results from 100 µg/mL to 300 µg/mL, but the errors within each experiment are less than ten, so this variation cannot be attributed to error in the assay. The area underneath a given peak in an FPLC trace is indicative of the concentration of the molecule. The FPLC traces were integrated and these areas were compared to the respective BCA results. Comparisons of these findings are displayed in Figure 7.

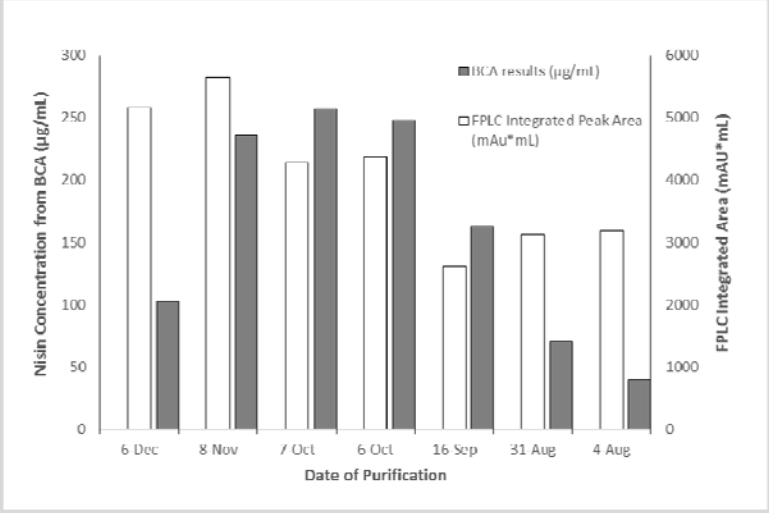

Figure 6: Comparison of BCA results with integrated peak areas from FPLC. In many cases the two correlate. These results suggest that there is variation in the stock nisin lyophilized powder.

Typically, when the BCA result was lower for a given experiment the integrated FPLC peak was as well. However, on December 6 this pattern did not hold true. There are many factors that could have caused this. Nisin was diluted two-fold three times and each dilution (1x, 0.5x, 0.25x, and 0.125x) was tested. It was noted that while the relationship between calculated concentration and dilution factor was typically linear, the slope was not as high as would be expected and changed dramatically between experiments. The experiments done on August 4 and August 31 did not show a perfectly linear relationship because their absorbance was at the low limit of detection for this assay. The constants from the linear fit are displayed in Table 2.

Table 2: Linear coefficients y=mx+b and the associated R-squared value for each experiment for calculated nisin concentration vs. dilution factor.

Date of Purification	m	b	R-squared
6-Dec	77.511	29.436	0.9976
8-Nov	189.94	49.818	0.9893
7-Oct	223.33	36.357	0.9955
6-Oct	210.73	42.086	0.9907
16-Sep	135.11	30.953	0.9867
31-Aug	95.162	-11.363	0.711
4-Aug	28.522	11.064	0.9648

During the BCA assay, peptides form a complex with cupric ions via the biuret reaction which then induces a color change. This can be done with any sequence of three or more amino acids, but the color change is enhanced by cysteine, tryptophan, and tyrosine, suggesting the BCA response for nisin (which lacks all three of these amino acids) may not be ideal. However, used in conjunction with FPLC, the efficiency of nisin recovery can be compared for each experiment set.

4.2.2 Alamar Blue MIC

A dose response study was done with the purified nisin to find the minimum inhibitory concentration (MIC) against *S. aureus.* According to Dosler et al. the MIC of nisin against clinical strains of a methicillin susceptible *S. aureus* and a methicillin resistant *S. aureus* is 2-32 µg/mL [17]. The BCA assay results suggest that purified nisin is recovered at 100-300 µg/mL. To investigate this, and to devise an effective particle formulation scheme, purified nisin was collected and serially diluted ten-fold across six orders of magnitude. Purified nisin at each dilution was cultured with *S. aureus* overnight followed by incubation with Alamar Blue reagent to quantify cell viability after exposure. Results are presented in Figure 8.

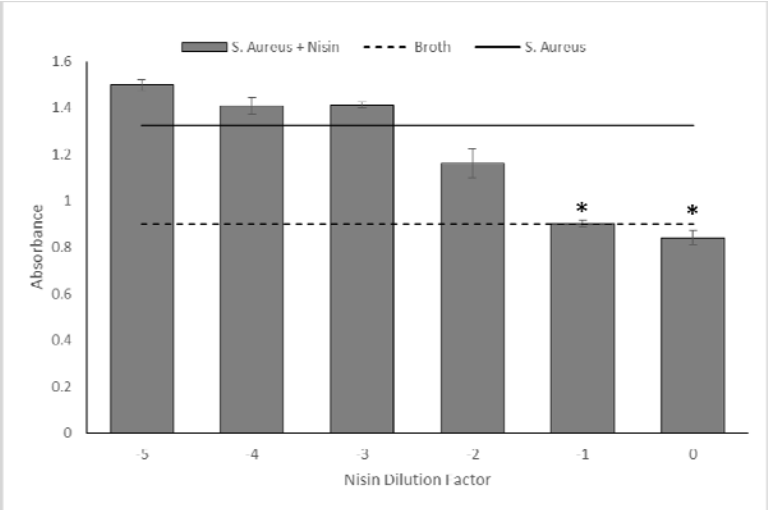

Figure 7: Alamar Blue results after overnight exposure to different concentrations of nisin. Asterisks on dilutions 0 and -1 indicate no statistical differences from blank broth, implying no cell viability after exposure. Dilution -2 demonstrates inhibition and dilutions -3 to -5 have no effect.

Samples treated with nisin were compared to a blank control, representing no viability, and an untreated control where *S. aureus* was allowed to proliferate uninhibited, representing 100% viability. Purified nisin at dilutions 0 and -1 exhibited no statistical difference from the blank control. Dilution -2 demonstrated growth inhibition. Dilutions -3 through -5 were equivalent to uninhibited *S. aureus*. The BCA assay suggests that Dilution 0 had a concentration on the order of 100 μg/mL. Applying that assumption, the concentrations for the dilutions were 100, 10, 1, 0.1, 0.01, 0.001 μg/mL. These data agree with the observations made by Dosler et al [17]. The dose response study supports that the BCA assay can be used as an indicator of nisin concentration. Additionally, these results established the minimum nisin concentration necessary for preparing active PCNs.

4.3 Particle Characterization

The previous studies were used to design the final particle preparation protocol. In the ultimate formulation, high molecular weight PAA (450 kDa) was used, the polymer concentration was reduced to 0.1 mg/mL to match the concentration of nisin calculated by BCA assay, and volume ratios were adjusted to maximize nisin availability. Four particle types, three containing nisin, were characterized and tested. These include, PAA:PEI, 2:1 (PP); PAA:Nisin:PEI, 2:1:1, (PNP); PAA:PEI:Nisin, 2:1:1, (PPN); and PAA:Nisin, 2:1, (PN). These compositions were calculated to provide sufficient nisin for antimicrobial activity according to the MIC study. Characterization results are listed in Table 3. PP are the smallest, have the lowest PDI, and the largest zeta potential. PPN are significantly larger than the others, have a mid-range PDI, are the only formulation to exhibit a positive zeta-potential, and demonstrate the lowest nisin loading efficiency. The PNP and the PN formulas have similar properties. Their sizes fall between that of PP and PPN, with PN being slightly smaller. They have the largest PDIs, but remain beneath the threshold for being considered polydisperse (0.7) [18]. They both exhibit negative zeta potentials and loading efficiencies above 90%.

Table 3: Characterization results for the four optimized particle formulations.

Particle Formulation	Effective Hydrodynamic Diameter (nm)	Polydispersity Index (PDI)	Zeta Potential (mV)	Loading Efficiency FPLC
PAA:PEI	150	0.11	-12 ± 0.5	NA
PAA:Nisin:PEI	380	0.65	-7.4 ± 3.5	91 ± 7%
PAA:PEI + Nisin	960	0.45	4.6 ± 2.7	31 ± 20%
PAA:Nisin	270	0.61	-5.0 ± 1.0	96 ± 3%

Particle formation and the resulting properties are influenced by polymer size, molecular weight, polyelectrolyte strength, pH, and the presence of salts [12, 13]. The property differences observed between particle formulations are due to these relationships. These can be used to tailor particles for various applications. A smaller particle size is desirable for use on a textile to increase surface area, and to minimize effects on color and breathability. Uniform particles are advantageous for product consistency. Formulation factors can also be used to control the

location and availability of the encapsulated nisin. Understanding the interplay between these elements is essential for particle design.

The PAA and PEI used both have a high molecular weight, and are weak polyelectrolytes. Particle formation is done near neutral pH, where both polymers exhibit approximately 50% ionization [19, 20]. The PEI is branched and thus unable to make conformation adjustments. The PAA, however, is flexible under these conditions, and can configure itself to accommodate binding with the PEI. This results in efficient ion-pairing and compact, monodisperse particles exhibiting a diameter of 150 nm and a PDI below 0.2, which is the accepted level to be considered monodisperse [15]. The PAA is in excess and assembles in a corona around the PEI core made evident by the negative zeta potential.

However, when nisin is added, the ionic strength of the solution is increased. The purified nisin elutes when sodium chloride concentration is between 0.1 and 0.4 M. The solution is then diluted four fold during particle preparation to less than 100 mM, but ionic strength effects are still manifested in particle properties. Almodovar et al. prepared polyelectrolyte multilayers at a buffer concentration of 0.2 M and observed large amounts of swelling when weak polyelectrolytes were used. Furthermore, Place et al. saw similar effects when forming complexes in 0.1 M buffer. Both were attributed to the antipolyelectrolyte effect, where insufficient ion-pairing results in the retention of counter-ions and an increased osmotic pressure [21, 22]. Weak polyelectrolytes will change in response to their environment. For example, when counterions are introduced the polymers may dissociate and preferably bind to the small molecules. This will alter osmotic pressure and result in swelling. This is evident in the increase in hydrodynamic diameter of the nisin-containing formulations.

Additionally, Muller et al. saw that the mixing order influenced particle size substantially with a PEI:PAA system. When the “majority” polyion was added to the “minority” polyion the system had a difficult time reaching equilibrium because ion-pairing cannot exceed a 1 to 1 ratio and resulted in fewer, larger particles [14]. In the case of the particle formulation PAA:PEI + Nisin, when nisin is added to PAA that is already complexed with PEI it may be difficult to find carboxylic acids available for binding in these large particles, resulting in very large particles with a hydrodynamic diameter of 980 nm. The presence of salt compounds this further. The PAA is restricted by its association with the PEI. As an ion pair dissociates it will preferably bind to the small molecule over the larger, weakly charged nisin which would result in the low loading efficiency observed. The change to a positive zeta potential confirms that any anionic groups that were available were consumed in this process. If higher nisin loading is desired, it would need to be desalted before exposure.

However, when nisin is introduced first, it has the opportunity to bind completely with the PAA, giving the high loading efficiencies seen in these two formulations. Furthermore, the PAA is in enough excess that the salt is able to bind as well, resulting in the increase in hydrodynamic diameter from 150 nm to 270 nm. The decrease in magnitude of zeta potential while not changing sign further illustrates this. The PAA is flexible and the species bound to it are much smaller, thus it retains the ability to change conformation in response to its environment. When less flexible PEI is added to the PAA nisin complex, the complex may adsorb around the PEI, or multiple PEI molecules may interact with one PAA nisin complex, producing a larger

supercomplex with a hydrodynamic diameter of 380 nm. More studies would need to be done to elucidate the mechanisms that produce this species.

4.4 Functionality Testing

The optimized particle formulations were tested for their activity against *S. aureus*. The results for a qualitative drop test are shown in Figure 9. The strategy behind the different formulations was that some would encapsulate nisin for slow release and others would display it on the surface for immediate use. In the drop test it is unclear if this strategy is successful. The lack of a defined zone of clearing for all nisin-containing particles suggests that there was either insufficient nisin or it was so tightly encapsulated that it is not available to bind to the bacteria. This makes sense since the three lysine residues in nisin (Figure 3) provide its cationic character and are thus responsible for the binding in the PCNs and for the electrostatic interactions with anionic lipids in bacterial cell membranes. Nisin kills bacteria by adhering to and perturbing the cell membrane [3,4]. If it is too strongly held by the particle it will not be able to dissociate and perform this function properly.

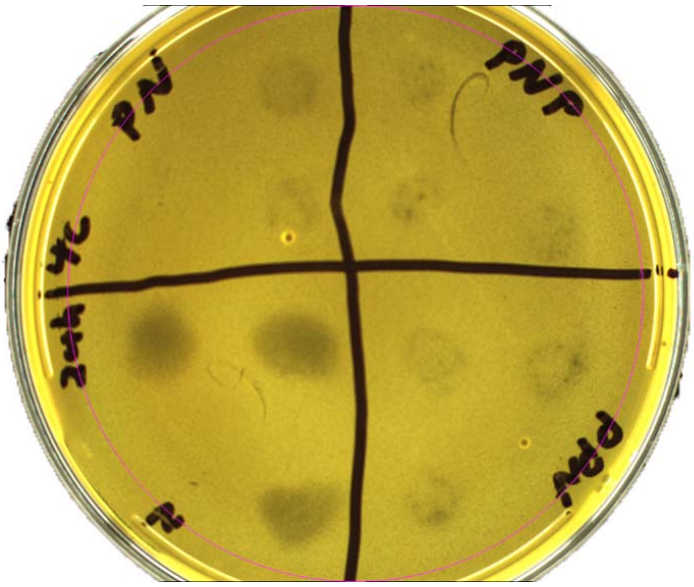

Figure 8: Drop test against *S. aureus* of the four nisin-containing particle types, and purified nisin at a concentration equivalent to the formula concentration of the particles. PN, PNP, and PPN all showed insufficient zones of clearing, whereas for purified nisin (N) they were better defined.

The drop test, shown in Figure 4, of unpurified nisin exhibits better defined zones of clearing. This could be due to decreased concentration of nisin during the purification process, low bioavailability, or stability issues. The contaminants may confer protection from denaturing conditions such as temperature and pH. It was hypothesized that polyelectrolyte complexation, with PAA in excess, would provide a microenvironment to protect the nisin, however this does not appear to be an effective strategy. Further analysis is necessary to determine whether the cause is stability, concentration, or availability.

Quantitative testing using Alamar Blue and a TEMPO™ Enumerator was done to further elucidate activity. The results of the Alamar Blue assay are displayed in Figure 10. The nisin concentrations listed were based on results from the BCA assay and the amount used in the

original formulation. It does not take into account loading efficiency. Thus particles contain less nisin than the nisin control. All nisin-containing conditions show activity for concentrations 2-50 µg/mL. The particles with 0.4 µg/mL of nisin all behave the same as the untreated control cultured with *S. aureus*. These results correlate with the MIC reported in the literature and shown by the dose response study. Pure nisin, however, continues to show activity at 0.4 µg/mL, but the cellular activity is still statistically higher than the blank control, indicating some viability. This is not surprising since there is more nisin here than in the particles and the BCA assay has limitations in determining absolute concentration.

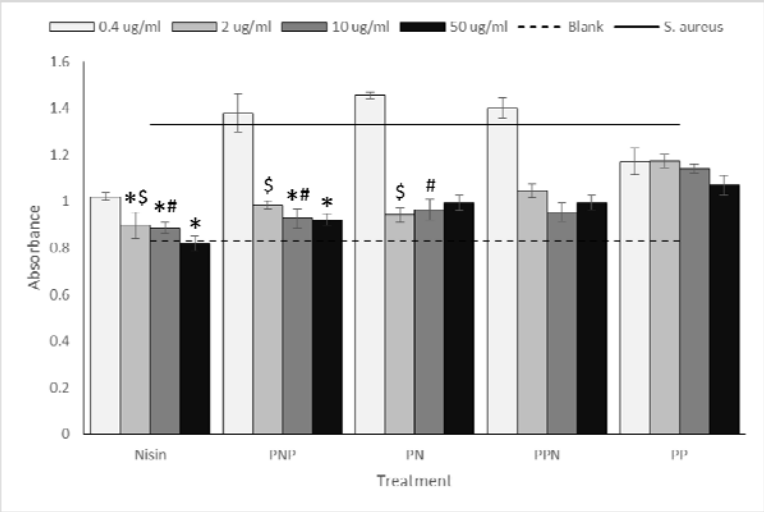

Figure 9: Alamar Blue results for particle formulations at different concentrations. Symbols indicate groupings. When all conditions were compared using Tukey's Multiple Comparisons Test, there were no statistical differences between particles types.

An asterisk indicates that the condition was not statistically different from the blank control, suggesting no cell viability for that sample. Bars marked with a dollar sign or a pound sign indicate that they are not statistically different from the nisin control at the given concentration. These statistical groups were determined using Dunnett's method for multiple comparisons to a control group. No statistical differences were observed using Tukey's method. While purified nisin induces a stronger cytotoxic response at 50 µg/mL than the particles, this difference is overcome for PN at 10 µg/mL, and at 2 µg/mL for PN and PNP. The PPN particles never perform as well as the purified nisin. These results correlate with the loading efficiency data: PN demonstrated the highest loading, followed closely by PNP, and PPN showed significantly less nisin loading. The PP particles exhibit less cell viability than the negative control, which may have resulted from insufficient ion-pair matching with the PEI, resulting in the inhibition of cell growing: PEI has been shown to have some antimicrobial properties [22]. These were not strong enough to manifest in the qualitative drop test, but did become apparent in this quantitative assay. While they appear to have some inhibitory effects, it is less than that exhibited by the nisin-containing formulas.

In terms of biological activity, the PPN formulation, which has much lower loading than the others, nevertheless had a similar activity profile. As stated earlier, if the nisin is too tightly bound to the encapsulating polymer it may not be biologically available. The characterization of the PPN implies that the nisin is on the outside and bound loosely. There may be less present, but

the higher availability may be enough to overcome this difference. Release studies are needed to determine if the nisin remains bound or is released over time by the other formulations. After quantitative analysis, antibacterial activity results are promising for the use of this system as a delivery vehicle.

5 Conclusions and Recommendations

Three formulations of nisin-containing particles were developed and tested against *S. aureus*. Crude nisin contains too many contaminants to be encapsulated through electrostatic interactions. Nisin can be purified using chromatography, but recovered amounts vary significantly due to heterogeneity of the lyophilized powder, making the concentration difficult to quantify with the tools used in this study. Additionally, after purification, the antimicrobial activity of nisin is significantly reduced. This could be due to changes in concentration, low bioavailability, or instability of amino acids important for nisin activity. The structures and resulting properties of each formulation type vary due to ion-pairing and salt effects. These different structures may be responsible for the reduced activity seen with PN and PNP and the emergence of activity with PPN. This could be harnessed to engineer release kinetics, but more studies are needed. Currently it does not appear that polyelectrolyte complexation with PAA confers any stability to purified nisin.

At the time that this proposal was written there was little literature on mechanisms of lantibiotic stability. It was observed that nisin in its crude form remained stable for long periods of time across many temperatures and maintained activity in some organic solvents. There is clearly a relationship between the contaminating salts and proteins and nisin that needs to be understood in order to develop an effective stabilizing vehicle. Encapsulating nisin in its crude form using a polymer system that does not rely on electrostatic interaction, but rather on physical entrapment may be successful. Nisin is hydrophilic and could be encapsulated with a hydrophobic polymer such as poly-lactic acid or poly(methyl methacrylate) using an emulsion process. Liposomes are another common technique, but would not be suitable for use on textiles. Changing nisin on the molecular level so that denaturing enzymes are unable to recognize it or so that it folds differently while maintaining its activity is another avenue in the realm of synthetic biology.

In this study, much was learned that can be utilized in future efforts to use polyelectrolytes for stabilizing small proteins, particularly about the nuances of ion-pairing with weak polyelectrolytes and the added challenges of crude protein. Due to its ability to change conformation in response to its environment, this system does show potential for triggered release applications. Additionally, PEI has strong buffering properties and both polymers contain functional groups that could be modified or crosslinked for additional functionality and stability. More studies are needed to understand the mechanisms on a molecular level and then to engineer an effective system. The long term goal is to functionalize materials to prevent wound infection. Basic research needs to be done to understand the specific proteins and enzymes that accomplish this.

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