CURRICULUM VITAE

Jennifer Klutts

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Education:

Master of Science in Toxicology May 2019
University of Maryland, Baltimore, Baltimore, Maryland

Bachelor of Science in Chemistry and Forensic Science May 2017
Trine University, Angola, Indiana

Relevant Experience:

Graduate Researcher

University of Maryland, Baltimore County: Baltimore, MD Aug. 2017- present

- Worked with zebrafish embryos to study the impact of poly (oxonorbornenes), an antimicrobial polymer
- Helped maintain adult zebrafish in fish facility
- Performed zebrafish embryo toxicity tests, immunohistochemistry and imaging, and microplate assays
- Spent 3 weeks on laboratory exchange at University of Wisconsin- Milwaukee to work with corroborators and learn gene expression techniques
- Designed and executed experimental procedures, troubleshooted problems, and analyzed data
- Attended multiple conferences to give poster and oral presentations
- Mentored and trained three undergraduate students

Teaching Assistant

University of Maryland, Baltimore County: Baltimore, MD Jan. 2019 – May 2019

- Supervised 30 undergraduate students during analytical chemistry laboratory twice a week
- Held weekly office hours for students in both laboratory sections
- Graded lab notebooks and lab reports
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Chemistry Laboratory Assistant

Trine University: Angola, IN Sept. 2014 – May 2017

- Assisted in preparing and testing chemistry experiments for laboratory classes
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Nuclear Chemistry Summer School Fellowship

Brookhaven National Laboratory: Upton, NY June 2015 – July 2015

- Department of Energy summer program in nuclear and radiochemistry
- Included six credits of lecture and lab

- Attended lecture every day and lab once or twice a week
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Skills/Techniques:
Computer: Microsoft Office, SPSS, FIJI
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Trine University Future Forensic Scientists
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Circle K
Vice President (2015-2016), Member (2013-2017)
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Student Advisory Committee
Member (2018- 2019)
Trine American Chemical Society
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Scholarships/Awards:
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Outstanding Student Award- Science Department, Trine University (2017)
President's List-GPA 3.75 or above, Trine University (2013-2017)
Trustee Scholarship, Trine University (2013-2017)

Conference Presentations:
Jennifer Klutts, April Laranang, Zheng Zheng, Julia Saar, Karen Lienkamp, Rachel Brewster, and Zeev Rosenzweig. Molecular level studies of the impact of poly (oxonorbornenes) on *D. rerio*. embryos. American Chemical Society National Meeting. August 19-23, 2018. Boston, Massachusetts. (Poster)

Jennifer Klutts, April Laranang, Zheng Zheng, Julia Saar, Karen Lienkamp, Rachel Brewster, and Zeev Rosenzweig. Molecular level studies of the impact of poly (oxonorbornenes) on *D. rerio*. embryos. Sustainable Nanotechnology Organization Conference. November 8-10, 2018. Washington, DC. (Oral)

ABSTRACT

Title of Thesis: Molecular level studies of the impact of poly (oxonorbornenes) on *D. rerio.* embryos

Jennifer Klutts, Master of Science, 2019

Thesis Directed by: Dr. Zeev Rosenzweig, Professor and Chair, University of Maryland, Baltimore County Department of Chemistry and Biochemistry

Poly (oxonorbornenes) (PONs) are amphiphilic cationic polymers that possess antimicrobial properties. Cationic polymers are proposed as an alternative to antimicrobial peptides and it is important to assess their impact on organisms. The two side chains of PONs that are responsible for these properties are a hydrophobic alkyl and a charged amine. In this study, we investigated how changing the amine/alkyl ratio and polymer length affects the activity of PONs on the model vertebrate organism, *D. rerio*. (zebrafish). Zebrafish embryo toxicity test were used to elucidate the LC_{50} of PONs. Whole-mount immunofluorescence with caspase-3 was used to analyze apoptotic cells. We hypothesize that PONs would interact with the cell membranes of embryos to induce toxicity and the level of toxicity would depend on the molecular structure of PONs. Our results indicate that increasing the hydrophobicity and polymer length decreases the viability of the embryos and number apoptotic cells in the embryos.

Molecular level studies of the impact of poly (oxonorbornenes) on *D. rerio*. embryos

by
Jennifer Klutts

Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, Baltimore in partial fulfillment
of the requirements for the degree of
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2019

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CHAPTER 1: INTRODUCTION

Cationic (positively charged) polyelectrolytes are an important class of polymers that are commonly used in water treatment, drug delivery, cosmetics, detergents and antimicrobial applications [1]. This thesis focuses on the potential use of cationic polymers as antibacterial agents towards antibiotic resistant bacteria. Antibiotic resistant bacteria have emerged as a significant threat to human health. This issue started from the over use and exposure of patients to sub-lethal doses of antibiotics, mainly in hospitals. Through evolutionary processes, bacterial cells develop resistance mechanisms. [2] New antibiotic agents have been developed to keep up with antibiotic resistant bacteria, but it isn't enough. Since traditional antibiotics mainly work by receptor binding mechanisms, bacteria can become resistant after creating a few mutations of the receptor site after exposure. [3] Therefore, there is a need for new classes of antimicrobial agents that work through different mechanisms. Cationic polymers are promising candidates for antimicrobial agents with a decreased potential for resistance development. [4] Synthetic mimics of antimicrobial peptides (SMAMPs) are polymers that are designed to act like natural antimicrobial peptides (AMPs). These polymers are made to be selective against bacteria, both Gram-positive and Gram-negative, but benign to mammalian cells. One class of these SMAMPs are called poly (oxonorbornenes) (PONs).

PONs are amphiphilic polymers that are made from oxonorbornene-derived monomers. [5] These monomers have two characteristic side chains attached: a hydrophobic alkyl and a charged amine. The hydrophobic alkyl can range from a methyl group to a hexyl group. In these experiments, a butyl group was used. The structure of PONs can be seen in Figure 1.

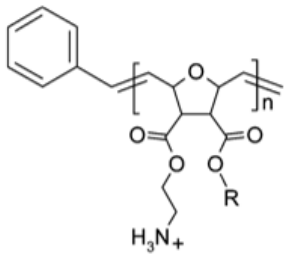


Figure 1 Molecular structure of poly (oxonorbornenes).

These side chains are responsible for these antimicrobial properties of PONs. They work though a mechanism different from receptor binding. The positively charged amine gets attracted to the negatively charge bacterial membrane and the hydrophobic alkyl group inserts into the membrane to cause membrane disruption which leads to cell death. [6] Since unspecific membrane disruption is the mechanism in which the PONs causes cell death, the development of resistance would be slower than traditional antibiotics because it would require much more complex changes to the bacterial membrane. [7] Since PONs are cationic, they can differentiate between the negatively charge bacterial membrane and mammalian cells, which are neutral in charge. [8] PONs can be synthesized with different amine/alkyl ratios to alter the activity against bacteria. The amount of alkyl and amine side chains on the PONs can be expressed by an amine/alkyl ratio percentage. The ratio percentages range from 50% to 100%. 50% amine/alkyl ratio PONs have half amine side chains and half alkyl side chains while 100% amine/alkyl ratio PONs have all amine side chains. This is shown in Figure 2.

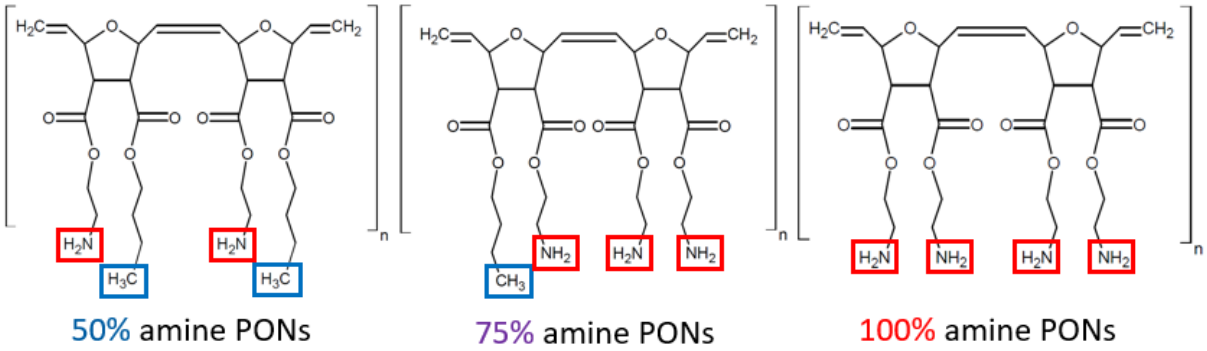


Figure 2 Differences in molecular structure and amine composition between the 50%, 75%, and 100% PONs.

The activity can also be affected by changing the polymer length of the PONs. Small PONs have 10 monomers and large PONs have 70 monomers. Previous work has been done on model membranes and bacteria that show their antimicrobial effectiveness. Studies using fluorescence-based liposome lysis assays showed that the 50%-75% PONs had high liposome lysis efficient but increasing the amine content to 95% and 100% significantly reduced the liposome lysis efficient. This indicates that higher hydrophobicity increases membrane disruption. [9] Biological assays were also done on *Escherichia coli* and *Staphylococcus aureus* that showed significant antibacterial activity and the low hemolytic activity when the hydrophobicity and polymer length is optimized. It was found that increasing hydrophobicity increases toxicity to both bacteria and mammalian cells. [10] Both of these studies indicate that lower amine content PONs has higher antibacterial activity.

Zebrafish are a common model organism used to investigate the acute effects chemical exposure in early life stages. Several characteristics make zebrafish embryos an ideal model organism for toxicity studies. The fish embryo toxicity test is considered a pain-free

in vivo test which makes them accepted as a replacement for other types of animal experiments. [11] The zebrafish embryo is transparent, so developmental changes and malformations are easy to visualize and measure. They are also small in size, easy to handle and maintain, and have rapid embryogenesis. Being small in size allows for high-throughput screening for toxicity testing. [12] Zebrafish are highly fecundity and can continuously reproduce. One pair of zebrafish are able to lay 200-300 eggs in one morning. [13] The zebrafish embryo also can be used as a model for human embryo development because of the similarities in embryogenesis between zebrafish and mammalians. [14] The genome of the zebrafish has been sequenced, which makes genetic information available and is rapidly growing.

In this study, we investigated how changing the amine/alkyl ratio and the polymer length of PONs affects the activity of PONs on the zebrafish embryo. Zebrafish embryo toxicity test are used to study the viability of the embryos after exposure. LC_{50} values were then calculated from the results obtained from these assays. To investigate the mortality seen in the embryos, whole-mount immunofluorescence with caspase-3 was utilized to visualize apoptosis occurring in the embryos. We hypothesized that PONs would strongly interact with cell membranes and induce toxicity towards zebrafish embryos due to the cationic nature of the polymer under physiological conditions (the amine functional groups are converted to ammonium ions under these conditions). We also hypothesized that the level of PONs toxicity would depend on their molecular structure, specifically the amine/alkyl ratio of the polymer and the length of the polymer chain. The long polymer chain would assemble into a secondary structure that would decrease the hydrophobicity and increase

the hydrophilicity of the polymer when dissolved in aqueous solution. Since PONs are considered for use as antibacterial coatings on surfaces, it is important to study their impact on human health and organisms.

CHAPTER 2: METHODS AND MATERIALS

Chemicals and Reagents

Poly (oxonorbornenes) were synthesized and purified by the Lienkamp group as previously described. [15] Characterization of poly (oxonorbornenes) done by Lienkamp group and done throughout studies at UMBC to monitor degradation of PONs.

Zebrafish maintenance and embryo collection

Wild-type AB strain zebrafish were used for all experiments in this study. All experiments were approved by the University of Maryland, Baltimore County's Institutional Animal Care and Use Committee (IACUC) and were performed according to national regulatory standards. Zebrafish embryos were obtained by transferring zebrafish in separate spawning tanks in groups of 2 females to 1 male in every spawning tank the evening before spawning. A divider was inserted in each spawning tank to separate the females and male. In the morning, the dividers were pulled out and the zebrafish were allowed to mate. Embryos were collected 15 minutes after pulling dividers.

Fish embryo toxicity test

180 healthy embryos were selected and divided into a 24 well plate where each well had 7 or 8 embryos and each exposure condition had 30 embryos. 24 well plate set up shown in Figure 3. Embryos screened right before 6 hpf to confirm all embryos were at the correct stage. Staging was done as previously described. [16] At 6 hpf, E3 water in the wells containing the embryos was withdrawn and replaced with either E3 water for the control embryos or the corresponding PONs exposure solution. Embryos kept in incubator at 28.5ºC and checked every 12 hours until 48 hpf for death. This study aimed for 6 replicates for each PONs but because of problems with control embryos, some of the PONs ended up

with less than 6 replicates but at least 3 replicates. Any experiment where the control embryo viability was less than 70% were not included.

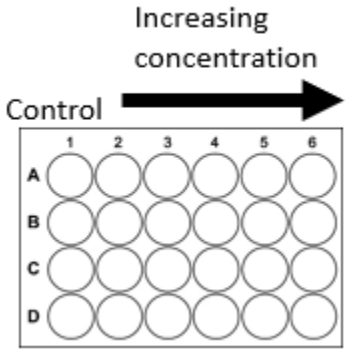


Figure 3 Experimental set up for fish embryo toxicity test. 24 well plate used as one replicate with a total of 30 embryos/exposure condition.

Whole-mount immunofluorescence to detect activated caspase-3

Apoptosis in the zebrafish embryos was analyzed using a rabbit polyclonal anti-caspase-3 antibody for the primary antibody and goat anti-rabbit IgG (H+L) cross-adsorbed secondary antibody. At 24 hpf, control and exposed zebrafish embryos were dechorionated, washed in 1X PBS, and fixed with 4% PFA overnight. The next day, the 4% PFA was removed and samples were washed with 1X PBS twice for 5 minutes each at room temperature. Samples were treated with l mL blocking solution at room temperature for 30 minutes. Blocking solution consist of 2.5 mL normal goat serum, 1 g BSA, 0.75 mL triton X, and 1X PBS filled up to 50 mL. Samples were then treated with 1:500 primary antibody diluted in blocking solution for two days at 4ºC on a shaker. Samples were then washed with 1X PBS three times and treated with 1:1000 secondary antibody diluted in blocking solution for two days at 4º C on a shaker. Samples were washed again with 1X PBS three times at room temperature and imaged using Zeiss Axio Zoom.V16 microscope. A HXP200C fluorescence lamp is used with a DsRed filter. A 1X objective lens with 32.0X

magnification and an exposure time of 450 ms was used to image. Z-stack images were taken and processed with extended depth of focus. Procedure was adapted and modified as previous described. [17]

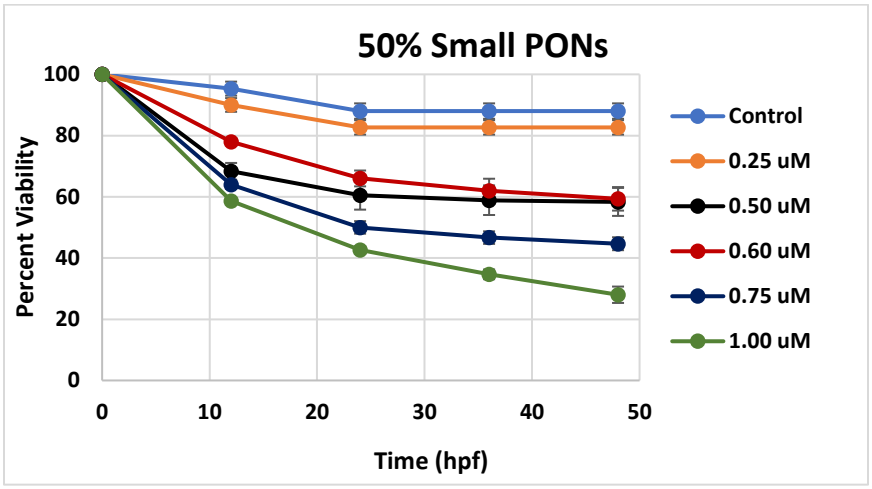
Statistical analysis

Excel was used for statistical analysis. Mean and 95% confidence intervals (CI) were calculated for each PONs and exposure concentration. Viability data was represented using line graphs with 95% CI for error bars. LC_{50} values were calculated using linear regression. LC_{50} values represented using bar graph with standard deviation for error bars.

CHAPTER 3: RESULTS AND DISCUSSION

To study the effects of molecular structure changes of PONs on zebrafish embryos, fish embryo toxicity test was used. Exposures were carried out until embryos were 48 hours post fertilization (hpf). Experiments were not carried out past this because the activity of the PONs on the zebrafish embryos were most active between 6-24 hpf, with minimal changes in viability after. A range of concentrations of each of the PONs was determined by a series of experiments testing different concentration until an optimal range was found. After an optimal range of concentrations was determined for each PONs, 3-6 replicates of n=30 embryos for each exposure condition was done and data points are an average of the total replicates done for each PONs. Figures 4-8 are shown as percent viability as a function of time. A change within the 95% CI is not a significant change. There is a concentration dependent relationship between the PONs and the viability of the zebrafish embryos. This can be seen in Figure 4.

A



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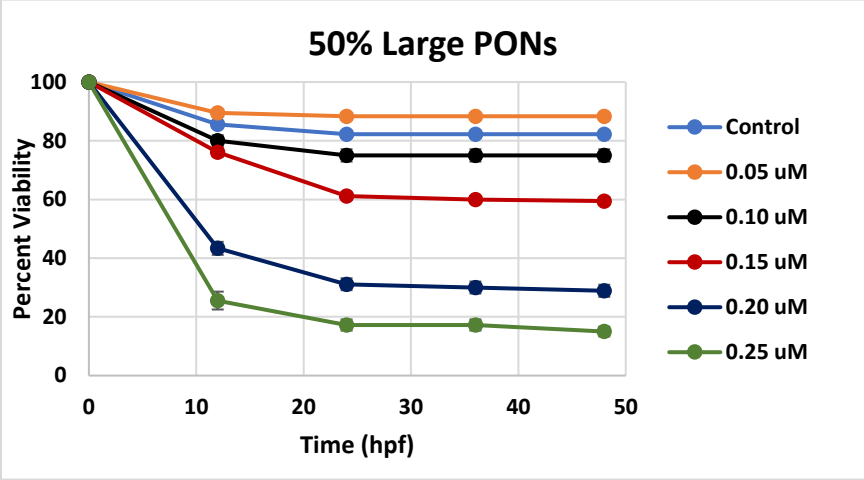

Figure 4 Mean percent viability of zebrafish embryos exposed to (A) the 50% small PONs over the course of 48 hours post fertilization (five replicates of n=30 zebrafish embryos) and (B) the 50% large PONs over the course of 48 hours post fertilization (six replicates of n=30 zebrafish embryos) Error bars shown as 95% CI.

Both the 50% small and large PONs follow this concentration dependent trend. One deviation in this trend can be seen with the 0.05 uM 50% large PONs, which has slightly higher viability than the control. It can also be seen that the concentration range that induces mortality in the zebrafish embryos in the 50% large PONs is around 4-5 times lower compared to the 50% small PONs. This indicates that the 50% large PONs is more active against the embryos compared to the small PONs. Similar trends are seen with both the 100% small and large PONs, shown in Figure 5.

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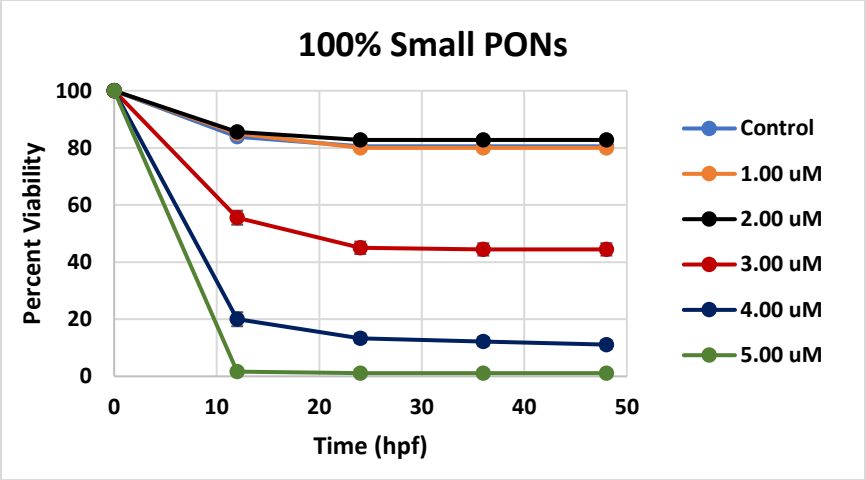

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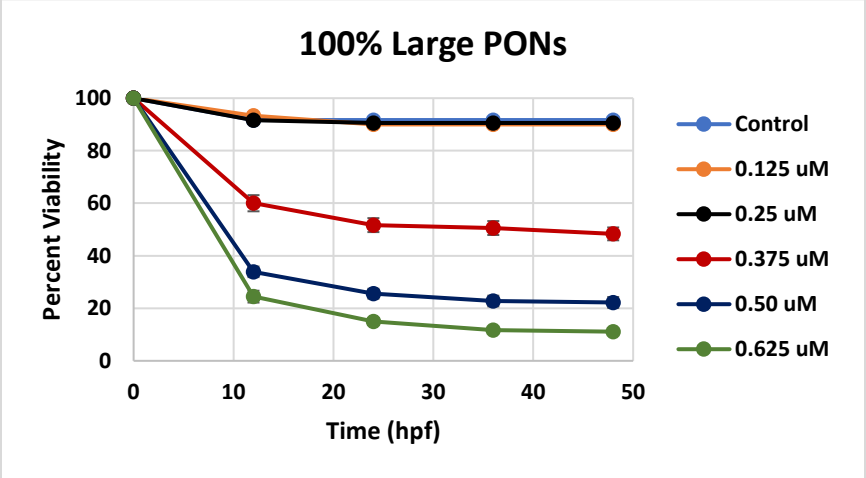

Figure 5 Mean percent viability of zebrafish embryos exposed to (A) the 100% small PONs over the course of 48 hours post fertilization and (B) the 100% large PONs over the course of 48 hours post fertilization. Error bars shown as 95% CI. Six replicates of n=30 zebrafish embryos done for both PONs.

Slight deviation from the concentration dependent trend is seen with first two low concentrations for both the small and large PONs. The viability between these concentrations and the control embryos do not differ. When higher concentrations are used, the concentration dependent relationship is seen. The 100% large PONs are also more potent than the 100% small PONs, with the concentration range for the large PONs being

around 8 times lower than the small PONs. These same trends are also seen in the 55%, 75%, and 95% small and large PONs, shown in Figures 6-8.

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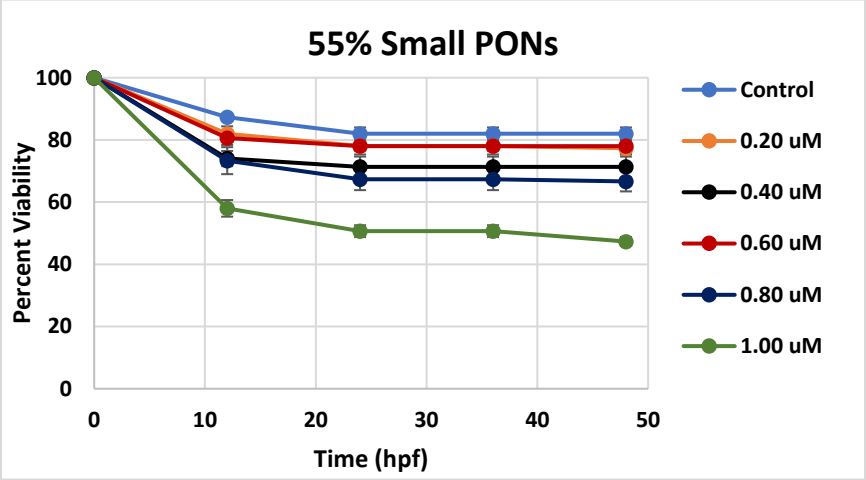

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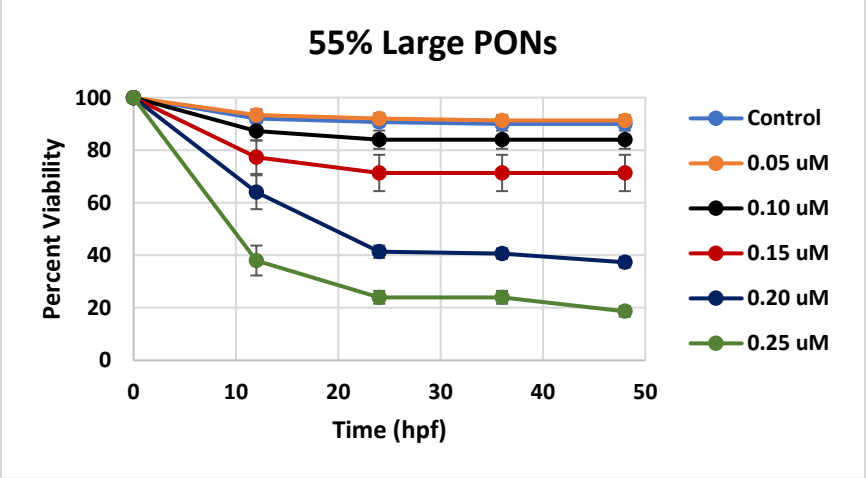
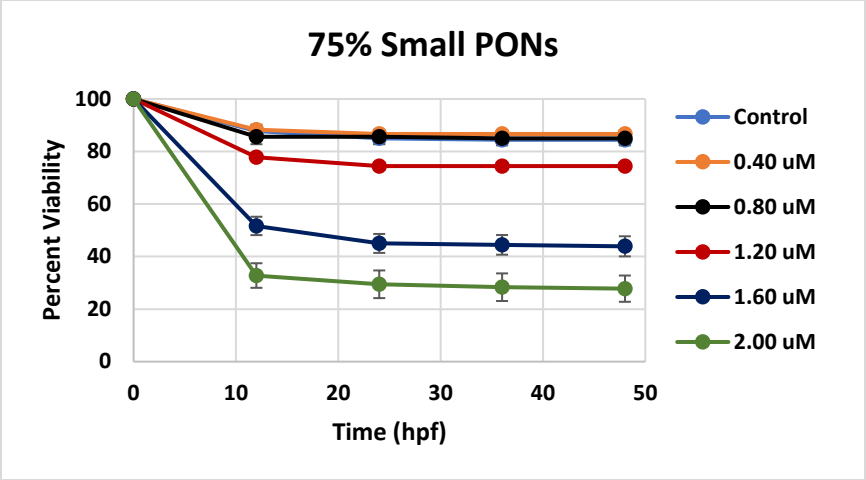


Figure 6 Mean percent viability of zebrafish embryos exposed to (A) the 55% small PONs over the course of 48 hours post fertilization and (B) the 55% large PONs over the course of 48 hours post fertilization. Error bars shown as 95% CI. Five replicates of n=30 zebrafish embryos done for both PONs.

A



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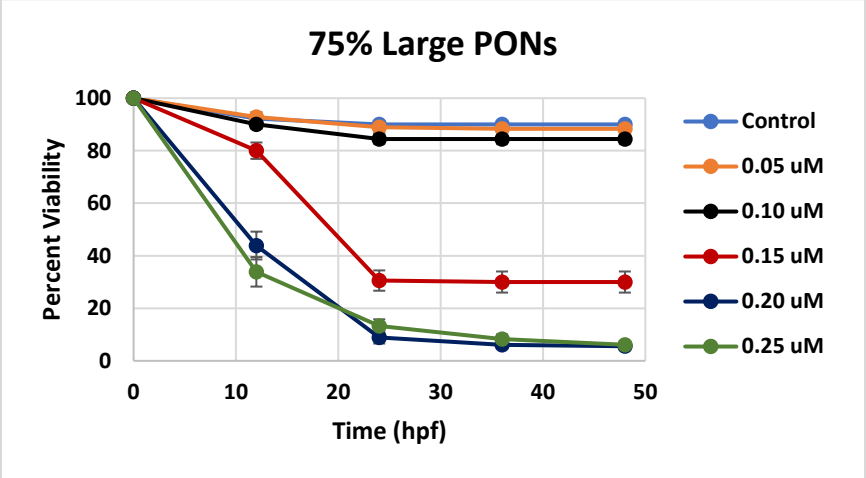
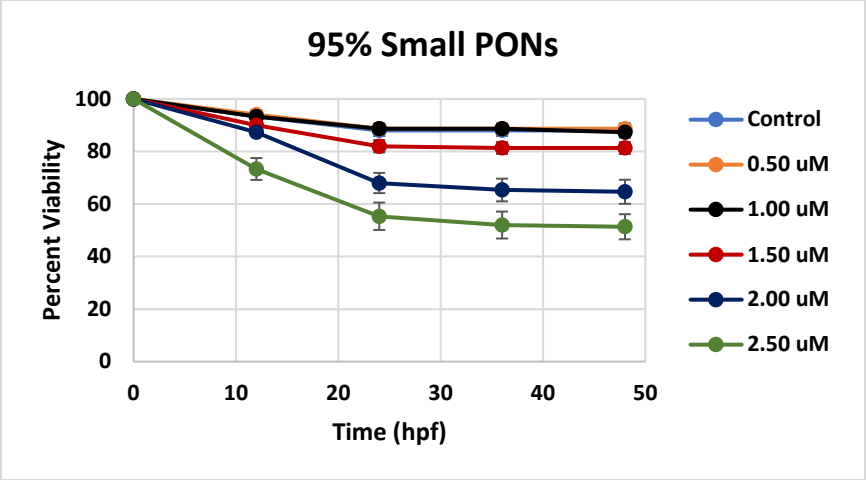


Figure 7 Mean percent viability of zebrafish embryos exposed to (A) the 75% small PONs over the course of 48 hours post fertilization and (B) the 75% large PONs over the course of 48 hours post fertilization. Error bars shown as 95% CI. Six replicates of n=30 zebrafish embryos for both PONs.

A



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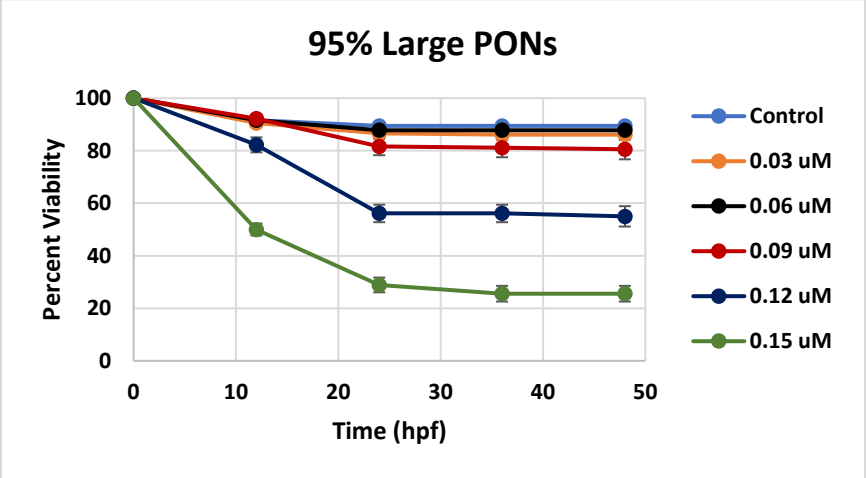


Figure 8 Mean percent viability of zebrafish embryos exposed to (A) the 95% small PONs over the course of 48 hours post fertilization (five replicates of n=30 zebrafish embryos) and (B) the 95% large PONs over the course of 48 hours post fertilization (six replicates of n=30 zebrafish embryos). Error bars shown as 95% CI.

When looking at the LC_{50} values, a better comparison can be seen between the different PONs. This is depicted in Figure 9.

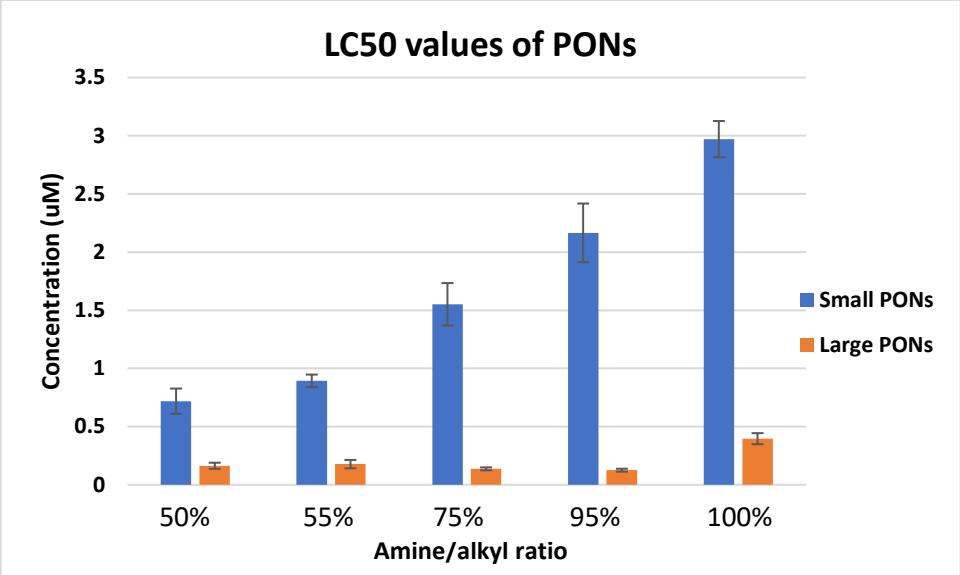


Figure 9 Mean LC_{50} values for the 50%-100% small and large PONs. Error bars shown as standard deviation.

Quantification of the values can be seen in Table 1.

	LC_{50}		
Amine/alkyl ratio	Small PONs	Large PONs	Relative Difference (Small PONs/Large PONs)
50%	0.72 ± 0.11 uM	0.16 ± 0.03 uM	4.50
55%	0.89 ± 0.05 uM	0.18 ± 0.04 uM	4.94
75%	1.55 ± 0.18 uM	0.14 ± 0.01 uM	11.07
95%	2.17 ± 0.25 uM	0.13 ± 0.01 uM	16.69
100%	2.97 ± 0.16 uM	0.40 ± 0.05 uM	7.43

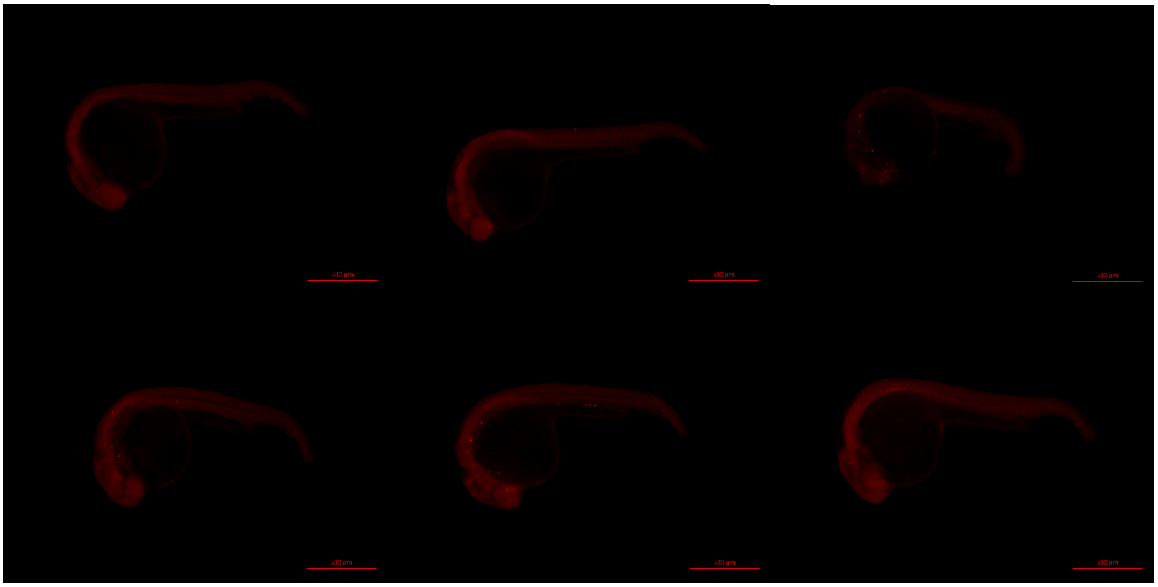
Table 1 Table shows LC_{50} values seen in Figure 14. Standard deviation is shown for error.

When comparing the different amine/alkyl ratios in the small PONs, the 50% PONs has a LC_{50} of 0.72 uM while 100% PONs has a LC_{50} of 2.97 uM. This indicates that the 50% PONs is more biologically active against the zebrafish embryos compared to the 100%. There are minimal changes in the LC_{50} values between the 50%-95% large PONs, but the LC_{50} for the 100% large PONs is higher than the latter. The 50% PONs has a LC_{50} of 0.16 uM while the 100% PONs has a LC_{50} value of 0.40 uM. This indicates the importance of the hydrophobic alkyl group. Without this alkyl group, the activity in both the small and large PONs is dramatically decreased. The length of these polymers is also important in their activity against zebrafish embryos. To further examine the difference in activity between the small and large PONs, the relative difference between the LC_{50} values was calculated. Since the large PONs have 7 times more monomers than the small PONs, they should be 7 times more active. If this were true, the relative difference would be 7. This is not the case for most of the amine/alkyl ratios. For the lower amine/alkyl ratios (50% and 55%), the relative difference is below 7, which indicates that the small PONs are more active than the large PONs. When the amine/alkyl ratio increases (75% and 95%), the relative difference is above 7. This is suggesting that the large PONs are more active than the small PONs. The 100% PONs has a relative difference of 7.43, which indicates that the small PONs and large PONs have about the same activity. There is an outlier in this data. The relative difference between 95% and 100% PONs is significantly different. The 95% PONs has a relative difference of 16.69 while the 100% PONs is 7.43. Further studies should be done to determine if this is an error because the activity of the PONs should not have such a large decrease in activity when the amine/alkyl ratio is only changing by 5%. This data suggests that there is a stronger interaction due to increased amine charge density

when large PONs associate with the embryos. Fine tuning the molecular structure of PONs can provide antibacterial agents that have maximum activity against bacteria while having no toxicity on host organism cells.

To identify cells undergoing apoptosis, activated caspase-3 was visualized in exposed zebrafish embryos using whole-mount immunofluorescence. Using this method identifies both intrinsic and extrinsic apoptosis occurring in the embryo. Apoptotic cells brightly fluoresce while nonapoptotic cells show minimal background fluorescence. Figure 10 shows representative images of a control embryo and embryos at 24 hpf expose to the following: 0.25 uM and 1.00 uM 50% small PONs, 0.15 uM and 0.25 uM 50% large PONs, and 1.00 uM 100% small PONs.

Control 0.15 uM 50% large PONs 0.25 uM 50% large PONs



0.25 uM 50% small PONs 1.00 uM 50% small PONs 1.00 uM 100% small PONs

Figure 10 Zebrafish embryos exposed to 50% small PONs, 50% large PONs, and 100% small PONs at 24 hpf analyzed by immunofluorescence to detect activated caspase-3.

The control embryo shows minimal apoptotic cells. It is normal for the control to have some apoptotic cells since the embryo is in the middle of development and apoptosis is a

normal part of development. All the exposed embryos show more apoptotic cells compared to the control. The 0.25 uM 50% large PONs shows a developmental delay and a significant increase in apoptotic cells compared to the control. This is consistent with the viability studies done since the 50% large PONs is the most potent form of the PONs tested. The same concentration dependent trend is seen from the viability studies. Increasing the concentration of the PONs increases the number of apoptotic cells in the embryos. Also, the trend of decreasing amine/alkyl ratio decreases the viability of the embryo is also confirmed with these results. At 1.00 uM, there are more apoptotic cells seen in the 50% small PONs compared to the 100% small PONs. At 0.25 uM, the 50% large PONs show more apoptotic cells compared to the 50% small PONs. This further confirms the polymer length trend seen in the viability studies. Additional studies need to be done to confirm the results shown here by quantifying the number of apoptotic cells in the embryos.

CHAPTER 4: CONCLUSIONS

In this study, we were able to elucidate the effect of changing the amine/alkyl ratio and polymer length on zebrafish embryos. Using the zebrafish embryo toxicity test, we were able to determine the impact on viability between the different types of PONs and calculate LC_{50} values. We were also able to visualize the impact on the zebrafish embryos by analyzing the apoptotic cells after exposure compared to an untreated embryo. Our results show that increasing the hydrophobicity (decreasing the amine/alkyl ratio) and the polymer length of the PONs decreases the viability of the embryos. However, increasing the polymer length does not always increase the efficiency of the individual monomers. This can be seen in the LC_{50} values increasing in concentration as the amine/alkyl ratio increases and decreasing in concentration when the polymer length increases. To visualize apoptosis occurring in the embryos, whole-mount immunofluorescence was used. The images further confirm the trend seen from the viability graphs and LC_{50} values. The number of apoptotic cells increases as the amine/alkyl ratio decreases and the polymer length increases. The most active form of the PONs has a negative impact on zebrafish embryo development. This is cause for concern because usually antibacterial polymers are only tested for their selectivity against mammalian cells. Since this study suggests that there is a negative impact on zebrafish embryo development, PONs and other antibacterial polymers should not only be tested on cells, but also on whole organisms. This study also suggests that slight variations in the molecular structure of PONs can significantly lower their impact on zebrafish embryos while still having strong antibacterial activity. It is therefore important to optimize the polymer structure to have the highest selectivity against bacteria while having no or minimal toxicity against organisms. Future work should include the

quantification of caspase-3 in the zebrafish embryos to confirm that apoptosis is being induced in the embryos and increases as the amine/alkyl ratio decreases and polymer length increases (as seen in the immunofluorescence images in this study). More amine/alkyl ratios should also be tested to investigate the huge difference in activity seen between the small and large PONs. Conjugation of these PONs to different nanomaterial, like gold nanoparticles, can also be done to study how conjugation affects the activity against zebrafish embryos.

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