FINAL REPORT
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Anti-Microbial Nanomaterials for Rendering Applications

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Duration of Project: 14 months

Lay Summary: This proposed project sought to build upon our very successful ACREC supported program toward the development of functional nanomaterials for rendering operations. We have successfully developed and continue to study biodegradable, functional nanomaterials that are capable of capturing or chemically modifying malodorant molecules that contribute to unsavory odors associated with raw material piles and rendering operations. In this off-shoot proposal, we began to investigate whether the same design strategies could be employed to develop related materials that exhibit antimicrobial properties. We envisioned that these novel materials might provide a safe and reliable method to 1.) decontaminate work surfaces at rendering sites (including raw material staging areas, raw material front-end loaders, raw-material trucks, grease collection bins), and 2.) as a potential non-toxic additive or filter treatment for rendered products, especially fats, to prevent bacterial recontamination during truck loading and off-loading as well as during the application of the fat coat on pet food kibble.

Briefly, the ultimate goals of this project were to pursue the synthesis of functional anti-microbial biodegradable nanomaterials and to confirm their ability to kill both Gram-Negative and Gram-Positive bacteria in culture broth.

Objective (s):

1. *To synthesize and characterize chlorinated polymeric nanoparticles for investigation as antimicrobial materials.*
2. *To investigate the antimicrobial properties of chlorinated nanoparticles against Gram-positive and Gram-negative bacteria in culture broth.*

Project Overview:

Introduction: The high temperature and pressure of cooking processes associated with rendering operations are well known to provide an efficient and nearly complete means for the decontamination of raw material from bacteria, parasites, viruses and fungi.[2] Nevertheless, bacterial recontamination, particularly from *Salmonella* sp., of rendered products after the cooking step during loading, transportation, *etc.* is a significant concern.[3-7] Further, there can be risk of contaminating pet food during or after the fat coat is added to kibble during processing if the rendered fat is accidentally contaminated in transit or after arrival at the pet food plant.

Over the past several years with the support of ACREC and FPRF, we have been actively developing a class of functionalized biodegradable nanoparticles that are capable of capturing malodorant volatile organic compounds (VOCs) associate with rendering operations. These efforts have culminated in the development of nanoparticles comprised of poly(lactic acid)-poly(ethylene glycol)-poly(ethyleneimine) block copolymer (*i.e.* PLA-PEG-PEI). We have demonstrated this novel material is capable of capturing malodorous VOCs of the carboxylic acid, aldehyde, and sulfide functional group classes.[1] Subsequently, we have demonstrated that similar performace can be realized with much cheaper, modified natural materials including aluminosilicate clays and cellulose nanocrystals. Further, we have demonstrated that these materials are capable of capturing targeted malodorous VOCs at a rendering plant in a pilot scale experiment. ACREC support has been parlayed into a grant from the Clemson University

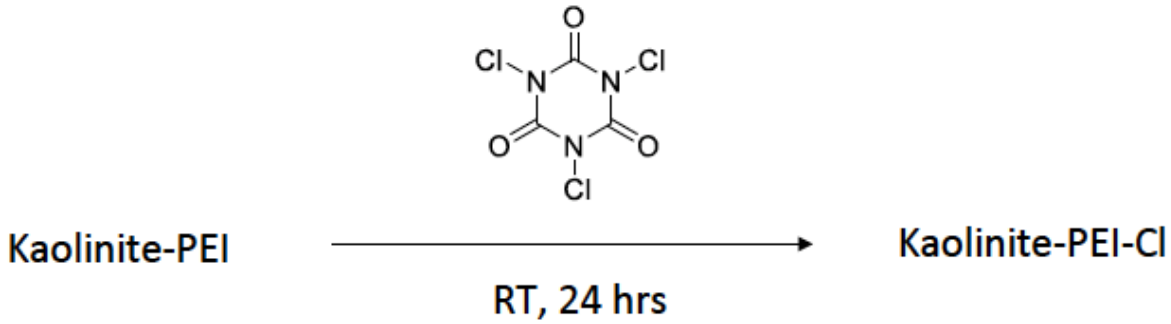
Research Foundation (CURF) to support scale-up efforts that will ultimately facilitate the commercialization of the material.

In this proposal, we sought to build upon our previous successes in the arena of functional nanomaterials in order to develop a related class of functional nanomaterials that exhibit antimicrobial properties. To accomplish this task, we set out to synthetically modify our amine-capped nanomaterials for odor control by treatment with halogenating agents. The resulting N-halo nanomaterials should eventually allow for the isolation of a new set of materials that exhibit potent and broad-spectrum microbicidal properties, since the incorporation of N-halo amines onto polymeric substrates has been demonstrated previously to be an effective method to impart antibacterial properties to materials.[8]

Our original proposal sought to achieve two aims: 1. to prepare and characterize potential antimicrobial materials, and 2. to demonstrate that the materials are capable of killing Gram positive and Gram negative bacteria in culture experiments. Our original proposal requested a sum of $70,000 to accomplish these two tasks. After the proposal was reviewed by the FPRF committee, the budget was reduced to $45,000. In the end, we set out to accomplish both tasks with this reduced budget, but we were only able to accomplish the first task: the successful synthesis of chlorinated materials for potential antimicrobial applications. We were poised to continue to optimize our preparation of the desired materials and to tackle their biological evaluation when the project was not selected for further support during the Spring ACREC meeting. Thus, this Final Report describes the current state of the project, highlights challenges and hurdles to overcome, and potential longer-term goals should the project resume at a later date.

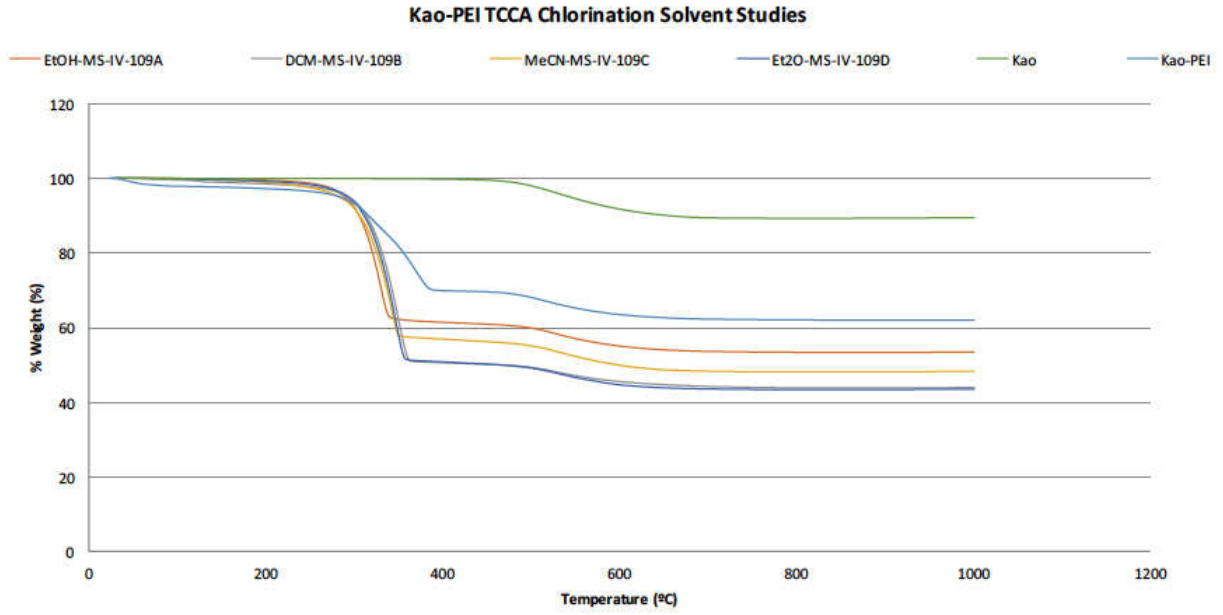
Results: Our initial efforts on this project focused on developing suitable conditions to carry out the chlorination of our previously developed poly(ethylenimine) modified kaolinite clay (*i.e.* Kao-PEI) utilizing the reactive chlorenium agent, trichloroisocyanuric acid (TCCA). To accomplish this take we undertook a very thorough investigation of reaction parameters including solvent (organic or aqueous conditions), solution pH (acidic, basic, neutral), Kao-PEI:TCCA ratio, reaction time, and isolation protocol. Taken together, assaying these parameters comprised over 100 individual experiments. After significant investigation, we realized a protocol for the successful chlorination of Kao-PEI with TCCA utilizing various organic solvents (Figure 1).

Figure 1. Chlorination of Kao-PEI with TCCA.



Ultimately, we discovered that Kao-PEI-Cl could be prepared efficiently in a variety of organic solvents including: ethanol, dichloromethane, acetonitrile, and diethyl ether. The newly synthesized Kao-PEI-Cl materials were first characterized by infrared spectroscopy (Data not shown), which did not provide many diagnostic changes as compared to the non-chlorinated materials. This result was not entirely unexpected given that the desired N-Cl stretching and bending frequencies tend to resonate in the already crowded and convoluted fingerprint region of the IR spectrum. Thus, we turned to thermogravimetric analysis (TGA) and Energy-dispersive X-ray Spectroscopy (EDS) in order to assess the degree to which we had successfully chlorinated the Kao-PEI samples. The TGA analyses of select samples are depicted below in Figure 2.

Figure 2. TGA analysis of Kao-PEI-Cl samples prepared in organic solvents.



Evidence for the desired chlorination event is apparent upon the emergence of changes in the degradation profile of the products after treatment with TCCA. Nevertheless, the TGA analysis did not provide quantitative evidence for the presence of chlorine after TCCA treatment. Thus we turned to EDS analysis in order to obtain elemental data that would conclusively confirm the incorporation of the halogen onto the Kao-PEI. We discovered that the degree to which chlorine was incorporated into the scaffold depended on the solvent that was used for the halogenation event. The percentage of chlorine available in the sample ranged from 0.5% Cl by weight in dichloromethane to 1.2 % chlorine by weight when acetonitrile was used as the solvent. Figures 3-6 depict the EDS data for each Kao-PEI-Cl formulation.

Although the %/wt Cl loadings were not very high, we were able to conclusively demonstrate the ability to modify our poly(amine) modified materials with Cl. If this project is revisited in the future, it would be worth exploring strategies to further enhance the chlorine loading of the resulting material in order to improve the synthesis and performance of the desired materials. It is possible that the loading of Cl is higher than the results provided by the EDS analysis, and we

attempted to carry out titration experiments with the Cl-modified clays to probe this question, but these efforts were unsuccessful.

Figure 3. EDS of Kao-PEI-Cl synthesized in ethanol.

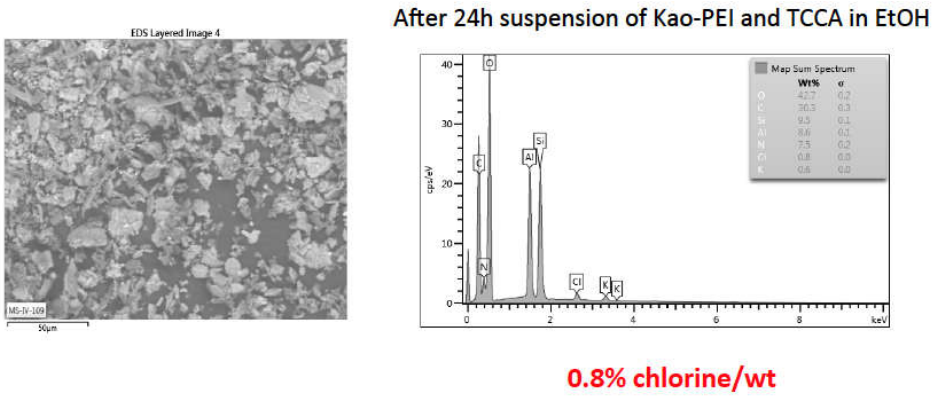


Figure 4. EDS of Kao-PEI-Cl synthesized in dichloromethane.

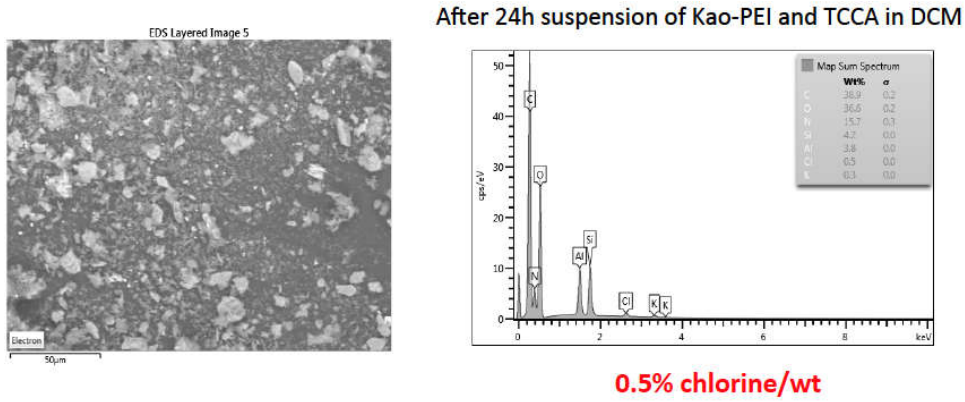


Figure 5. EDS of Kao-PEI-Cl synthesized in acetonitrile.

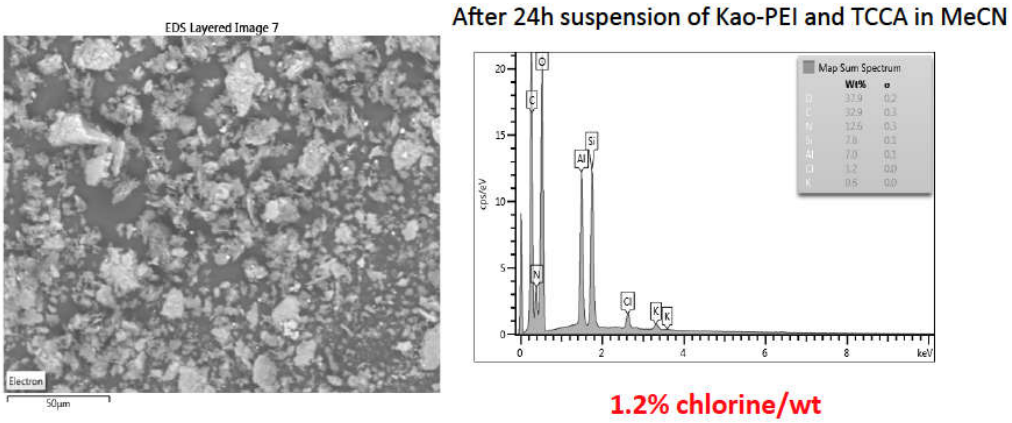
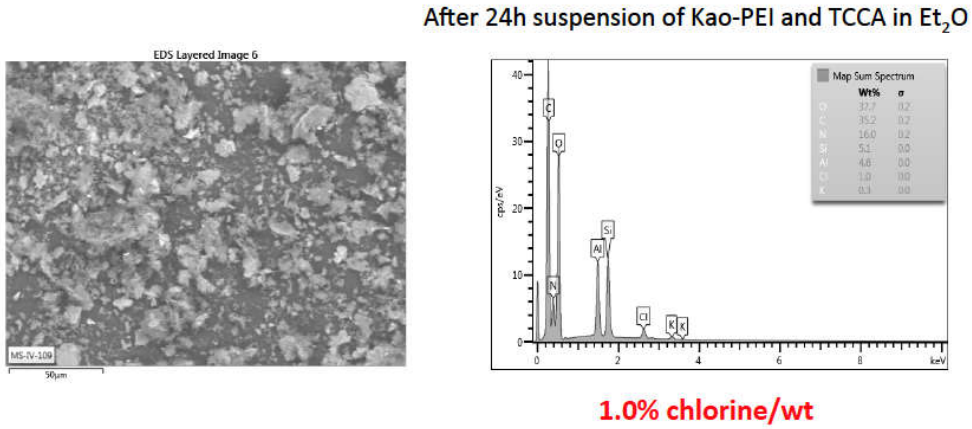


Figure 6. EDS of Kao-PEI-Cl synthesized in diethyl ether.



Additionally, since aluminosilicate clays possess pores between the stacked aluminosilicate crystal lattices, we wondered whether it might be possible to impregnate unmodified clay substrates with a halogenating microbicidal compound without installing the polyamine cap. In this effort, we simply treated suspensions of unmodified clays with trichloroisocyanuric acid (TCCA), filtered and washed the clay. In this formulation, any chlorinating agent present would be absorbed onto the clay substrate without conducting a chemical reaction. Similar to the process described above for the chlorination of polyamine clays, we characterized these new materials utilizing FT-IR, TGA, and EDS analysis. Figure 7 shows a representative EDS analysis of the kaolinite clays impregnated with TCCA using this strategy. As is evident from this approach, we are able to incorporate more of the chlorinating agent into the clay materials using this strategy.

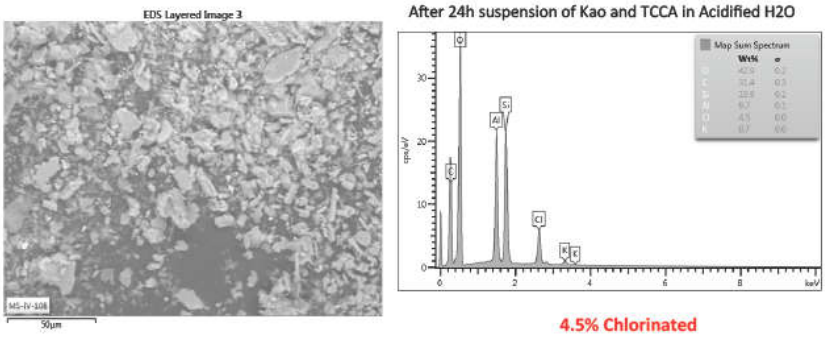


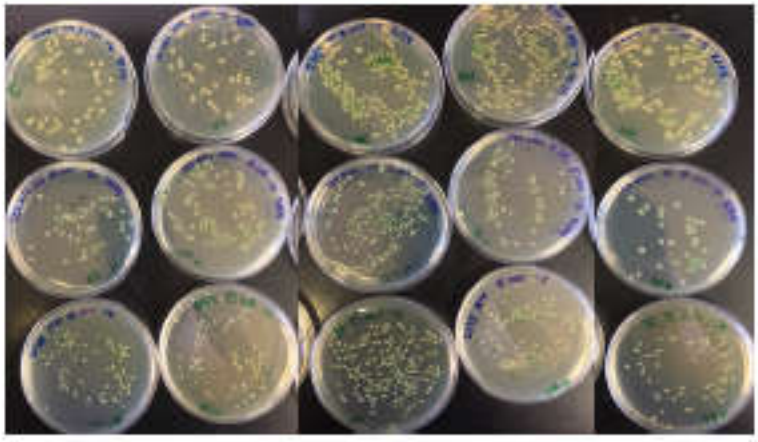
Figure 7. EDS analysis of TCCA-impregnated kaolinite clay. 4.5% chlorine incorporation.

Having successfully synthesized a few examples of Kao-PEI-Cl and Kao clay impregnated with TCCA, we next turned to beginning the biological evaluation of the materials against relevant Gram positive and Gram negative bacteria in culture experiments. Our initial microbicidal assays involved the treatment of mid-log phase bacterial cultures with 10 mg/mL samples of functionalized and control clay materials. The treated cultures are then incubated for 2 hours and then an aliquot of cells is removed for dilution plating to assess the number of colony forming units arising from the treated cultures as compared to untreated controls. We conducted initial experiments with *Escherichia coli* (Gram negative) and *Staphylococcus aureus* (Gram positive). We are also equipped to evaluate *Salmonella enterica* serovars *typhimurium* and *enteritidis* if this project commences again. Figure 8 depicts representative data from an initial evaluation of Kao-PEI-Cl against *E. coli*. At a 10 mg/mL loading of the modified clay relative to bacterial culture, we did not observe any microbicidal activity. Nevertheless, remain confident that our

strategy will bear fruit after trouble shooting. Unfortunately, these initial results were obtained shortly before the project was not renewed for a subsequent year of funding.

Figure 8. Preliminary evaluation of Kao-PEI-Cl against *E. coli* at 10 mg/mL loading.

	0 hrs.	2 hrs.	19 hrs.
E. coli	6.20×10^8	8.20×10^8	2.16×10^9
E. coli MPC	4.90×10^8	2.96×10^9	1.86×10^9
E. coli M	3.48×10^8	5.14×10^8	4.96×10^8
E. coli KPC	3.02×10^8	1.50×10^8	4.12×10^8
E. coli K	2.83×10^8	9.00×10^8	1.46×10^{10}



Conclusions: The goals of this project were partially realized. After a significant number of experiments we successfully developed two methods for the successful incorporation of halogenating microbicides into modified clays. These materials were fully characterized by a number of spectroscopic and analytical techniques. Thus, Kao-PEI-Cl materials were prepared with Cl loadings ranging between 0.5%/wt and 1.2%/wt as judged by EDS analysis. Additionally, an intercalation strategy yielded Kao-Cl materials that bore over 4%/wt of chlorine.

We also began to investigate the potential of these new materials in the key experiments for this project: assessing whether they were capable of killing relevant Gram-positive and Gram-negative bacteria. Nevertheless, at that time, we failed to provide a compelling case to justify further support from FPRF. We still maintain that this project could be successful, and may ultimately result in a broadly useful bacterial disinfectant for rendering applications.

References:

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Impacts and Significance: This project has the potential to deliver a novel platform for the development of functional, biodegradable nanomaterials that exhibit microbicidal properties. Such materials could provide an efficient and effective means to prevent bacterial contamination of rendered materials during loading, transportation and delivery of rendered products. The materials have the potential to serve as a useful, biodegradable disinfectant for fat tanker trucks, meal trucks, and plant equipment. Additionally, the material might also be used as a preventative treatment to prevent contamination of pet food as or after the fat coat is applied to kibble. Since this project was not selected for further funding after the preparation of the materials had been realized but before they could be adequately vetted as microbicides, these potential uses, though appealing, remain speculative.

Publications: Since we were only able to complete the first aim of the proposal (*i.e.* the synthesis of the proposed chlorinated nanomaterials), and had only just begun the second aim focusing on antimicrobial assays when the project was not selected for renewal, the project has not progressed to the point where we have obtained publishable data.

Outside funding: We continue to see support from the National Science Foundation and the United Stated Department of Agriculture to pursue the work described in the proposal as well as the related work on nanomaterials for odor remediation and wastewater purification.

Future Work: This project essentially stopped right before the optimization and evaluation of the key experiments that would demonstrate the feasibility of our strategy. Our progress was delayed initially by a more difficult time than anticipated in accessing the chlorinated materials. Nevertheless, we overcame these obstacles and successfully made a series of chlorinated poly(amine) functionalized clays. We also invested significant time and effort in setting up and validating an operationally useful laboratory assay for microbicidal effects. We remain poised to further explore this project if interest from FPRF returns. While our first round of microbicidal testing did not yield promising results it is entirely possible that either a higher loading of the Kao-PEI-Cl material per mL of culture could exhibit microbicidal activity. Similarly, it is also highly likely that further investigation will allow us to prepare a modified nanomaterial that carries a higher chlorine load relative to our first generation materials described in this Final Report. We believe these future studies are worth pursuing. Finally, if our materials can be validated in laboratory culture experiments, the investigation of the intentional inoculation of rendered materials (both meal and fat products) would be warranted, with the goal of demonstrating that our materials can decontaminate rendered products that have been affected by bacterial exposure.

Acknowledgments: A number of students worked on this project including: Anthony Santilli (D. Whitehead graduate student), Maria Swasy (D. Whitehead Graduate Student), Fernanda Guerra (F. Alexis graduate student), Kyleigh Connelly (K. Whitehead undergraduate student), and Jonnise Macomson (K. Whitehead undergraduate student). Additional assistance was provided by Mrs. Kim Ivey (staff scientist in Materials Science and Engineering).