Application and Toxicity Assessment of Antimicrobial *N*-halamines for Food Safety Controls

by

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Abstract

Cross-contamination from food-associated environments accounts for a big portion of microbial contamination in food products. The objective of this study is to develop *N*-halamine based antimicrobial coatings on various food-contact surface materials for food safety controls and to evaluate the safety of *N*-halamines for food-associated applications. There are three projects covered in this dissertation:

The first project is to modify thermoplastic polyurethane (TPU) with rechargeable antimicrobial function using polyelectrolyte-based *N*-halamines through a compounding method. Chlorination condition, antimicrobial efficacy, storage stability, rechargeability and physical property were investigated.

The second project is to modify stainless steel 316 with antimicrobial/anticorrosion multifunctional coating using conducting polymer-based *N*-halamines through an electroplating method. Surface characterization, antimicrobial efficacy, storage stability and anticorrosion property were investigated.

In the third project, the toxicity of *N*-halamine compound was evaluate to assess the safety for food-related applications using *in vitro* toxicity testing methods. Cytotoxicity and mutagenicity were investigated using cell culture and nutrient deficient bacteria. The possible mechanism of cytotoxicity for *N*-halamine compound was also illustrated.

Results from these studies indicate that *N*-halamines may have great potential as high-effective, low-cost and safe antimicrobials for food safety controls in the food industry.

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"Imagination is more important than knowledge. For knowledge is limited, whereas imagination embraces the entire world, stimulating progress, giving birth to evolution."

- *Albert Einstein, What Life Means to Einstein (1929)*

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Chapter 1

Applying Antimicrobial *N*-halamines for Food Safety Controls: Advances, Opportunities and Challenges

Abstract: Food microbial contamination has been a big concern for the food industry for both food safety and economic reasons. In recent years, novel antimicrobial technologies are continually introduced to the food industry for controlling food safety. Among these, *N*-halamines, as a group of emerging antimicrobial agents, have shown great promise for several advantages such as potent, broad and rechargeable antimicrobial function, adjustable stability and low-cost. Currently, *N*-halamines are researched for non-food applications such as biomedical devices, textiles and water treatments. However, *N*-halamines will find broad applications throughout the food chain for food safety preventive controls. *N*-halamines have been studied for both monomers and polymers; they can be applied as either antimicrobial pesticides or protective coatings for food-contact and environmental surfaces. However, applying *N*-halamines for food safety controls may have the challenges to pass safety regulations in the future.

Keywords: Antimicrobials; *N*-halamines; food safety; advances; opportunities; challenges

1.1 Introduction

Foodborne illnesses have become a big burden to both public health and the food industry. The US Centers for Disease Control and Prevention (CDC) estimates that, in the United States, about 48 million sicknesses, 128,000 hospitalizations and 3,000 deaths are resulted from foodborne diseases per year (Scallan and others 2011). The total economic loss was estimated as $15.6 billion per year by the US Department of Agriculture (USDA) (USDA 2014). Most of these foodborne illnesses were caused by consuming foods contaminated with harmful microorganisms such as fungi, bacteria, virus, etc. For instance, 91% of foodborne illnesses were contributed from five top pathogens including Norovirus, *Salmonella* (nontyphoidal), *Clostridium perfringens*, *Campylobacter spp.* and *Staphylococcus aureus* (Scallan and others 2011). Foodborne pathogens also contribute significantly to the ever increasing food recalls happened in the food industry: usually almost half of these recalls are due to microbiological contamination (Kowitt 2017). In 2016, there were 764 food recalls reported from USDA and US FDA (Food and Drug Administration) and 43% of them were caused by microbiological contamination recalls (Maberry 2017). On average, about $10 million direct costs were associated with a single recall to a food company, not including brand damage and losses of sales (TIS 2012). The total cost to the food industry can be extremely expensive and threatens the profitability (Kowitt 2017; TIS 2012). All these factors combined demand the food industry to re-examine current food safety control strategies and to introduce novel technologies for controlling the food safety challenges caused by microbiological contamination.

Cross-contamination is one of the major causes for microbial contamination in food products which can easily occur in multiple stages across food chain: food production,

processing, distribution and handling (Larsen and others 2014; Muhterem-Uyar and others 2015; Gorman and others 2002; Kaneko and others 1999; Carrasco and others 2012). The contamination may happen in various food-associated environments including food-contact surfaces and non-food contact surfaces. Since microorganisms can attach and colonize on almost any solid surfaces, if not eliminated timely, they can quickly proliferate, excrete extracellular biopolymers and form a non-porous 3-D matrix: biofilm (Ryu and Beuchat 2005). Once mature biofilms are formed, microorganisms are more difficult to be removed since the biofilms are more resistant to common sanitation treatments including aggressive sanitizing/cleaning agents (Corcoran and others 2014). The bacteria embedded in the biofilm may potentially contaminate the food products that get contact with the surfaces (Faille and others 2014; Kumar and Anand 1998; Brooks and Flint 2008). Besides, non-food contact surfaces, including machinery parts, floors, ceilings, walls, and sewage systems may also cause contamination to final food products indirectly (Muhterem-Uyar and others 2015). In addition, environmental factors such as water or air quality and human contact can also introduce pathogenic microorganisms into the final food products (Gil and others 2009; Lianou and Sofos 2007). Therefore, food safety controls should not only focus on decontaminating the food products, the preventive controls of associated environments and human are also of great importance.

To mitigate or eliminate the microbial cross-contamination on solid surfaces and associated environments, several controlling methods have been established by the food industry based on physical, chemical or biological principles (Niemira and others 2014; Kumar and Anand 1998). Among these, chemistry methods such as antimicrobial interventions are indispensable tools due to their high efficiency and low cost. Antimicrobial pesticides are widely used in sanitation and environment controls; however, the are several limitations of existed

chemistry-based antimicrobial control strategy. First, the antimicrobial activity of major antimicrobial pesticides cannot last long, therefore microbial contamination may still happen during processing or handling (Corcoran and others 2014). Second, since sanitation depends heavily on human workers, there is a risk of insufficient or improper sanitation due to human error. Third, microorganisms are developing more and more resistance to currently used antimicrobial agents, resulting in insufficient sanitation (Condell and others 2012; Corcoran and others 2014). Therefore, the food industry needs to introduce more novel antimicrobial control chemicals and materials that can be used for sanitation or environmental controls. In addition, nowadays the consumers are demanding for food products with improved nutritional value and convenience such as minimally-processed, ready-to-eat or clean-label products. All these trends will result in the continue trimming of traditionally used intervention technologies such as thermal processing and antimicrobial additives. Finally, these will create more challenges for the food industry to maintain or improve food safety. Therefore, there is a need to create a secondary or multiple prevention strategies in compensation with current sanitation systems to minimize the risk of microbial contamination. New chemistry or material engineering methods should be continually introduced to the food industrial and domestic settings to solve the ever increasing food safety challenges.

N-halamines, as a group of emerging antimicrobial agents, have drawn more and more interest in recent years (Hui and Debiemme-Chouvy 2013; Dong and others 2017). All *N*-halamines share the same nitrogen-halogen functional groups that are formed from transferring nitrogen-hydrogen bond through halogenation (Figure 1.1). The antimicrobial function depends on the oxidative halogen atoms in the nitrogen-halogen moieties. Current research in *N*-halamines mainly focus on basic research areas of organic chemistry and polymer chemistry and

application areas of biomedical devices, water treatments and textiles. Several recent reviews discussed the potential applications of *N*-halamines in various areas, including in the food industry (Dong and others 2017; Hui and Debiemme-Chouvy 2013; Kenawy and others 2007). The applications of *N*-halamine in food systems were also highlighted within the food science society (Bastarrachea and others 2015; Qiao and Huang 2016). There are several reasons that make *N*-halamines appealing for food applications. First, *N*-halamines have potent biocidal activity against a broad spectrum of microorganisms including fungi, bacteria, virus, etc. Second, *N*-halamines have adjustable antimicrobial activity and stability to fulfill specific application requirements. Third, the antimicrobial activity of *N*-halamines is rechargeable by simply treating with chlorine bleach (Figure 1.2), which can be well incorporated with current sanitation practices in the food industry. Finally, *N*-halamines can be synthesized and fabricated on various materials using low-cost methods. All these characteristics make *N*-halamines especially attractive for the food industry.

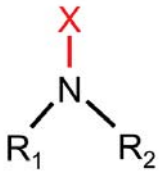

Figure 1.1 Structure of *N*-halamines. R_1, R_2 = H or organic groups. X = Cl, Br or I.

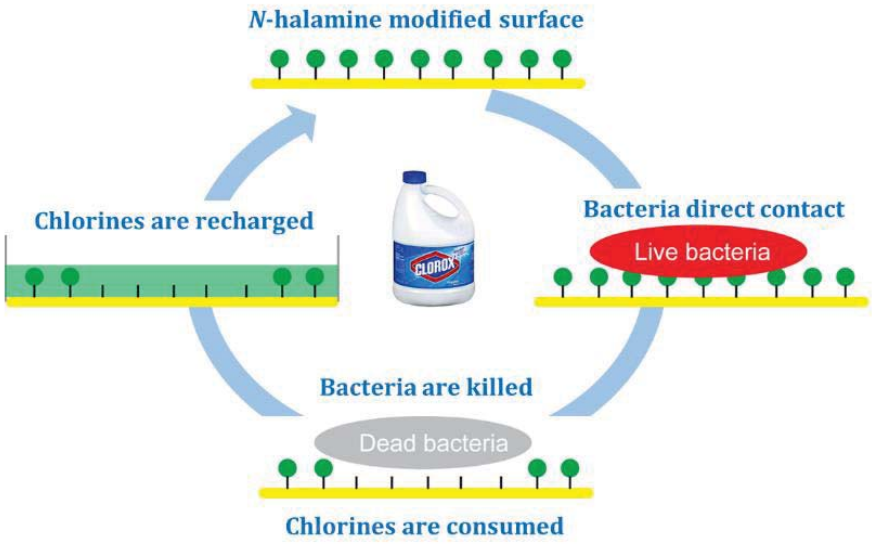

Figure 1.2 Rechargeable antimicrobial function of *N*-halamine modified surfaces.

Some recent research have already started to investigate the potential applications of *N*-halamines in the food industry (Denis-Rohr and others 2015; Bastarrachea and Goddard 2015; Bastarrachea and others 2014; Bastarrachea and others 2013; Bastarrachea and Goddard 2013; Qiao and others 2017b); however, no reports focuses on analyzing how to apply *N*-halamines in the food industry. Systematic review of special requirements and technologies desired by the food industry are needed to direct the research of applying *N*-halamines for food safety controls. This review will give insight information about some niches in current food safety controls and how *N*-halamine technology can be used to fit the need of food industry. Firstly, a brief overview of current food safety control strategies based on chemistry will be mentioned. Secondly, introduction of important properties and current research status of *N*-halamines will be provided. In addition, detailed descriptions of the feasible applications of *N*-halamines in food production,

processing, distribution and handling will be presented. Finally, potential challenges and pitfalls about applying *N*-halamines in food systems will also be discussed.

1.2 Antimicrobial chemistry for food safety controls

To prevent microbial cross-contamination on solid surfaces, there are two fundamental strategies: one is to apply cleaning/sanitizing agents on the surface to detach or kill attached microorganisms; another one is to make the surface with antimicrobial/antifouling function, preventing the attachment of microorganisms continuously (Chmielewski and Frank 2003). Here we will focus on antimicrobial compounds that can be applied for either sanitation or equipment design including antimicrobial pesticides and antimicrobial coatings.

1.2.1 Antimicrobial pesticides

Antimicrobials pesticides are pure or mixed chemicals that can kill or inhibit harmful microorganisms on inanimate objects and surfaces. According to US EPA (Environmental Protection Agency), there are more than 15 different classes of antimicrobials based on chemical structures and mechanisms of action; and about 275 different active ingredients are registered as 4,000 products in the marketplace (EPA 2017). Currently, approximately one billion dollars are spent on various types of antimicrobial products each year (EPA 2017). Many of these products

are used to control infectious microorganisms in food-related environments including processing. Generally, antimicrobials can be divided into natural and synthetic compounds and they can be formulated as sprays, liquids, concentrated powders and gases to fulfill different application requirements. Detailed description of antimicrobial pesticides used in food systems can be found in elsewhere (Cords and others 2005). This article will focus on synthetic antimicrobial sanitizers for solid surfaces in food associated environments.

The basic action mechanism of major sanitizers used in the food industry can be summarized into three categories: oxidative effect, surfactant effect and pH effect. Most of antimicrobial agents used currently are based on either single or a combination of these mechanisms (Figure 1.3). Each of these sanitizers its advantages and disadvantages, and they are selected and used in case-by-case scenarios. The limitations of some major sanitizing agents currently used in the food industry and potential niches that need to be fulfilled will be discussed briefly. Most of antimicrobial pesticides applied in food premises are liquid sprays such as chlorines (bleach), iodophors, peroxyacetic acid (PAA), quaternary ammonium compounds (QACs), etc. Extensive reviews of common sanitizers can be found from the report done by Schmidt (2009) and Fraser and Pascall (2010). Here, we just provide an overlook about advantages and limitations of these sanitizing agents.

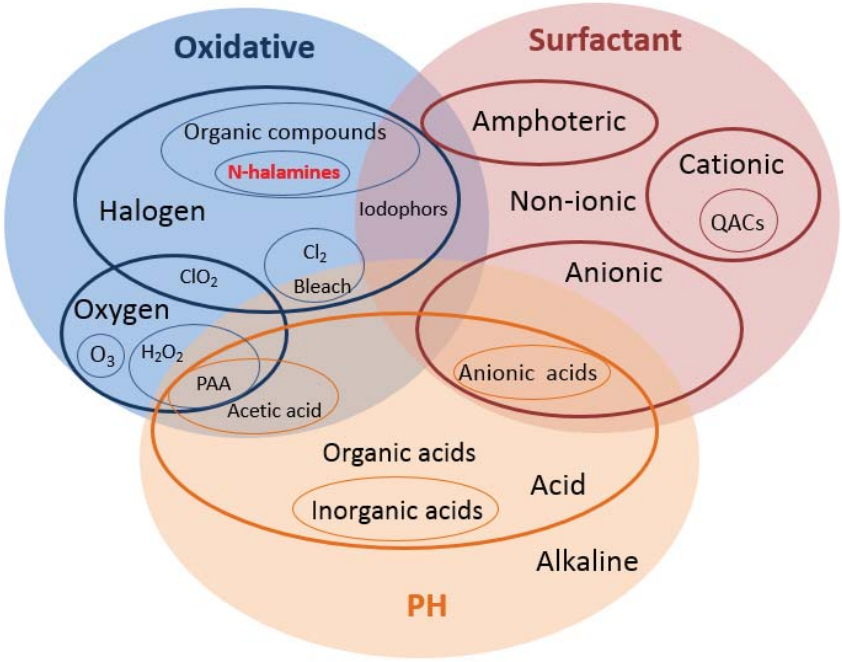

Figure 1.3 Major antimicrobial agents for food-contact surface applications.

Chlorines are currently the most widely used sanitizers due to their high effectiveness against a broad spectrum of microorganisms and low-cost. However, the applications of chlorines are limited due to their corrosion effects to equipment, irritation to human skin, poor stability and deteriorated activity in higher pH and organic matters (McGlynn 2004). Quaternary ammonium compounds (QACs), which are non-toxic, odorless, colorless, non-corrosive, non-irritating and stable to heat and organic matter and active over a wide range of pH, are usually used in the situation where chlorines are not preferred. However, QACs are not compatible with anionic detergents and hard water salts, and they are not effective against certain microorganisms such as coliforms and bacteriophages (Fraser and Pascall 2010). Iodophors are the complex of iodine and surfactants, and they are less irritation to human with improved stability. However, iodophors will lose their antimicrobial activity rapidly at higher pH and they are less effective against bacteria spores and bacteriophages. In addition, iodophors are usually expensive and may

discolor equipment and surfaces. Peroxyacetic acid (PAA) is the combination of acetic acid with hydrogen peroxide. As a sanitizer on hard surfaces, it will be decomposed into nonhazardous products. However, PAA has strong skin irritation and can cause corrosion to the equipment.

Some gaseous antimicrobial agents such as chlorine dioxide and ozone are applied to the whole facility including environmental and food-contact surfaces. They are highly effective against biofilm and vegetative cells; however, they are usually unstable, which may cause explosion risk and irritation to workers. They are also possible to cause corrosion to food equipment due to strong oxidative effect in wet environment (Pascual and others 2007; Singh and Singh 2002). There are also some other antimicrobial agents based on either combination of these mechanisms and/or size effects (nanoparticles). Examples and reviews about these antimicrobial pesticides can be found elsewhere (Rai and others 2009; Taglietti and others 2012; Ochomogo and others 2006) and will not be discussed in this review. Most of existed antimicrobial pesticides are considered as one-time use sanitizers and their antimicrobial activities only last for a short period of time. There is a lack of long-lasting and high-effective agents that are suitable for either food-contact or environmental surface applications without having those limitations as mentioned above. *N*-halamine will provide promising keys to address these challenges either as novel antimicrobial pesticides or antimicrobial coatings.

1.2.2 Antimicrobial coatings

The other strategy to protect hard surfaces from microbial contamination is to coat the surface with an antimicrobial protection layer based on either repelling or killing mechanism

(Figure 1.4) (Siedenbiedel and Tiller 2012). Generally, the repelling mechanism based coatings are called antifouling coatings and the killing mechanism based coatings are named antimicrobial coatings. Only killing-based antimicrobial coatings will be discussed in this review. There are two major technologies for making the surface with antimicrobial killing capacity. One method is to embed antimicrobial agents such as triclosan and metal particles into the substrate polymer (Lyutakov and others 2015; Petersen 2016; Palza 2015). The antimicrobial function relies on the antimicrobial agents migrating onto the surface of embedded materials. This method is most widely researched and adapted for biomedical applications (Singha and others 2017). Some commercial products for food applications such as conveyor belts also base on this mechanism (Cediel and others 2006). However, this method has many drawbacks for food applications. Usually, the concentrations of antimicrobial agents are regulated to under certain levels that do not have enough dose to provide antimicrobial activity on the surface. Although there are some products in the market, research indicated these migration-based antimicrobial belts did not work well as expected in real application environment because of the chelating or fouling effects from the complex of food components (Chaitiemwong and others 2010). The other method is to coat antimicrobial polymers on the surface (Liu and others 2016), which one has shown more and more promise. Since the polymers are fixed on the surface and will not migrate into food products (Bastarrachea and others 2015), this method is expected to have better antimicrobial activity and less safety regulation obstacle.

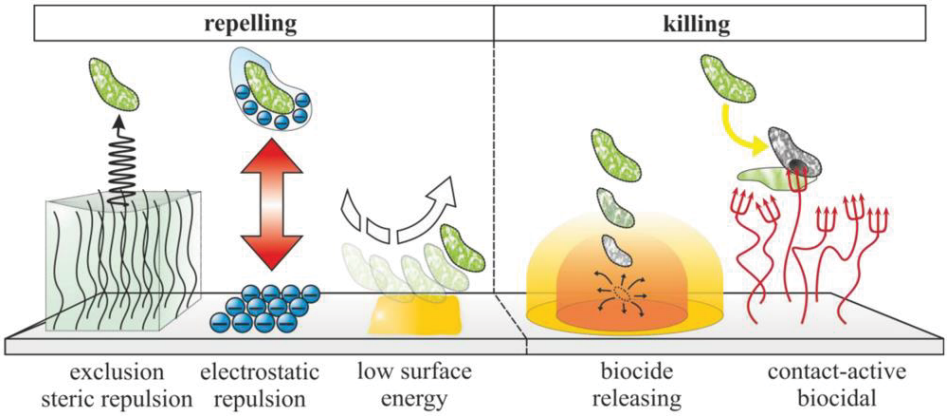

Figure 1.4 Antimicrobial/antifouling coatings based on mechanisms of action. Reproduced with permission from reference of Siedenbiedel and Tiller (2012). Copyright 2012 Multidisciplinary Digital Publishing Institute.

Generally, antimicrobial polymers can be divided into natural and synthetic polymers. Although natural antimicrobial polymers have drawn more attention in recent years due to regulation and consumer preference reasons, synthetic polymers still show great promise for earlier being commercialized and applied in the food industry due to their low-cost and better quality control for large-scale production. For example, antimicrobial peptides (AMPs) are highly effective and have been researched extensively to coat biomedical device surfaces in recent years (Yu and others 2017; Yazici and others 2016; Tan and others 2014); however, the high cost will prevent the wide applications as antimicrobial coatings for the food industry. Therefore, many synthetic polymers are preferred for using as surface coating materials in food-associated environments (Bastarrachea and others 2015; Mérian and Goddard 2012). In addition, the polymer synthesis should be simple, straightforward and inexpensive. Currently, most of synthetic antimicrobial polymers are based on cationic groups such as quaternary ammonium, quaternary phosphonium, guanidinium or tertiary sulfonium (Liu and others 2016; Siedenbiedel and Tiller 2012). However, they also experienced the problems of limited antimicrobial function

against certain microorganism, compromised antimicrobial activity in certain application environments and developing antimicrobial resistance. Detailed reviews of synthetic antimicrobial polymers can be found elsewhere (Timofeeva and Kleshcheva 2011; Hui and Debiemme-Chouvy 2013; Kenawy and others 2007).

The research in antimicrobial surface coatings mainly focuses on biomedical areas; however, there are some differences between food and biomedical applications. The application environments in food systems are more aggressive than these in biomedical settings. There are more soil and organic loads on the coated surfaces including lipids, proteins, polysaccharides and tissue fluids (plasma, blood) from food components. Therefore, many coatings based on migration mechanism are not suitable for food-contact surface applications although they are widely accepted for biomedical applications. Besides, the cost of coating technologies for food applications should be low enough since the profit margin in the food industry is always much lower than the biomedical industry. Thus, there is a need to develop new classes of antimicrobial polymers that are high-effective, low-cost and safe for the food industry applications. *N*-halamine polymers have the potential to fulfill this niche well.

1.3 *N*-halamine antimicrobials and current research status

In this part, an overview of *N*-halamines chemistry, antimicrobial efficacies and mechanisms, surface modification methods and application research areas will be provided.

1.3.1 *N*-halamine chemistry

The core component of *N*-halamine chemistry is the nitrogen-halogen bond, which is the fundamental of *N*-halamine with antimicrobial function. The structure of *N*-halamine molecule has direct impact on its antimicrobial activity and stability. One of the most attractive features of *N*-halamines is that their activity and stability can be designed by manipulating the molecule structure and there are two ways to achieve this goal. One way is to manipulate the halogen atom in the nitrogen-halogen bond, which can be chlorine, bromide or iodine. The antimicrobial activity follows the order of iodine > bromine > chlorine and the stability is reversed (Dong and others 2017; Tsao and others 1991). However, chlorine is more studied due to i) it is more stable compared with iodine and bromine, ii) the halogenation process can be achieved through the treatment of chlorine bleach, which is a cheap and widely used sanitizing agent. The other strategy is to modify the adjacent groups of nitrogen-halogen bond or spatial structures of *N*-halamines. Currently, almost all *N*-halamines are transferred from amide, imide or amine through halogenation (Figure 1.5). Generally, the activity follows the order of imide > amide > amine based *N*-halamines while the stability is reversed (Kenawy and others 2007). Cyclic or acyclic structures in *N*-halamine molecules also have impact on stability and activity of functional groups. All these combined will create a great deal of possibilities to design suitable molecules for specific applications depending on the requirements of activity and stability.

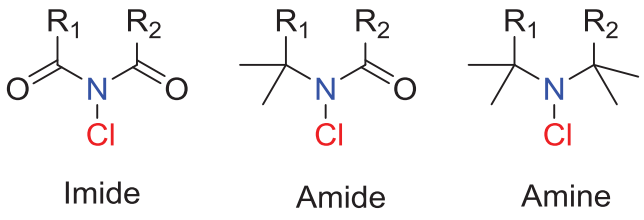

Figure 1.5 Molecular structures of imide, amide and amine based *N*-halamines. R_1, R_2 = H or organic groups.

The stability of *N*-halamines is also influenced by environmental factors such as temperature, light and pH. Usually, *N*-halamines have lower stability under UVA light compared with fluorescent light and dark (Liu and others 2016). This phenomenon has been intensively studied for textile applications since *N*-halamine coated textile clothes need to be exposed to sunlight; however, for food application this is not a serious issue since most of applications are indoors without direct exposure to sunlight. The stability of *N*-halamine can also be improved with TiO_2 (Li and others 2013a). Due to its oxidative nature, *N*-halamines are susceptible to certain reducing agents such as SO_3^{2-}, HSO_3^-, SO_2, ascorbic acid, iron metal, and thiols in amino acids (Dong and others 2017). All these are important factors that need to be considered for designing and applying antimicrobial *N*-halamines in food systems.

Another attractive property of *N*-halamines is that their antimicrobial activity can be recharged. The nitrogen-halogen bond can revert to nitrogen-hydrogen bond without damaging the molecular structure and the nitrogen-hydrogen bond can be transformed into nitrogen-halogen bond again (Figure 1.6) (Dong and others 2017). Theoretically, this process can be repeated many times. Just like the rechargeable battery; however, chlorines are immobilized in *N*-halamine molecules instead of electrons in rechargeable battery. This will add tremendous benefits as antimicrobial coatings: improving antimicrobial efficacy, reducing the cost and protecting the environment through reducing the discharge of many other antimicrobial agents.

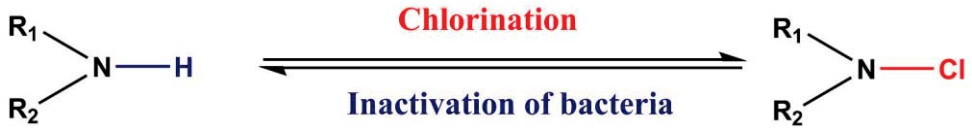

Figure 1.6 Rechargeable antimicrobial activity of *N*-halamine functional groups.

N-halamines include both monomers and polymers. As monomers, *N*-halamine compounds can provide long-lasting and strong antimicrobial activities to fulfill special requirements. The monomers are more suitable to apply as novel antimicrobial pesticides. The solubility or water hydrophobicity of *N*-halamines can also be adjusted through introducing different functional groups, creating the convenience of designing suitable chemical molecules for specific applications. The structures of some *N*-halamine compounds are shown in Figure 1.7. As for polymers, the antimicrobial activities of *N*-halamines can be recharged. They can be used for antimicrobial coatings on solid surfaces. In most cases, *N*-halamine precursor polymers were prepared first and coated on the surface, then the coatings were transferred into *N*-halamines by treating of chlorine bleach. Some polymerizable *N*-halamine precursor monomers and synthesized *N*-halamine precursor polymers are shown in Figure 1.8 and 1.9. More details about modification and application of these compounds will be discussed in the following sections of this review.

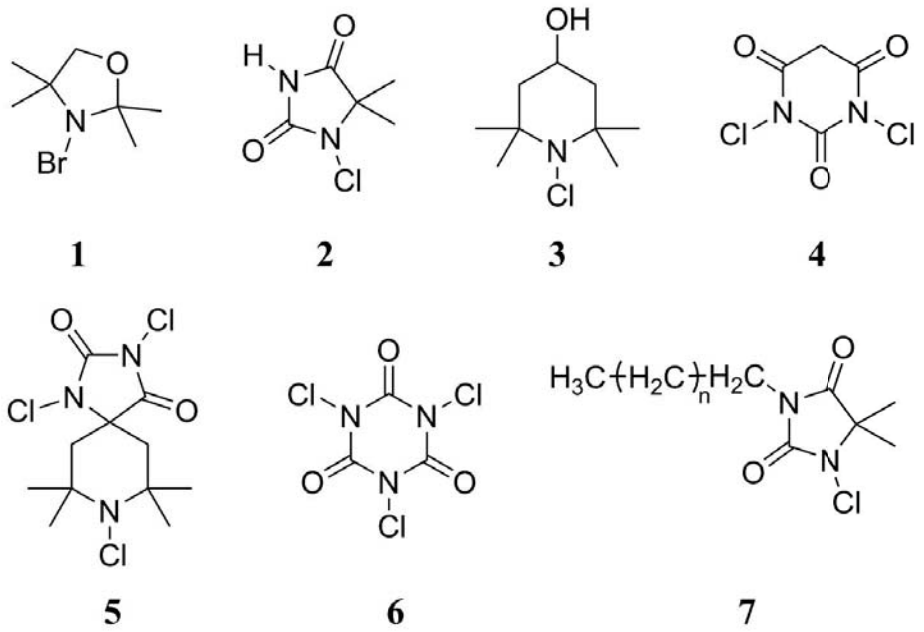

Figure 1.7 Structures of *N*-halamine monomers.

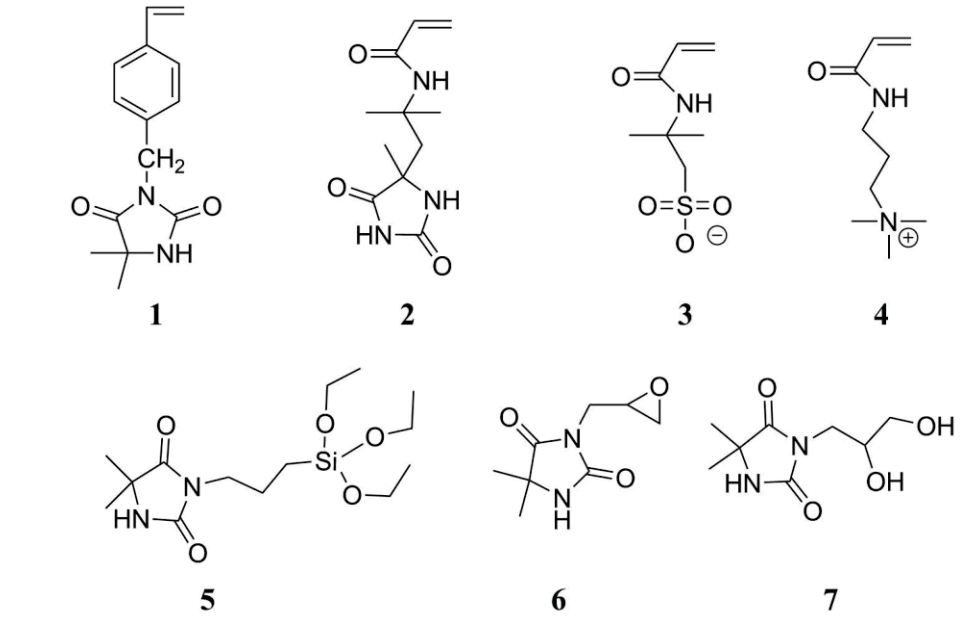

Figure 1.8 Structures of polymerizable or graftable *N*-halamine precursor monomers.

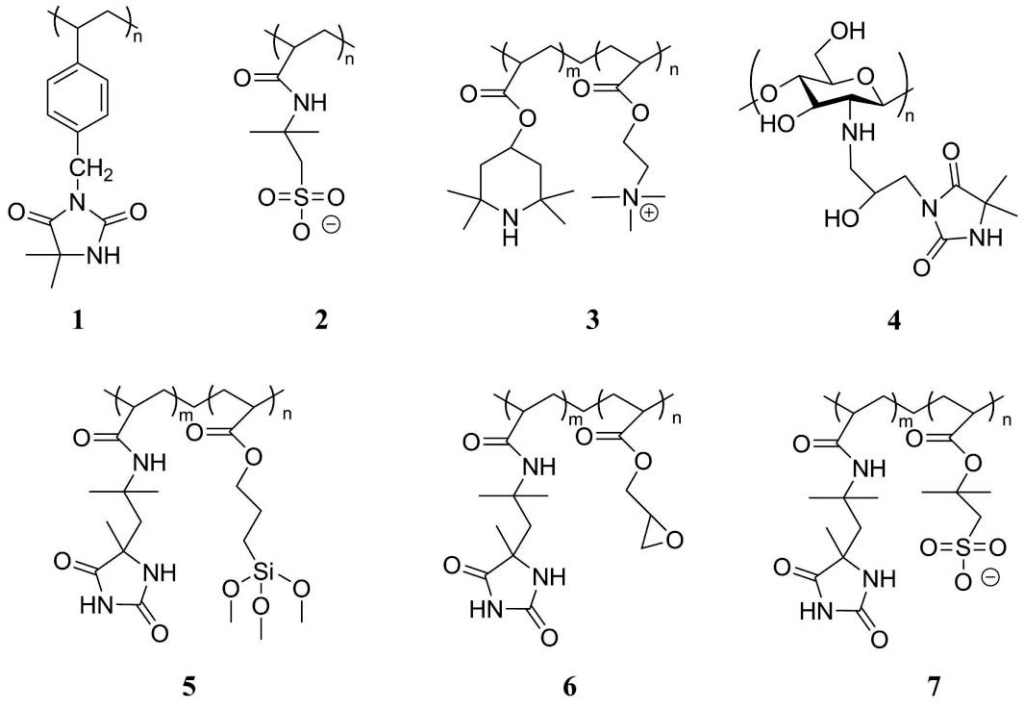

Figure 1.9 Structures of some *N*-halamine precursor polymers.

1.3.2 Antimicrobial efficacies and mechanisms

The antimicrobial function of *N*-halamine is potent and able to against a broad spectrum of microorganisms (Hui and Debiemme-Chouvy 2013). Antibacterial activity of *N*-halamines are comparable to other agents based on metal ions, metal oxides, and cationic compounds. The MIC and MBC was reported to range from 1 to 200 μg/mL against *E. coli* and *S. aureus* (Dong and others 2017). Usually, for *N*-halamines, more than 6-log CFU bacterial kill can be achieved within seconds or minutes. For example, hydantoinyl acrylamide based copolymers inactivated 7 logs of bacteria within 10 seconds of contact time with coated silica particles that are packed into a column (Jiang and others 2016). In another study, hydantoinyl acrylamide based *N*-halamine polymer completely killed 6 logs of *S. aureus* and *E. coli* within two min of contact time as coatings on fabrics (Cerkez and others 2016). *N*-halamines have broad and strong antimicrobial activititives against bacteria, fungi, virus, spores, etc. Besides *S. aureus* and *E. coli*, *N*-halamines have been reported to against many other microorganisms that are of great importance to medical, household and foods (Table 1.1). *N*-halamines are also reported to be highly effective against multi-drug resistant species such as methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant enterococcus (VRE) and *E. coli* 29214 (Cao and Sun 2009; Chen and others 2007). All these characteristics combined make *N*-halamine have great potential as alternative agents to current antimicrobial agents.

Table 1.1 Antimicrobial function of *N*-halamines besides against *S. aureus* and *E. coli*.

Microorganisms	Species	References
Bacteria (Gram-positive)	*Staphylococcus epidermidis*, *Clostridium difficile, Streptococcus pyogenes*, *Bacillus subtilis*	(Worley and others 1983; Luo and others 2006)
Bacteria (Gram-negative)	*Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Klebsiella pneumoniae*, Shigella dysenteriae, *Shigella boydii*, *Salmonella enterica* (Serotypes: Chlorerasuis, Typhimurium, Gallinarum, Enteritidis), *Serratia marcescens*, *Proteus vulgaris*, *Sphaerotilus natans*, *Enterobacter cloacae*	(Worley and others 1983; Williams and others 1985; Lauten and others 1992)
Fungi	*Candida albicans*, *Candida tropicalis*	(Cao and Sun 2009; Chen and others 2007; Deng and others 2008)
Virus	*E. coli* bacteriophage MS-2	(Cao and Sun 2009; Chen and others 2007)
Spores	*Stachybotrys chartarum* spore, *Bacillus atrophaeus* spore	(Cao and Sun 2009; Chen and others 2007)

The biocidal mechanism of *N*-halamines is still not completely understood. Generally, there are three mechanisms proposed: contact killing, release killing and transfer killing (Figure 1.10). The contact kill mechanism is widely agreed as the main effect (Li and others 2012) and the release killing is also well accepted and observed (Hu and others 2014). However, the transfer killing is relatively less reported and accepted. The dissociation constant of imide, amide and amine based *N*-halamines are 10^{-4}, 10^{-9} and 10^{-12} respectively in water (Dong and others 2017) while sodium hypochlorite can be completely dissociated in water solvent. The free chlorines in the solution are much lower in *N*-halamines than current those in the chlorine based antimicrobial agents. Therefore, *N*-halamine based antimicrobial agents can minimize the adverse toxicological effects due to free chlorines found in current chlorine based antimicrobial

agents. *N*-halamines have been reported to inhibit the formation of biofilms on various materials because of potent and long-lasting antimicrobial function (Sun and others 2012a; Luo and others 2006). This biofilm prevention property is of great importance for food applications (Chmielewski and Frank 2003; Brooks and Flint 2008).

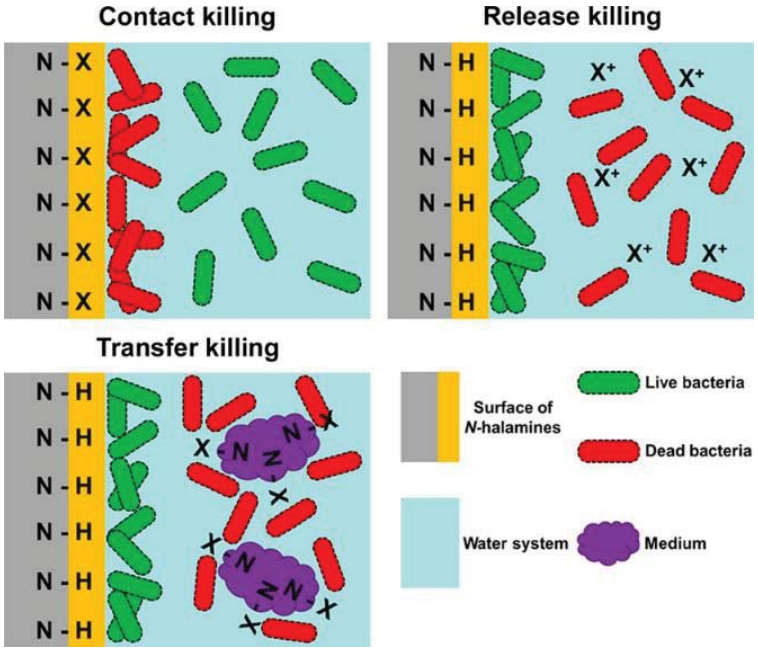

Figure 1.10 Antimicrobial mechanism of *N*-halamines. Reproduced with permission from reference of Dong and others (2017). Copyright 2017 American Chemical Society.

1.3.3 Surface modification methods

The other important advantage of *N*-halamines is that they have versatile application methods. In the past few years, many different surface modification methods have been exploited for *N*-halamine polymers; however, there are more promising methods that have not been explored. Here, we will provide some modification methods that have potential to apply in the food industry including compounding, grafting, painting and copolymerizing.

Bulk modification. Bulk modification methods such as compounding or in-situ copolymerization are usually simple and low-cost, thus they are widely used in the polymer and material industries. Compounding is a straightforward method for processing antimicrobial films or elastomers for surface applications. *N*-halamines can be manufactured as polymer additives or fillers adding into plastic materials such as polyurethane, polystyrene and polyethylene (Chen and Sun 2006; Sun and others 2010; Lin and others 2015; Qiao and others 2017a), or other materials, such as polyvinyl alcohol (Yin and others 2016), cellulose acetate (Liu and others 2017), etc. The advantage of compounding method is its excellent resistance to abrasion. Another method to modify the bulk structure is based on copolymerization. *N*-halamine modified monomers are used to substitute the monomers for block copolymers such as polyurethanes and hydrogels (Worley and others 2003; Hicyilmaz and others 2017). The advantage of this method is that functional groups of *N*-halamine are evenly distributed within the whole structure. However, this require more research for optimizing the formulation and the substitution of certain components that should not affect the bulk properties for application such as mechanical strength. These bulk modification methods are especially beneficial for food-contact surface applications including food conveyor belts or containers. These methods have more potential to be adapted for large-scale industrial production of antimicrobial coatings for the food industry applications due to robust and low-cost.

Painting. Painting is another low-cost method to produce antimicrobial coatings. Previously, *N*-halamines have already been studied for painting systems. One feasible method is to synthesize *N*-halamine copolymers first and to disperse into existed commercial painting systems (Kocer and others 2011; Cao and Sun 2009). *N*-halamines modified antimicrobial paints with reported to be developed from commercial paints through modifying the additives such as

TiO_2 pigments (Worley and others 2005). These *N*-halamine modified paints can be easily coated on various surfaces and will achieve potent antimicrobial activity after chlorine bleach treatment. They are suitable for protecting the surfaces of non-food contact equipment and facilities from microbial contamination. Therefore, this method is especially useful for facilities such as floors, walls, ceilings, sewage systems in both food processing and production environments. Some paints can also be applied to food-contact surfaces through mixing with food-grade paints. However, many commercial painting companies try to avoid the claim for frequently food-contact surface applications such as conveyor belt and working tables. This painting method is cheap but not as robust as bulk modification or surface grafting.

Grafting. Another commonly used method for fabricating surface with a thin-layer polymeric film is chemical grafting or finishing. Since the molecules are attached to the surface through covalent bonds, they will have better adhesive effects. But, this method is not resist to abrasion due to the fact that the coatings are usually very thin (10-1000 nm). Therefore, it is suitable to apply this method in the situation where less or no abrasion will be experienced such as inner side of pipes and tanks, machinery parts, containers, etc. Two basic methods are available to achieve this coating: "grafting to" and "grafting from" methods. Detailed explanation of these two methods can be found in elsewhere (Minko 2008; Bastarrachea and others 2015; Siedenbiedel and Tiller 2012). Briefly, for "grafting to" method, functional polymers are synthesized first and attached to the surface through chemical reaction; for "grafting from" method, initiators are tethered to the surface first and work as anchors for further polymerization from monomers in solutions. Each method has advantages and disadvantages; however, the "grafting to" method has been more explored for *N*-halamine coatings. Various reactive moieties such as epoxide, hydroxyl groups (diol), and alkoxy silane (siloxane) can be

added into *N*-halamine polymers (Figure 1.7) or polymerizable monomers (Figure 1.8) through copolymerization or substitution reactions. These molecules can be used for "grafting to" and/or "grafting from" attaching methods. The grafting methods are suitable for fabricating *N*-halamine coatings on materials with reactive groups (e.g. -OH, -COOH, -NH_2) in cellulose (Jiang and others 2017), polyurethanes (Sun and others 2012b), polylactic acids (Cerkez and others 2013), silica (Jiang and others 2016), and chitosan (Li and others 2013b). For inert surfaces, it can be activated through treatment using high energy electron beam, plasma, UV, ozone, etc. However, these treatments will increase the cost and are not suitable for fabricating large piece of equipment in the food industry. In addition, chemical grafting method usually requires the use of toxic agents or rigorous environment (e.g. oxygen free), which will create additional barrier for real applications in the food industry due to high cost and safety regulations. In recent years, non-toxic agents and mild reaction conditions are exploited for chemical finishing method to tackle these drawbacks. However, all these elaborate techniques are still constrained in the research stage and are not expected to be applied soon in the food industry.

Coating. In recent years, more simple, green and cost-effective methods are explored for surface coatings, such as layer-by-layer (LBL), dip/spray coating, self-assembled mono layer (SAM), particles with polymer, electroplating, etc. (Bastarrachea and others 2015). Many of these methods have already been reported for *N*-halamine research (Cerkez and others 2011; Liu and others 2015). Although dip coating is widely studied, spray coating methods are preferred for the food industry applications due to its high production efficiency (Denis-Rohr and others 2015). This method showed great promise for direct food-contact surface coating. Some other coating methods such as electroplating are suitable for metal materials including stainless steel.

1.3.4 Current research status and application areas

Currently, *N*-halamines are still in the research stage. To depict the whole picture of the status and trend in *N*-halamine research, we searched three databases: *Google Scholar*, *ScienceDirect* and *Web of Science*. "*N*-halamine" was used as keyword and the result was included in Figure 1.11. *Google Scholar* includes all results of the research in this area such as journals, books, patents, and/or meeting abstracts while the latter two include only peer-reviewed journals, books, reference works and/or meeting abstracts. Although these results may not reflect the accurate number to include all research in this area, the trend provide valuable information which clearly showed that the research of *N*-halamine increased rapidly in recent years, especially in recent five years. Most of these studies focused in basic research areas such as polymer/material science, chemistry, engineering. Research which is directly related to food application takes only 2-3% of all studies and appeared in the past 5 years. In summary, *N*-halamines is an emerging field of research and has great potential for the application in the food industry.

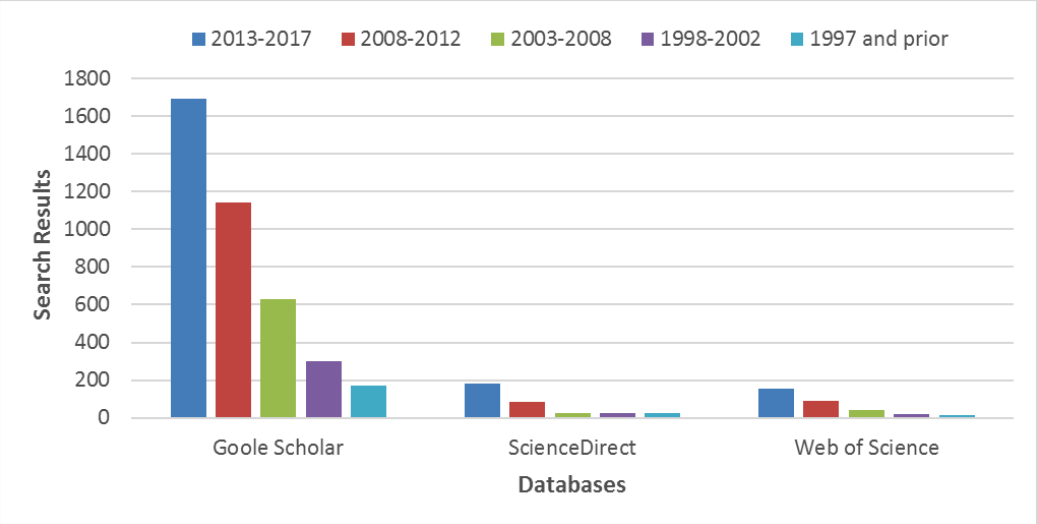

Figure 1.11. Current research status of *N*-halamines. "*N*-halamine" was used as Keyword in all search conducted. Accessed 06/13/2017.

N-halamines have been researched on various materials such as textiles (cellulose, non-woven), films, paints, and food equipment. As for the areas of application, biomedical, water treatment and textiles are the top three. Healthcare applications including textiles, films and equipment tubes have been intensively studied in recent years. At the early stage, *N*-halamine was extensively researched for water filtration, and has been commercialized as water cartridge or swimming pool treatment. A company produces water filtration and treatment based solely on *N*-halamine technology has been established (HaloSource Inc., Seattle, WA) and many patents were granted regarding *N*-halamine application. Recently the air filtration was also researched and showed high promise. More research is expected for food safety applications soon.

1.4 Potential applications of *N*-halamines for food safety control

Appropriate application of *N*-halamines should consider the application environments and properties of the chemicals and materials. For example, for food production environments, the surface should be robust enough to withstand heavy inorganic and organic load and severe abrasion or wearing; however, the cost should be kept low. In this situation, antimicrobial compounds spray or antimicrobial polymers embedded within the substrate material are preferred. In the processing environment, it is free of soil, but still have abrasion issue during sanitation and fouling issue due to organic loads from food components. However, the cost can be higher since the processing equipment is relative expensive. Therefore, elaborate technologies such as surface finishing can be considered as additional choice to increase the added value of these high-cost equipment or materials. Contamination of microorganisms can happen from facilities, equipment, utensils, environment, and humans. *N*-halamines can be found potential applications as preventive control methods across the food chain from farm to table: food production, processing, distribution and handling (Figure 1.12).

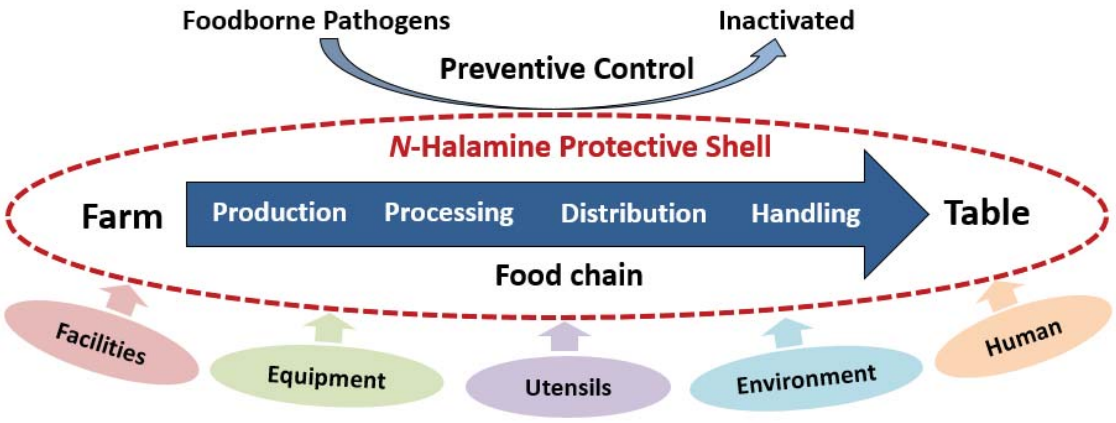

Figure 1.12 Potential applications of antimicrobial *N*-halamines for food safety controls.

1.4.1 Antimicrobial pesticides/sanitizers

N-halamine compounds can be used as sanitizers. Compared with chlorine based agents, the major advantages of *N*-halamine compounds are their improved stability and low toxicity. The most promising application is in the production stage of raw agricultural commodities and poultry. It can be used as spraying agents on utensils for produce or in other raw agricultural production environments where the container and picking machine can be major contamination sources. This will be a cost-effective way to control cross-contamination on the farm level. Another application is in chicken house, which *N*-halamine compound can be applied onto the construction materials to control the cross-contamination of major pathogens such as *Salmonella* and *Campylobacter* (Ren 2015). *N*-halamine compounds will not have inhalation or irritation hazards. Several *N*-halamine compounds with different activity and stability have been synthesized (Tsao and others 1991; Worley and others 1983) and part of them are shown in Figure 1.7. One of the most exploited *N*-halamine compound is MC (1-chloro-2,2,5,5, -tetramethyl-4-imidazoidinone) (Figure 6 molecule 2) due to high antimicrobial activity and stability, e.g. as a spray agent for non-food contact surfaces in the food processing plant. Some polymeric *N*-halamines can also be explored to apply as spraying agents on surfaces. Whether *N*-halamine sanitizing agents can be used on food-contact surface is ambiguous since it will leave residues on the surface without portable water rinsing. At this stage, it is more reasonable and safe to apply *N*-halamine sanitizing agents on non-food contact surfaces. *N*-halamines can find some niches for special applications. One interesting example is to apply antimicrobial agent for controlling *Salmonella* on shell eggs (Worley and others 1992).

1.4.2 Protective coatings for food equipment and facilities

One the promising area for applying *N*-halamine polymers is to manufacture protective coatings for food-contact and non-food contact surfaces in food production, processing and handling environments.

Food-contact equipment and utensils. Equipment and utensils are widely used in food processing and handling environment such as conveyor belts, working tables, containers, machinery parts, cutting knife, hooks, pipes, etc. Most of them have direct contact with food and considered as food-contact surfaces. They are usually the most important sources for microbial cross-contamination (Chmielewski and Frank 2003; Brooks and Flint 2008; Fraser and Pascall 2010). The most common materials for food equipment and utensils are plastics and stainless steels. Most conveyer belts are made of thermoplastic materials such as PP and TPU. However, modular plastic belt usually has the problem of insufficient sanitation such as the connecter niche. They are not only a burden for sanitation team, but also create potential hidden point for biofilm formation and cross-contamination (Scheffler 2009; Heide 2007). Therefore, in recent years, thermoplastic polyurethanes are getting more and more popular. However, TPU is not resistant to sanitizers such as bleach (Scheffler 2009). The other fact is that the scratch on plastic containers such us picking bin for raw agricultural commodities are also potential area for biofilm formation (Chaturongkasumrit and others 2011). Previous research showed that stainless steel is prone to be attached by microorganisms and form biofilms (Rosado de Castro and others 2017; Ryu and Beuchat 2005). However, unlike plastic materials, stainless steel is easy to be corroded by oxidative sanitizers such as bleach, peroxide, organic acids (Schmidt and others 2012). Only certain non-corrosive antimicrobial agents such as QACs can be used to treat

stainless steel. This will limit the selection of sanitizing agents and increase the cost. On the other hand, the use of oxidative sanitizing agents to other materials will also cause the potential corrosion issue for stainless steel.

Both plastic and stainless steel materials can be modified with *N*-halamines for antimicrobial coatings; however, the strategies may be different. The most economical way to modify plastic materials such as PP and TPU are compounding, which distributes *N*-halamine polymers into the whole structure. For TPU, three methods can be used for *N*-halamine coating: dip coating, copolymerization and compounding. It should be noticed that some TPU (polyester-based) is not resistant to chlorine bleach; therefore, low concentration of chlorine bleach should be used to routinely treat the surface or to develop more bleach resistant TPU. There is already some research of coating stainless steel with *N*-halamine polymers either through grafting or layer-by-layer. However, it should be noticed that repeated chlorine treatment may cause corrosion to stainless steel. One potential strategy to solve this problem is to coat the surface with anti-corrosion polymers such as conducting polymers through electrochemical deposition or painting. Other methods can be used for stainless steel coating are dip-coating and painting.

Non-food contact plant facilities. To achieve preventive control for food safety, the facilities such as floor, walls, and ceilings should be cleaned and sanitized periodically. From previous research, the contamination on non-food contact surface can be transmitted into food-contact surfaces such as condensed water on the ceilings (Barnes and others 1999; Bower and others 1996; Chmielewski and Frank 2003). Therefore, it is important to make these surfaces clean and sanitary. Painting is the most cost-effective way to reach this purpose. Paint can be epoxy or polyurethane based systems. Different polarity of *N*-halamine copolymers can be synthesized and mixed thoroughly with current food grade painting systems to produce

antimicrobial paints (Kocer and others 2011). The other method is to synthesize the monomer containing *N*-halamine precursor functional groups, for substituting the component of commercial painting systems (Worley and others 2003). Refrigeration equipment is another important cross-contamination source, especially for *Listeria monocytogenes*, which can survive and form biofilms in low temperature (Evans and others 2004). In general, the refrigeration equipment is not cleaned as often, which will create the opportunity for biofilm formation. Usually, the antimicrobial function of *N*-halamines can last for at least 6 months (Worley and others 2003).

Water and air control. Water is one of the most important ingredients and processing aiding agents for food production. *N*-halamines can be used in water filtration units for treating and recycling water. Previously, *N*-halamines have been intensively studied for water filtration such as in columns, beads, and nanotubes. *N*-halamine based water filtration products are available in the market. The air quality is another important issue in food processing plant especially for ready-to-eat (RTE) foods. Air plays an important role in microbial dissemination (Kornacki 2014; Autio and others 1999). Making the air filtration parts with *N*-halamine functional groups will enable the filter to kill microorganisms during air circulation. Currently, one of the most important factor to prevent the wide application of air filtration units is the cost since expensive filters need to be changed frequently. *N*-halamine treated filters can be recharged periodically and reused thus the cost will be much lowered.

1.4.3 Personal protective equipment

Human is another important contamination source for foodborne pathogens (Todd and others 2008). More importantly, pathogens carried by human have higher risk to infect human, thus the consequences can be even more serious. Good personal hygiene and sanitation can mitigate this problem, but it is challenge to get all workers done in a right way. This is one of the overlooked parts, which can also be improved quickly. This may happen in production, processing, retailing and handling. On the farm level, the workers' hands are the contamination source for fresh produce and fruit production due to poor access to toilet or sanitation facilities. For example, in 2016, the multi-state Hepatitis A Virus (HAV) outbreak in frozen strawberry in U.S. was traced back to the HVA infected field picker in a Egyptian farm (Bill 2017). This also happens in the food processing plant, especially for fresh-cut or ready-to-eat products that require intensive labors. Although Sanitation Standard Operating Procedure (SSOP) requires the good handwashing, in reality it is difficult to get fully enforced and will impose contamination risk to the processed products. Therefore, secondary preventive measures should be used to reduce microbial contamination. One of the cost-effective methods is to coat the person protective equipment (PPE) such as gloves, working clothes, facial masks and boots with antimicrobial compounds (Todd and others 2008). For antimicrobial textiles, there are a large amount of research have been done. *N*-halamine treated clothes will exhibit antimicrobial function. This is also important to food service industry, especially some fast food stores involved preparing ready-to-eat foods for consumers using hands. The employees usually touch non-food contact and food-contact surface areas, and they are the potential risks for cross-contamination of pathogens. Therefore, *N*-halamines modified PPE can be applied in various stages throughout food chain.

1.4.4 Kitchen equipment and utensils

Food handling at kitchen is the last one mile of thousand miles of the food safety control marathon throughout food chain; however, this step also has many risks. Kitchen equipment and utensils in domestic or commercial food service kitchens have been reported to be important sources for microbial cross-contamination. Cutting boards, utensils, cleaning clothes, sponges, and refrigerators have been reported to be the sources of various foodborne pathogens (Gibson and others 2012). Although in recent years, a lot of training have been offered to consumers for good handling practices for food safety controls, the outcome is poor. Therefore, secondary strategies for preventing microbial cross-contamination should be applied in the kitchen to ensure food safety. Like the PPE mentioned above, kitchen utensils made of plastics, woods and stainless steels can be modified with *N*-halamine functional groups. The consumers only need to periodically treat the surfaces with diluted chlorine bleach to maintain antimicrobial function.

1.4.5 Food packaging

The research of *N*-halamine for food packaging is not clear at this stage due to the strict regulation of food packaging materials. However, it still can find some applications in special situations. One example is the antimicrobial food absorbent pads in packaging. Since the absorbent core in the pad is non-food contact and the water flow is one way, *N*-halmine monomers such as MC can be incorporated into the core of the absorbent pad as shown in Figure 1.13. Similarly, *N*-halamine modified hydrogel copolymers also have potential application in the

absorbent pad (Hicyilmaz and others 2017). However, the application of natural compounds or polymers for antimicrobial packaging is the main trend. It will be more feasible to modify biopolymers with *N*-halamine functional groups. For plastic films, natural polymers such as chitosan (Li and others 2013b) or polylactic acid (PLA) (Cao and Sun 2008) can achieve strong antimicrobial functional after being modified with cyclic hydantoin moieties and halogenated.

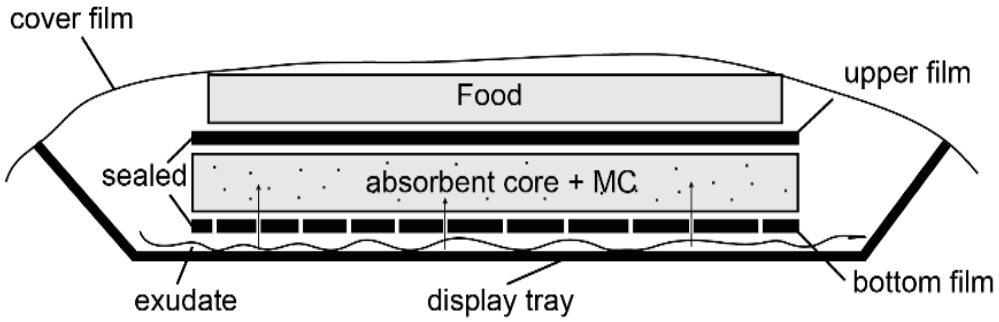

Figure 1.13 Illustration of *N*-halamine modified food absorbent pad for food packaging.

1.5 Challenges

Although there are many potential opportunities, for the wide application of *N*-halamines in the food industry, several challenges are existed to overcome. The biggest obstacle will be regulation. For any novel chemicals or materials that are proposed for food applications, the cost, benefits, potential risks on human health, and environmental impact should be thoroughly considered and well balanced to minimize risks. Pesticides are regulated by various state, federal and international agencies. In the US, pesticides are regulated by EPA, FDA and USDA. Usually, chemicals used in food-contact surfaces have stricter regulations than those used in non-

food contact surfaces. The sanitizers applied directly to food-contact surfaces need to be approved from FDA and USDA, and usually, no residues should be retained on the surface. Food-contact surface applications are regulated by FDA under 21 CFR Part 175 Subpart C. For non-food contact surface applications, the regulation is less strict, and it focuses on the occupational safety and cost. Although *N*-halamines are claimed to be low-toxicity, few information of toxicity is available. More research should be conducted to investigate the toxicity in animal models prior to applications.

The development of new chemicals for the food industry use is quite long process, because there are some requirements to limit the choices of antimicrobial chemicals. The foremost factor is safety and the cost is another important issue, since food industry has relatively low profit margin. Other factors such as effectiveness, many of new technologies recently developed that work well in other industry do not work well in food systems. Comparing with pharmaceutical or biomedical areas, the investment of research in foods is relatively low. Therefore, more research about applying chemistry and advanced materials for the food industry should be explored and funded, eventually the benefit will compensate the cost.

1.6 Conclusions

Food safety is usually complicated and one technology cannot solve all problems. The right strategy for applying "hurdle" concept is to select a suitable technology based on the specific requirements of the application. More research on the *N*-halamine chemistry and engineering technologies will help us make better use of the nature against the threaten from

foodborne illnesses to human health. This concept of disease prevention rather than treatment caused by foodborne pathogens will be more and more widely accepted. Through integrating different technologies, we can reduce outbreaks and withdraw unnecessary chemicals use in a low-cost and environmental-friendly way. *N*-halamines are able to play an important role at several stages throughout the food chain to control foodborne pathogens from the standpoint of prevention. Therefore, it is of great importance to include *N*-halamine in the toolbox for food safety controls. More research should be done in this area; however, researchers should always be prepared to answer two questions: Do they really work? And are they necessary?

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Chapter 2

N-Halamine Modified Thermoplastic Polyurethane with Rechargeable Antimicrobial Function for Food-Contact Surface

Abstract: A polymer blend of two *N*-halamine precursors was prepared and homogeneously incorporated into TPU structure *via* a solvent casting method, and an *N*-halamine modified TPU film with rechargeable antimicrobial activity was resulted after treating with chlorine bleach. Antimicrobial efficacies were evaluated against both *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* O157:H7 (*E. coli*). Results showed that the *N*-halamine modified TPU film caused 6 log CFU reduction of bacteria reduction within 2 hours of contact. Moreover, the *N*-halamine modified TPU displayed desirable rechargeability and stability, which maintained sufficient antimicrobial activity after 20 cycles of "discharge-recharge" process and over 4 weeks of storage. Besides, tensile strength and surface tension of TPU were not adversely affected by *N*-halamine modification. The *N*-halamine modified TPU with rechargeable antimicrobial function exhibited great potential as cheap, safe and effective food contact surface materials for preventing food microbial cross-contamination.

Keywords: *N*-halamines; polyurethane; rechargeable antimicrobial; food-contact surface

2.1 Introduction

Centers for Disease Control and Prevention (CDC) estimated that about 48 million people get sick, 128,000 are hospitalized and 3,000 die of foodborne diseases annually in the United States (Elaine and others 2011). The majority of these foodborne illnesses were caused by consuming foods contaminated with harmful microorganisms such as fungi, bacteria, virus, etc. (CDC 2010). Several outbreaks can be traced back to the microbiological cross-contamination from food contact surfaces during food preparation or processing (Jackson and others 2011). Therefore, in the food industry, cross-contamination was controlled and managed predominately in many hazard analysis-based systems. Microorganisms can attach and survive on food contact surfaces due to either poor design of equipment or insufficient sanitation. Even worse, these pathogens can accumulate on the surfaces and form biofilms, which make it even more difficult to be removed by established sanitation practices (Chmielewski and Frank 2003). Therefore, making the contact surface materials with antimicrobial activity is of great importance to reduce microbial cross-contamination and so as to ensure food safety.

Currently, one of the most popular methods to produce antimicrobial food contact surfaces is through compounding small molecular antimicrobial additives such as triclosan and metal ions (copper, silver, etc.) into the carrier materials (Chung and others 2003; Lee and others 2011). However, since their antimicrobial effectiveness depend on migration of antimicrobial agents to the contact surface, there are limitations of this method, such as insufficient effectiveness, diminishing of antimicrobial agents with time, higher cost, safety concern, etc. Another potential method is through grafting antimicrobial molecules onto surface of carrier materials (Bastarrachea and others 2015). However, this method is not suitable for food contact

materials used on the surface of processing equipment. Because the heavy abrasion during food processing and sanitation practices will damage or even remove the grafted functional molecules, resulting in the removal of the antimicrobial function layer. Until now, there are very few antimicrobial surface materials that are suitable for food processing applications in the markets. Therefore, there is a continuing need to introduce novel antimicrobial technology to the food industry for solving microbial cross-contamination problems.

N-halamine, as an emerging antimicrobial technology, has attracted a great deal of interest in recent years due to their superior antimicrobial efficacy against a broad spectrum of microorganisms, non-toxicity to human, good stability and low cost (Bastarrachea and others 2015; Kenawy and others 2007; Luo and others 2011). *N*-halamines are a group of compounds containing one or more nitrogen-halogen covalent bonds (N-Cl) that are normally formed by the halogenation of imide, amide or amine groups; and the antimicrobial activity of *N*-halamines was due to the oxidation state of halide atoms in chloramine or bromamine groups (Demir and others 2015). One important feature makes *N*-halamines different from any other biocides is that their antimicrobial activity can be recovered after consuming through simply treating with chlorine bleach, which can be perfectly incorporated into current sanitation practices established in the food industry (Kocer and others 2011b). All these features make *N*-halamines an attractive antimicrobial technology for food contact surface materials. A recent review by Bastarrachea and others (2015) also highlighted the great potential of applying *N*-halamine polymers on equipment coating in food industry. Previously, intensive research focused on the application of *N*-halamine technology in textiles (Lin and others 2016; Cheng and others 2015), biomedical materials (Ren and others 2009; Demir and others 2015), paints (Kocer and others 2011a) and water treatment materials (Jiang and others 2016; Worley and others 1988) by collaborators

within our group. However, applications of these *N*-halamine compounds for direct food contact surfaces have seldom been explored.

Food-contact surfaces can be made of various materials such as modular plastics, metals, fabrics and polyurethanes based on specific applications. In recent years, polyurethanes are attracting more and more interest in food processing industry especially for food conveyor belting (Chaitiemwong and others 2010). This is mainly because polyurethane belts are easy to clean, which can reduce both water consumption and sanitation cost. Therefore, TPU was selected as a representative material for food contact surfaces in this research. Previously, Julie M. Goddard's group worked intensively on applying *N*-halamines to food contact surfaces; however, they mainly focused on grafting method and on some other materials including polypropylene (PP) (Denis-Rohr and others 2015; Bastarrachea and Goddard 2015), polyethylene (PE) (Bastarrachea and others 2013), stainless steel (Bastarrachea and Goddard 2013), etc. Although Yuyu Sun's team worked intensively on polyurethane, they also focused on grafting method and their research targeted to biomedical applications (Liu and others 2013; Luo and others 2011). Our group focused on synthesizing new *N*-halamine compounds and many new *N*-halamine polymers have been successfully developed and tested in the past few years.(Cerkez and others 2012; Kocer and others 2011c; Ren and others 2010; Ren and others 2009; Liu and others 2015; Yin and others 2016; Fan and others 2016; Li and others 2015; Ma and others 2013) This allows us to select the suitable *N*-halamine polymers for food contact surface applications based on efficiency, safety and cost.

Recently, Liu and others (2015) from our group synthesized two novel *N*-halamine polyelectrolytes that have shown strong antimicrobial efficacy. Compared with previous non-electrolyte *N*-halamine precursor polymers, these two *N*-halamine polyelectrolytes were

synthesized from low-cost acyclic monomers that were already commercially available in the market and the procedure was simple and clean. This will help to lower the cost and make it easier to pass future safety examinations for food-contact surface applications. Also, the combined polymer was found to be not dissolved in either water or many organic solvents, which means it is not easy to leak out after long time use in food processing environment containing both water and lipids. Moreover, compared with hydrophilic antimicrobial compounds, this polymer blend will also not have expected to lower the surface energy too much after compounding into hydrophobic carrier materials. All these properties are quite attractive for food contact surface applications. Therefore, we explored a new approach to apply these two new N-halamine precursor polymers in TPU elastomer aimed for food contact surface applications, and we addressed the rechargeable antimicrobial function of the new *N*-halamine modified TPU elastomer. This research is the first time to investigate the potential of *N*-halamine modified TPU as an effective antimicrobial surface material and highlighted the advantage of *N*-halamines to provide rechargeable antimicrobial function.

2.2 Materials and methods

2.2.1 Materials and instrumentation

Cationic monomer, (3-acrylamidopropyl) trimethylammonium chloride (APTMAC, 74-76 wt. % in H_2O), was purchased from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). Anionic monomer, 2-acrylamido-2-methylpropane sulfonic acid sodium salt solution (AMPSS,

50 wt. % in H_2O), was purchased from Sigma-Aldrich (St. Louis, MO). Sodium persulfate ($Na_2O_8S_2$) was purchased from Acros Organics (Fair Lawn, NJ). Sodium thiosulfate ($Na_2O_3S_2$) was purchased from Alfa Aesar (Heysham, LA3 2XY, England). Potassium iodide (KI) and isopropyl alcohol were purchased from Fisher Scientific (Fair Lawn, NJ). Tetrahydrofuran (THF) was purchased from EMD Millipore Corporation (Billerica, MA). Chlorine bleach was purchased from Walmart (Great Value™, Sodium hypochlorite: 8.25%). Polyether-based thermoplastic polyurethane pellets (TPU, IROGRAN® A 85 P 4394) was kindly supplied by Huntsman Corporation USA (Derry, NH). Nuclear Magnetic Resonance (NMR) spectra were collected using an AVANCE III 400 MHz Digital NMR spectrometer (Bruker AXS GmbH, Karlsruhe, Germany). NEXUS 470 spectrometer (Nicolet Instrument Corporation, Madison, WI) with an attenuated total reflectance (ATR) accessory was used to collect Fourier Transform Infrared (FTIR) data.

2.2.2 Preparation of *N*-halamine precursor polymer modified TPU

A cationic polyelectrolyte, poly[(3-acrylamidopropyl) trimethylammonium chloride] (pAPTMAC) and an anionic polyelectrolyte, poly (2-acrylamido-2- methylpropane sulfonic acid sodium salt) (pAMPSS), were synthesized by free radical polymerization method following the protocol of Liu *et al*.(Liu and others 2015) Briefly, for cationic polyelectrolyte (pAPTMAC), 27.56 g (0.1 mol) of (3-acrylamidopropyl)trimethylammonium chloride, 0.1 g of $Na_2O_8S_2$ and 300 mL of DI water were stirred at 65 °C for 5 h under the protection of nitrogen gas in a one liter three-neck flask. A yellowish cationic homopolymer with a yield of 95% was resulted after separation, washing with isopropyl alcohol and drying in the vacuum overnight. For anionic

polyelectrolyte (pAMPSS), 20.72 g (0.1 mol) of 2-acrylamido-2-methylpropane sulfonic acid sodium salt solution, 0.1 g of $Na_2O_8S_2$ and 300 mL of DI water were reacted under the same polymerization conditions as mentioned above. A white homopolymer with a yield of 90% was obtained after separation, washing with 95% ethanol and drying in the vacuum overnight. The structures of two polymer products were confirmed by NMR characterization as indicated in the same literature(Liu and others 2015) and were shown in Figure 2.1. The cationic polymer (pAPTMAC) has $M_\eta = 6.6\times10^4$ Dalton and the anionic polymer (pAMPSS) has $M_\eta = 7.4\times10^4$ Dalton. Then these two *N*-halamine precursor polymers were dissolved into deionized water to final concentration at 200 mg/mL, and equal volume of these two polymer solutions were mixed together with stirring. A new precipitate polymer blend was obtained and recorded as N-halamine polymer precursor (NPH) in this study, and the blending mechanism was shown in Figure 2. After that, this new polymer was dried completely in oven at 80 °C for about 6 h and grinded into fine powder (diameter ≤ 53 μm).

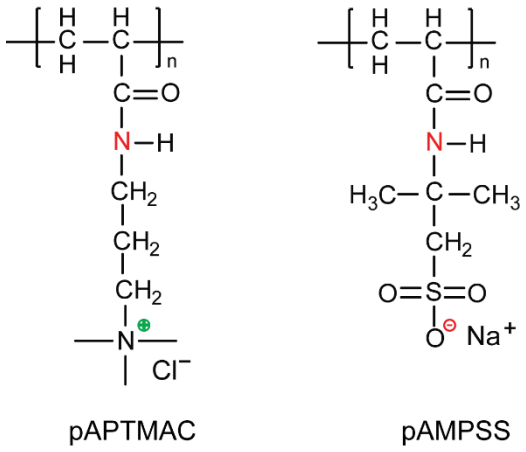

Figure 2.1 Structures of synthesized cationic (pAPTMAC) and anionic (pAMPSS) polyelectrolytes.

An *N*-halamine precursor polymer modified TPU film was prepared via a solution casting method following the protocol of Luo and others (2011) with modification (Figure 2.2). Briefly, 4.5 g of pre-dried polyether-based TPU pellets were dissolved in 45 mL of THF at room temperature and stirred until homogeneous. Then pre-weighed polymer particles were added into the TPU THF solution with vigorous stirring to disperse the particles homogeneously. The solution was vacuumed to remove air bubbles, quickly poured into a glass mold (size: 6×12×0.5") and dried in a fume hood overnight. The film was uniform with a thickness around 0.30 mm and further cured in a vacuum oven at 45 °C for 24 h to remove any residual solvent. The obtained *N*-halamine precursor polymer modified TPU was recorded as TPU-NPH. Three different content of *N*-halamine precursor polymer modified TPU were prepared in this study (1%, 2% and 4%). TPU control film was prepared following the similar procedures except that no polymer particles were added into the TPU solution. The presence of *N*-halamine precursor polymers in the modified TPU was confirmed with FT-IR.

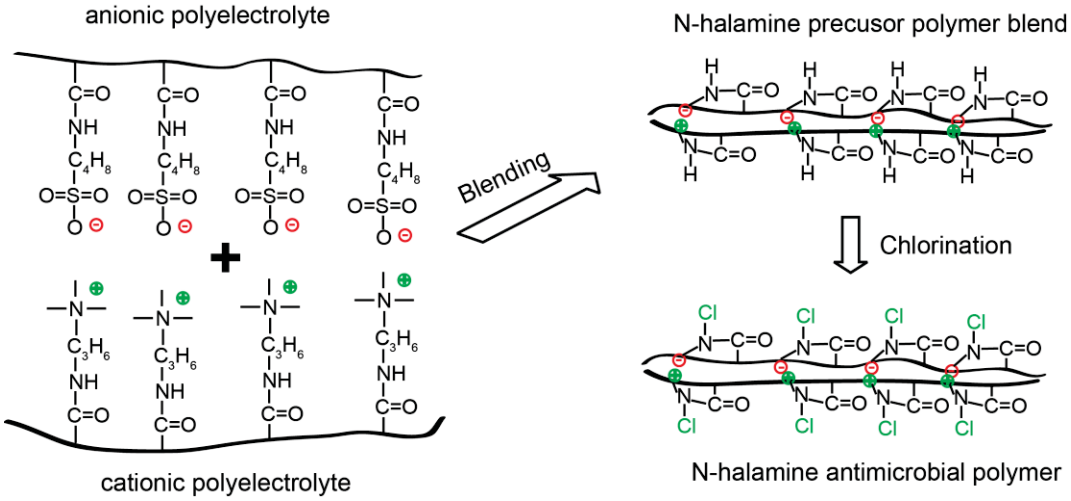

Figure 2.2 Preparation of *N*-halamine precursor polymer blend and formation of *N*-halamine structure through chlorination.

2.2.3 Chlorination and titration

The *N*-halamine precursor polymer modified TPU films (TPU-NPH) with different polymer contents (1%, 2% and 4%) were chlorinated by soaking in a 5% commercial aqueous bleach solution (1058±102 ppm of available chlorine, pH=7.0/HCl) for 1 h. After chlorination, the films were washed thoroughly with deionized water and dried in a 45 °C vacuum oven overnight to remove any free chlorine residuals. The chlorinated film containing N-halamine functional group (N-Cl) was recorded as TPU-NPCl. The original unmodified TPU film was treated with the same procedures to serve as chlorination treatment control and recorded as TPU-Cl. The presence of oxidative chlorines was confirmed with iodometric/thiosulfate titration as described in the following procedure. The chlorination conditions at 1% and 0.2% bleach, and treatment time of 1 h and 5 h were also investigated, respectively.

The oxidative chlorine contents of these TPU films were determined using an iodometric/thiosulfate titration method (Liu and others 2015). Briefly, chlorinated TPU films were cut into the size of one square inch pieces (6.45 cm^2). One piece of each film was put into a flask containing 20 mL of water, 1 mL of 0.1 N of acetic acid, and 0.25 g of KI, and stirred at room temperature for 1 h to form I_2. Then 0.5% starch solution was added into the sample and titrated by 0.001 N of sodium thiosulfate aqueous solution from blue to colorless. The active chlorine contents in the chlorinated films were calculated using the following formula: $[Cl^+]$ ($\mu g/cm^2$) = $(N \times V \times 35.5)/2A$, where N and V are the normality ($equiv \cdot L^{-1}$) and volume (L) of the titrant sodium thiosulfate and A is the total surface area of the chlorinated samples (cm^2).

2.2.4. Antimicrobial efficacy test

The antimicrobial efficacy of *N*-halamine modified films was performed using a "sandwich" test method as described previously (Liu and others 2015). A gram negative *E. coli* O157:H7 (ATCC 43895) and a gram positive *S. aureus* (ATCC 6538) were used as challenge bacteria in this study. Briefly, a single colony of each strain was transferred into 15 mL of trypticase soy broth (TSB, BD Co., MD) and incubated at 37 °C, 120 rpm for 16 h. The culture was washed twice with Butterfield's phosphate buffer (BPB) through centrifugation and re-suspended in the BPB buffer. Bacterial population was estimated by the spectrometer at $O.D._{640\ nm}$ and the inoculum with appropriated population was prepared. Then, an aliquot of 25 μL of the inoculum was added into the center of a square film, and a second identical film was placed on the top. A sterile weight was placed on the top to ensure complete contact with inoculated bacteria. At the contact time of 30 min, 1 h, and 2 h, the films were put into with $Na_2O_3S_2$ solution (0.05 N) and vortexed to remove any oxidative chlorine residuals. Ten-fold serial dilutions were made for all samples and each dilution was plated on Trypticase agar (TSA) plates. The plates were incubated at 37 °C for 24 h and bacterial colonies were enumerated and recorded for antimicrobial efficacy analysis. All experiments were performed three times. Antimicrobial efficacy test was performed on unchlorinated (TPU) and chlorinated control films (TPU-Cl), unchlorinated (TPU-4%NPH) and chlorinated N-halamine precursor polymers modified TPU (TPU-1%NPCl, TPU-2%NPCl and TPU-4%NPCl).

2.2.5 Rechargeability and stability test

Based on the results from antimicrobial efficacy test, the 4% *N*-halamine modified TPU film (TPU-4%NPCl) was selected for the rechargeability test. To simulate the real application, the chlorinated film (TPU-4%NPCl) was immersed into 0.1 N of $Na_2O_3S_2$ solution for 1 h to remove all the oxidative chlorines in the N-Cl structure of the film. This process was named "discharge". Then some of these films were washed thoroughly with DI water and chlorinated with 5% bleach for 1 h to form the N-Cl structure. This process was named "recharge" and the film was recorded as R1. These "discharge-recharge" procedure was repeated for 5 cycles, 10 cycles and 20 cycles and they were recorded as R5, R10, and R20, respectively. The rechargeability were evaluated by both antimicrobial efficacy and total available chlorine content.

The TPU-4%NPCl films were selected for stability test under three different conditions of fluorescent light, dark, and water. For each condition, the TPU-4%NPCl films were cut into the size of one square inches (6.45 cm^2) and stored in dark, under florescent light, or immersed in 100 mL of deionized water, respectively. For each condition, triplicate films were taken out and titrated for oxidative chlorine content at day 1, 3, 7, 14, 21 and 28. The chlorine content of immersion water was also titrated at each day. At day 28, all films were chlorinated with 5% bleach for 1 h, washed, and titrated to determine the oxidative chlorine content recovered from each group.

2.2.6 Physical properties test

The physical properties considered in this study are those related to the film performance and durability during use. On the other hand, to evaluate the deterioration of the film due to the additives and charging/recharging processing. These properties are strength and contact angle. Tensile strength was determined using an Instron® Universal Testing Machine Model 5565 (Instron®, Norwood, MA) at standard conditions of temperature and relative humidity of 21°C ± 2°C and 65% ± 5%, respectively. Rectangular specimens (76.2 mm × 25.4 mm) were stretched until breaking at a gauge length of 20 mm and a crosshead speed of 100 $mm \cdot min^{-1}$. Tensile strength as the maximum load divided by the specimen's cross-sectional area was recorded in MPa. Nine independent measurements were performed for each type of samples.

The contact angle was measured using a ramé-hart Standard Contact Angle Goniometer Model 200 (ramé-hart, Inc. Mountain Lakes, NJ). This system consists of a microscope, camera, x-y-z moving stage, dropping mechanism and a computer equipped with DROPimage software. The water contact angle values were measured using the sessile drop method. The measurement error of this system is specified as ±2°. Three independent measurements were performed for each type of samples. Contact angle is a measure of the hydrophobicity of the film.

2.3 Results and discussion

The synthesized *N*-halamine polymer precursors and their modified TPU elastomer were confirmed with optical observation and FT-IR. Compared with control TPU film, small particles were observed homogeneously dispersed in the TPU-4%NPH TPU film structure. FT-IR spectra of N-halamine polymer precursors (NP), unmodified TPU control film (TPU) and N-halamine

precursor polymer modified TPU (TPU-4%NPH) were collected and included in the supporting information (Figure S2.1 and S2.2). All these results confirmed that the *N*-halamine precursor polymer blend was successfully incorporated into the TPU film. It should be highlighted that this N-halamine precursor polymer blend was not dissolved in either water or any tested organic solvents (THF, phenol, DMSO, petroleum ether, acetonitrile, benzene), which is ideal for food-contact surface applications. In addition, water was the only solvent used through the whole procedure for preparing the polymer blend and sodium persulfate as water soluble catalyst was easily to be removed after synthesis procedure. This process is clean and simple, which means good potential for scale-up industrial production and fulfills the trend of green chemistry.

The TPU film modified with different contents of *N*-halamine polymers and under different chlorination conditions were shown in Figure 2.3(a). The control TPU film was determined to have 0.03 μg/cm^2 oxidative chlorines on the surface. This is because polyurethane also has amine group (N-H), which can also be halogenated into *N*-halamine (N-Cl). However, usually the chlorine content in halogenated polyurethane was very low and not stable, which is in good agreement with the observation from Luo and others (2011). After modified with 4% *N*-halamine polymers, the oxidative chlorine content increased dramatically to 2.7 μg/cm^2. According to previous research, this concentration of oxidative chlorine was enough to make the surface with potent antimicrobial activities (Luo and others 2015; Liu and others 2015).

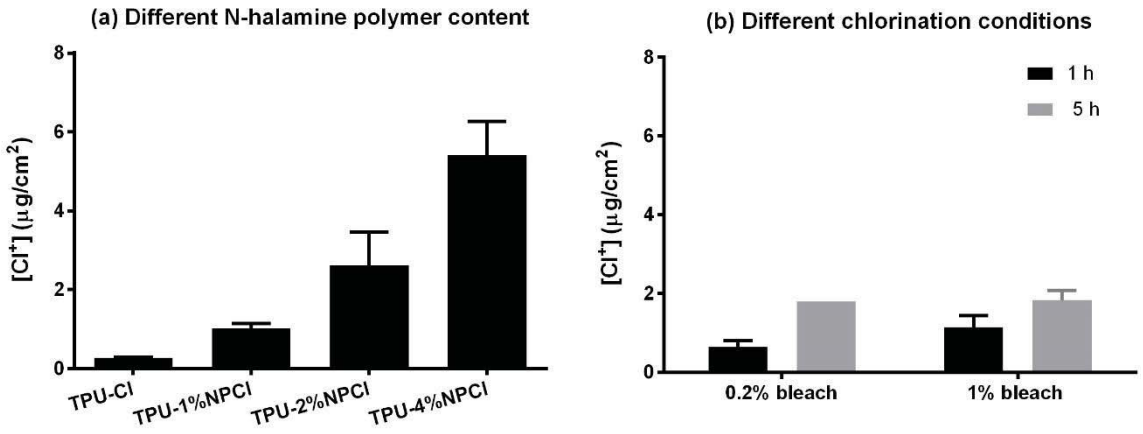

Figure 2.3 Titration of oxidative chlorine content: (a) TPU films with different *N*-halamine polymer content (0%, 1%, 2% and 4%) treated with 5% bleach (pH=7.0, 1058±102 ppm of available chorine); (b) films with 4% *N*-halamine polymer content (TPU-4%NP) chlorinated with different concentrations of bleach (1% and 0.2% bleach) and treatment time (1 h and 5 h).

In this research, however, TPU film was observed not resistant to high concentration of bleach, which damage could be observed in the TPU structure after 20 rechlorinating cycles (5% bleach for 1 h of each rechlorinating cycle). Therefore, the film was also chlorinated using lower concentrations of bleach (1% and 0.2%). The oxidation chlorine contents were titrated at 278 ± 42 ppm [Cl^+] (1% bleach) and 53 ± 0 ppm [Cl^+] (0.2% bleach), respectively. As shown in Figure 3(b), TPU-4%NPH could achieve enough oxidative chlorines (0.643 μg/cm^2) on the surface even chlorinated with as low as 0.2% bleach (53 ± 0 ppm [Cl^+]) for 1 h. After the time extended to 5 h, the oxidative chlorine content could reach at 1.79 μg/cm^2. These two bleach concentrations (1% and 0.2%) were addressed in this research because they were of great importance to the food industry. According to federal regulations (21 CFR Part 178), the 200 ppm of available chlorine is the maximum concentration that can be used for sanitizing food contact surfaces without further rinsing step. In the real application environment, even lower concentration, usually between 20

and 50 ppm, was preferred by industry because it can minimize the potential corrosive effects of bleach to contact materials including TPU and stainless steels (Moayed and Golestanipour 2005). Based on the results, this *N*-halamine modified TPU film could be chlorinated to achieve enough antimicrobial activity even at very low concentration of bleach (0.2%, 50 ppm of [Cl^+]) for using in food processing plant.

Antimicrobial efficacies of the *N*-halamine modified TPU were shown in Figure 2.4. As shown, unchlorinated control film and unchlorinated TPU-4%NPH film did not display antimicrobial activity within 2 h of contact for either *E. coli* O157:H7 (Gram-) or *S. aureus* (Gram+). Although TPU control film showed slight increase of antimicrobial reduction after 2 h contact time for both bacteria, the efficacy was not satisfactory. For *N*-halamine modified TPU films, a dose-response phenomenon could be observed between NP content and antimicrobial efficacy in both of tested bacteria: higher NP content resulted in better antimicrobial efficacy. When the NP content increased to 4%, it had a total kill of the inoculum (about 10^6 CFU/6.45 cm^2) for both bacteria within 2 h of contact.

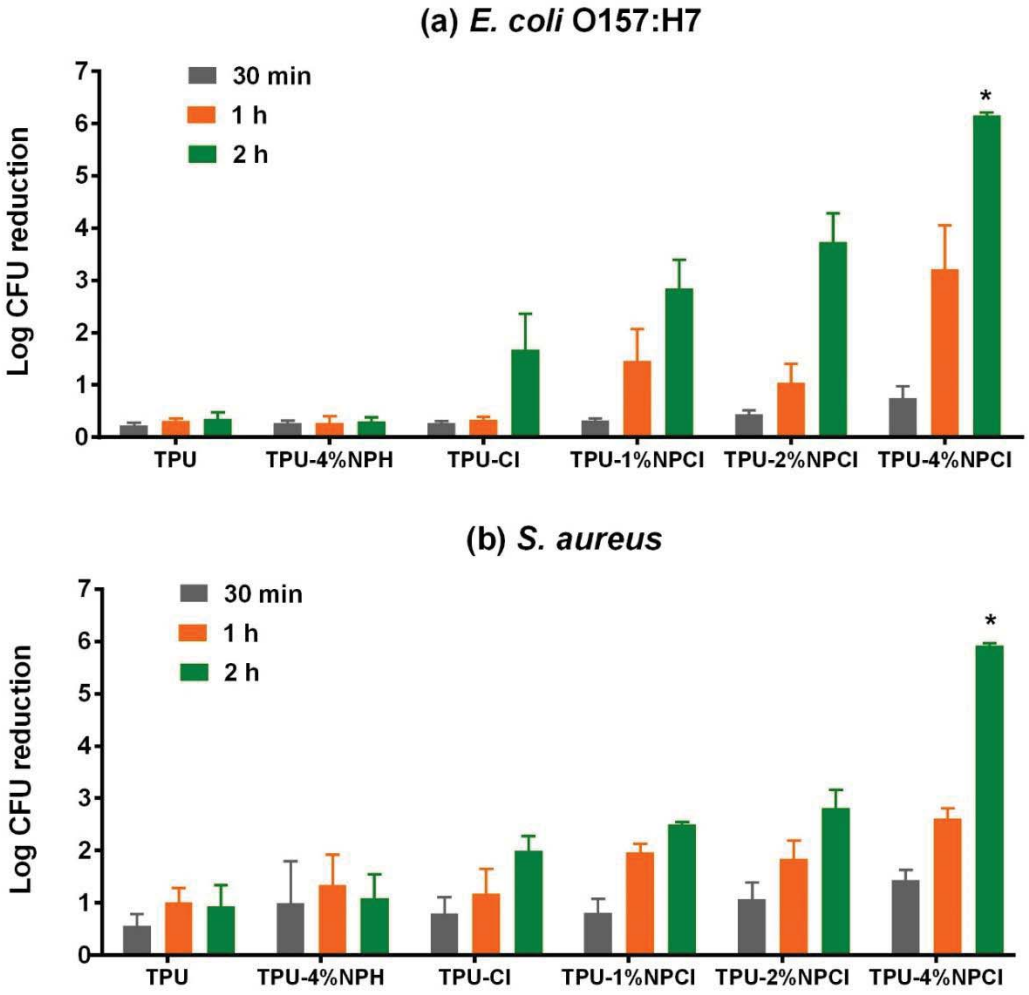

Figure 2.4 Antimicrobial efficacy of *N*-halamine modified TPU film against (a) *E. coli* O157:H7 (inoculum: 6.15 ± 0.06 log CFU/sample) and (b) *S. aureus* (inoculum: 5.84 ± 0.13 log CFU/sample). * indicates a total kill of all inoculated bacteria.

These results are in good accordance with previous research which *N*-halamines usually have very potent antimicrobial efficacy for killing of all inoculated bacteria (10^6-10^8 CFU/6.45 cm^2) within 30 min or even shorter (Jiang and others 2014; Liu and others 2013; Cerkez and others 2013; Liu and others 2015; Hui and Debiemme-Chouvy 2013). However, TPU was very

hydrophobic, which would prevent the sufficient contact of all bacteria with antimicrobial functional groups on the surface within a short period of time. Therefore, it needs longer time to have a 100% kill. For an ideal antimicrobial surface material, both high surface tension (hydrophobic) and strong biocidal activity are desired. In this way, the overall ability to prevent microbial contamination will be optimized. This *N*-halamine modified TPU film has both hydrophobic and potent biocidal activity.

The rechargeability was investigated using both titration and antimicrobial activity testing methods. As shown in Figure 2.5, *N*-halamine modified TPU could still have a total kill of 6 log CFU of inoculated *E. coli* O157:H7 within 2 h of contact after 20 cycles of "discharge-recharge" process. While the quenched film did not have any antimicrobial activity, which confirmed that the antimicrobial activity of *N*-halamine modified TPU film was due to the *N*-halamine functional groups (N-Cl). This rechargeability was also confirmed with chlorine content titration. After 20 cycles of "discharge-recharge" process, only a slight decrease of oxidative chlorine content was observed. The results indicated that this *N*-halamine modified TPU film has desirable rechargeability, which will create great potential for food-contact surface applications. In this way, the contact surface will always keep high antimicrobial activity after treating with diluted chlorine bleach during sanitation.

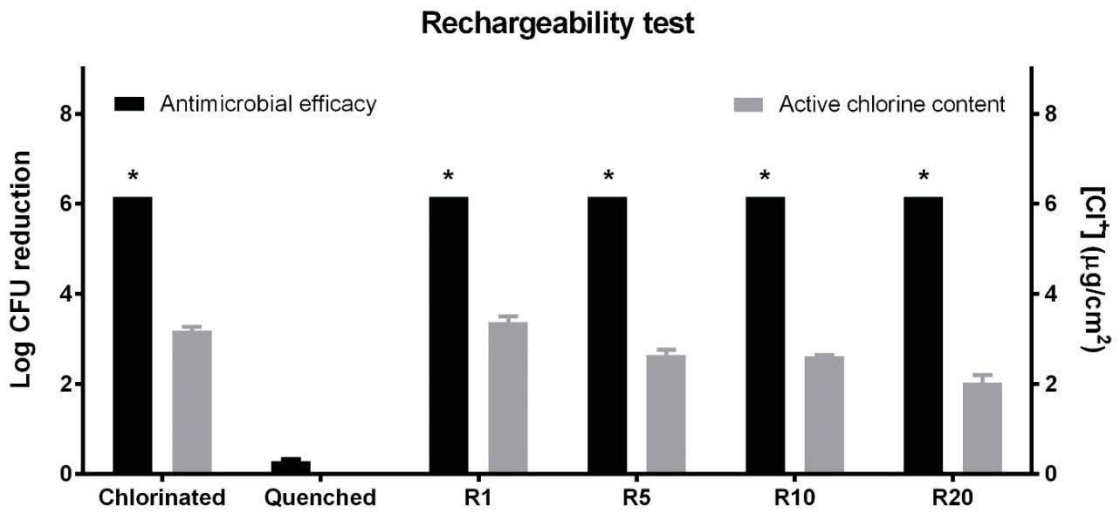

Figure 2.5 Antimicrobial efficacies and oxidative chlorine contents of rechargeable *N*-halamine modified TPU films. Inoculum: *E. coli* O157:H7 (6.15 log CFU/6.45 cm^2). * indicates a total kill of all inoculated bacteria.

Generally, *N*-halamine functional group (N-Cl) was reported to be not very stable under light or in water environment (Hui and Debiemme-Chouvy 2013), therefore the stability of antimicrobial functional groups of this *N*-halamine modified TPU was also investigated. As shown in Figure 2.6, the N-Cl bond in the *N*-halamine modified films remained unchanged after storage in dark for 4 weeks. Under fluorescent light, it experienced a slight decrease after 3 weeks. This result was in good accordance with previous research performed by Liu and others (2015) which these two *N*-halamine polyelectrolytes were quite stable under fluorescent light. However, the intensity of *N*-halamine functional group (N-Cl) decreased faster in the water environment: the oxidative chlorine content decreased to less than 1.0 μg/cm^2 in the water after 28 days of storage. However, the N-Cl bonds could almost be totally recovered after rechlorinating at day 28 (28R). The other fact is that many food processing plant will perform the

sanitation every day, which means rechlorinating (or "recharge" process) can be performed daily. Therefore, when combined with rechargeability result, this stability is far more enough to support its application as food antimicrobial surface material, which will always keep sufficient antimicrobial functional groups after regular treatment of diluted bleach during sanitation.

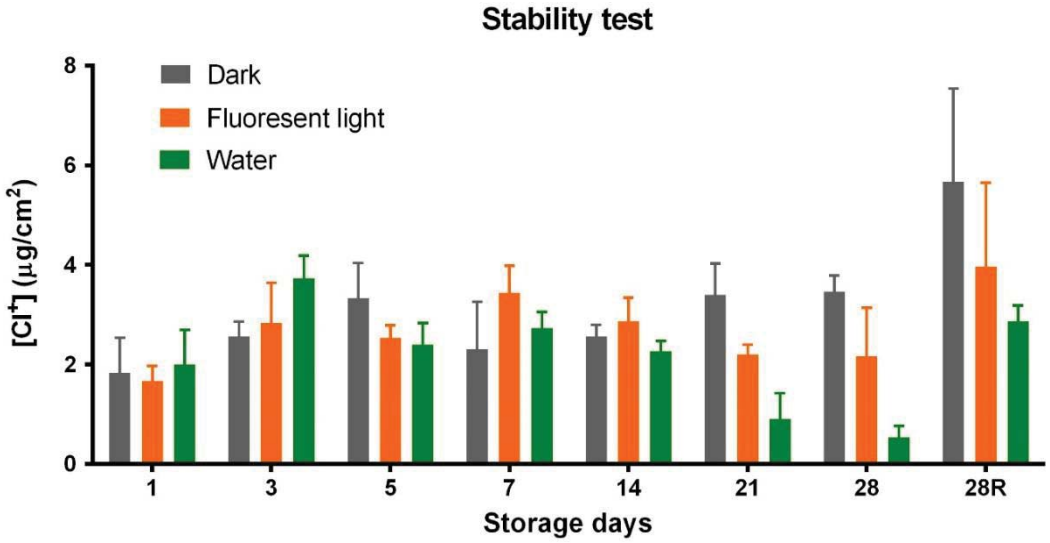

Figure 2.6 Oxidative chlorine contents of *N*-halamine modified TPUs stored in dark, under fluorescent light and in water for 28 days and rechlorinated after 28 days of storage (28R).

A successful antimicrobial functional modification should not sacrifice other important application functions of food contact materials. For example, tensile strength and hydrophobicity of conveyor belts should not be compromised too much to achieve the antimicrobial activity. Therefore, in this research, we also tested some physical properties of this *N*-halamine modified TPU at four different cases: TPU only (TPU-Ctrl) as a control, TPU charged with chlorine (TPU-Ctrl-Cl), *N*-halamine modified TPU (TPU-4%NP), and *N*-halamine modified TPU charged with

chlorine (TPU-4%NP-Cl). As shown in Table 2.1, the tensile strength did not decrease evidently after mixing with 4% NPH polymers. Compared to the TPU only control film, *N*-halamine modified films lost around 10% of the tensile strength. After treatment/charging with chlorine (5% bleach), films lost around 20% of their strength. However, this loss is not considered a sever deterioration and the change in tensile strength would be expected to be further minimized through using low concentration of bleach (0.2%) in food processing application environment. For contact angle, there was a certain decrease in values observed for *N*-halamine modified TPU after chlorination. This could be explained by the compounding method applied in this research.

Table 2.1 Physical properties of *N*-halamine modified TPU films.

Samples	Thickness (mm)	Tensile strength at maximum load (MPa)	Contact angle (deg)
TPU-Ctrl	0.301 ± 0.033	20.9 ± 6.2	90.3 ± 1.0
TPU-Ctrl-Cl	0.319 ± 0.055	14.3 ± 6.1	100.5 ± 3.3
TPU-4% NP	0.311 ± 0.039	18.3 ± 3.7	89.9 ± 1.8
TPU-4% NP-Cl	0.340 ± 0.045	14.5 ± 6.3	77.5 ± 1.2

For research convenience, we chose the solvent casting method in this study. However, this method also has drawbacks: the surface was not very smooth due to solvent evaporation and the particles were not distributed evenly in the film as those made in the industry through heat extrusion method. All these would contribute to lower number of contact angle as well as big variance of chorine content between different films. However, the surface tension test indicated this *N*-halamine modified TPU was still hydrophobic. The contact angle is also expected to be

improved after better engineering method such as hot blending and extrusion method was applied in the future when a large quantity of production was desired. All these characteristics will be positive and accepted by industry for the application as food contact materials.

For these materials proposed for food-contact surface applications, several requirements should be comprehensively considered and well balanced: the raw materials should be cheap and easily accessible; the synthesizing procedure need to be simple and clean, and less toxic processing aid agents should be used; the effectiveness and durability should be good enough; the method of application should also be compatible to current established practices as well as other materials in the environment. This *N*-halamine modified polyurethane can adequately fulfill all these above-mentioned requirements. It should be remarked here that only water was used during the whole synthesizing procedure of *N*-halamine precursor polymers, which means safe and green. This will also contribute to making it easier for passing the regulatory examination for new materials proposed for food contact surfaces in the future. Most importantly, the antimicrobial activity can be recharged, which can fulfill both effectiveness and durability requirements of antimicrobial material as desired by food industry.

2.4 Conclusions

In this study, a simple, clean and efficient method to modify thermoplastic polyurethane (TPU) with rechargeable antimicrobial function is reported. This *N*-halamine modified TPU film caused 6 log bacteria reduction within 2 hours of contact. The antimicrobial *N*-halamine functional groups were also stable enough for the application. Moreover, the *N*-halamine

modified TPU displayed desirable rechargeability, which maintained sufficient antimicrobial activity after 20 cycles of "discharge-recharge" process. Therefore, this new *N*-halamine modified TPU has great potential for food contact surface materials to make equipment with rechargeable antimicrobial function. Ultimately, antimicrobial functionalized food contact surface will help to prevent microbial cross-contamination during food processing and improve food safety.

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2.6 Supporting information

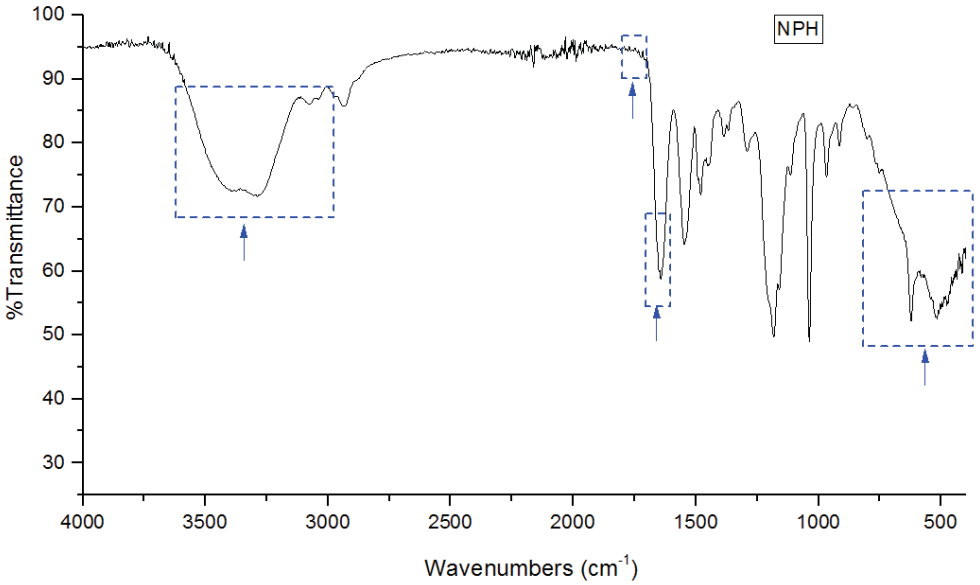


Figure S2.1 FTIR spectra of *N*-halamine precursor polymer blend.

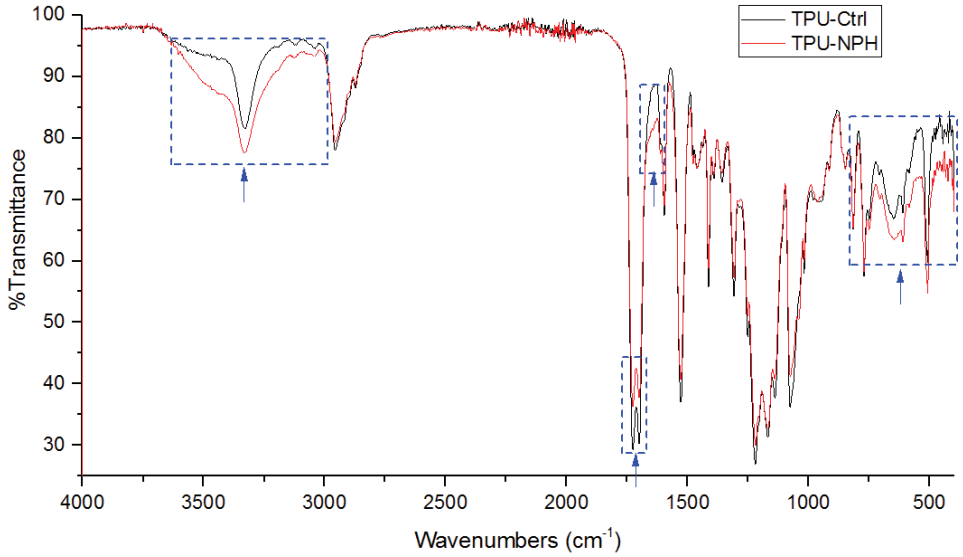


Figure S2.2 FTIR spectra of control TPU (TPU-Ctrl) and *N*-halamine precursor polymer modified TPU (TPU-NPH).

Chapter 3

Conducting Polymer Based *N*-halamines as Antimicrobial/Anticorrosion Multifunctional Coatings for Stainless Steel

Abstract: The goal of the current study is to develop a multifunctional coating material which possesses both electrical conducting and antimicrobial properties. Polypyrrole (PPy) was proved to be transformed into *N*-halamine after treating with chlorine bleach. This PPy based *N*-halamine was tested to have superior antimicrobial efficacy - its coating on tape inactivated more than 6 log CFU of both *Staphylococcus aureus* and *Escherichia coli* O157:H7 within one min of contact time. The stability of PPy based *N*-halamine was also satisfactory, maintaining 50% of functional groups after one week storage under fluorescent light. PPy *N*-halamine coating was successfully synthesized on the surface of stainless steel 316 L through electrochemical deposition and chlorination. This PPy *N*-halamine coating on stainless steel inactivated 6 log CFU of *S. aureus* within one minute of contact and the antimicrobial activity remained unchanged after a "recharge" cycle. In addition, the PPy *N*-halamine coating significantly improved the anticorrosion functionality of stainless steel through shifting the corrosion potential +245 mV to positive. This reported method of preparing antimicrobial/anticorrosion coating is simple, green and highly effective, and has potential to protect steels in harsh environments.

Keywords: *N*-halamines; conducting polymer; antimicrobial; anticorrosion; coatings

3.1 Introduction

Stainless steel is widely used in the food industry for equipment fabrication such as pipework, tanks, conveyor belts, worktables, machinery parts, etc. (Schmidt and others 2012). However, previous research has shown that stainless steel is prone to harboring pathogenic microorganisms that form biofilms, making it vulnerable as a source for microbial cross-contamination (Kreske and others 2006; Chmielewski and Frank 2003; Brooks and Flint 2008; Ryu and Beuchat 2005). Each year in the United States, 48 million people get sick with 3,000 deaths from foodborne illnesses, with an estimated economic loss of $15.6 billion (Elaine and others 2011; Hoffmann and others 2012). Foodborne pathogens from food contact surfaces, including stainless steel, can contribute as one of the important sources of infection (Lianou and Sofos 2007; Kumar and Anand 1998). In recent years, developing non-migrate antimicrobial coatings for stainless steel through exploiting advanced materials is a promising solution to tackle microbial cross-contamination problems (Bastarrachea and Goddard 2013; Falentin Daudré and others 2012; Ignatova and others 2009; Jampala and others 2008; Madkour and others 2009). Among all these explored antimicrobial agents, *N*-halamines showed a great potential for applications in the food industry due to their superior antimicrobial efficacy against a broad spectrum of microorganisms, low-toxicity, good stability and low cost (Bastarrachea and others 2015; Kenawy and others 2007; Dong and others 2017).

N-halamines are defined as a group of compounds containing one or more nitrogen-halogen bonds that are transformed from nitrogen-hydrogen bonds in groups of imide, amide or amine (Chen and Sun 2006). The antimicrobial activity of *N*-halamines is contributed from the oxidation state of halide atoms in nitrogen-halogen bonds (Hui and Debiemme Chouvy 2013).

One important feature makes *N*-halamines distinct from other existing biocides is that the antimicrobial activity can be recovered after halide atoms are consumed through treating with halogenation agents such as chlorine bleach. This "recharging" procedure can be easily incorporated into current sanitation practices applied in the food industry. These advantages make *N*-halamines an attractive antimicrobial technology for the food industry. Recent reviews and research articles intensively highlighted the great potential of applying *N*-halamine polymers for equipment coatings or packaging in the food industry (Bastarrachea and others 2015; Hui and Debiemme Chouvy 2013; Dong and others 2017; Kenawy and others 2007; Qiao and others 2017; Bastarrachea and Goddard 2015; Bastarrachea and others 2014; Bastarrachea and others 2013; Denis-Rohr and others 2015; Denis-Rohr and others ; Bastarrachea and others 2011). Some current research has grafted *N*-halamine polymers onto the surfaces of stainless steel (Bastarrachea and Goddard 2013); however, to maintain *N*-halamine antimicrobial functional groups (e.g. >N-Cl), chlorine bleach is needed to repeatedly treat the coatings, which may cause corrosion to stainless steel.

The corrosion of stainless steel is also an important concern for the food industry (Schmidt and others 2012). Although higher grades of stainless steel with better anti-corrosion properties (e.g. 304 and 316 types) are usually selected for food equipment fabrication, the food processing environments are still harsh for high-grade stainless steel through extended exposure to corrosive agents such as chloride in processing or cleaning agents, chlorine and oxidizing sanitizers, and even food components (e.g. meat/blood). Microbial biofilms will also attack the surface of the stainless steel through excreting oxidative or corrosive metabolites (Ibars and others 1992). All these factors are able to accelerate the corrosion process of stainless steel and cause enormous economic loss to the food industry. One promising strategy to protect stainless

steel from corrosion is to coat the surface with electroactive conducting polymers such as polypyrrole and polyaniline (Spinks and others 2002; González and Saidman 2011; Moraes and others 2003). Compared with other anticorrosion technologies on stainless steel, the synthesis and coating procedures for conducting polymers are usually simple and green. Conducting polymers, such as polypyrrole, have been reported to be safe for biomedical applications (Wang and others 2004). These are important criteria for applications in food contact materials.

The goal of the current study is to develop a conducting polymer based *N*-halamine coating material to address the issue of microbial contamination and corrosion in stainless steel simultaneously. Polypyrrole (PPy), one of the most thoroughly investigated conductive polymers, contains nitrogen-hydrogen structure, which can be transformed into *N*-halamines through treating with chlorine bleach. This study also aims to prove that polypyrrole can be transformed into *N*-halamine and to test the antimicrobial activity and stability of this polypyrrole based *N*-halamine as coating material. In addition, a potential application of this conducting polymer based *N*-halamine as an antimicrobial/anticorrosion coating on stainless steel 316 L was illustrated.

3.2 Material and methods

3.2.1 Chemicals and reagents

Pyrrole, platinum (Pt) gauze (100 mesh, 99.9% metal basis), sodium thiosulfate, and ammonium persulfate were purchased from Alfa Aesar (Heysham, England). Sodium

dodecylbenzenesulfonate (SDBS) was obtained from Spectrum Chemicals (New Brunswick, NJ). Potassium iodide was purchased from Fisher Scientific (Fair Lawn, NJ). House bleach was purchased from Walmart (Great Value^{TM}). Stainless steel 316 L 24 G (0.6 mm of thickness) was supplied by Stainless Supply Inc (Monroe, NC). All chemicals were used as received. *Staphylococcus aureus* ATCC 6538 and *Escherichia coli* O157:H7 ATCC 43895 were obtained from American Type Culture Collection (ATCC, Rockville, MD).

3.2.2 Preparation of PPy coating on tape and chlorination

PPy polymer was synthesized by a chemical oxidative polymerization method (Poyraz and others 2015). Briefly, one mL of pyrrole monomer was gently added into 60 mL of HCl (1 M) and stirred for 10 min. After that, 1.15 g of ammonium persulfate (APS) was added into the solution and the change of the color from yellowish brown to black in solution indicated PPy formation. The sample was filtrated and vacuum dried to obtain PPy dry powder. The PPy powders were coated onto 3M™ 600 Scotch Transparent Tape (2×2 cm) and pressed to obtain a compact and evenly distributed PPy coating on the surface of the tape. The PPy-coated tape films (Tape-PPy) were treated with different concentrations of bleach solutions (5, 1, and 0.5%) with different treatment times (5, 10, and 30 min) to find out the optimal chlorination condition. After chlorination, the tape films were washed thoroughly with deionized water and dried in a vacuum oven overnight to remove any free chlorine residuals. The uncoated tape film was treated with the same procedure to serve as chlorination control. The formation of nitrogen-halogen bonds (>N-Cl) from nitrogen-hydrogen bonds (>N-H) in PPy was confirmed by X-ray photoelectron spectroscopy (XPS) using a load-locked Kratos XSAM800 surface analysis system equipped

with a hemispherical energy analyzer, and Fourier transform infrared spectroscopy (FT-IR) using a Thermo Scientific Nicolet™ 6700 Spectrometer.

3.2.3 Determination of chlorine content and stability test

The oxidative chlorine contents of these chlorinated PPy-coated tape films (Tape-PPy-Cl) were determined using an iodometric/thiosulfate titration method (Liu and others 2015). Briefly, one piece of Tape-PPy-Cl film was put into a flask containing 20 mL of water, 10 μL of 0.1 N acetic acid, and 0.2 g of potassium iodine, and stirred at room temperature for 20 min. Then, the solution was added with 0.5% starch solution and titrated by 0.001 N sodium thiosulfate. The active chlorine content was calculated using the following formula: $[Cl^+]$ ($\mu g/cm^2$) = $(N \times V \times 35.5)/2A$, where N and V are the normality ($equiv \cdot L^{-1}$) and volume (L) of the titrant sodium thiosulfate and A is the total surface area of titrated sample (cm^2). The stability of PPy *N*-halamine under three different conditions (dark, fluorescent light and Ultraviolet) was determined based on chlorine content measured by the titration method. After a certain period of storage time, oxidative chlorine content of three Tape-PPy-Cl films from each storage condition were measured.

3.2.4 Antimicrobial efficacy test with PPy *N*-halamine coated tape

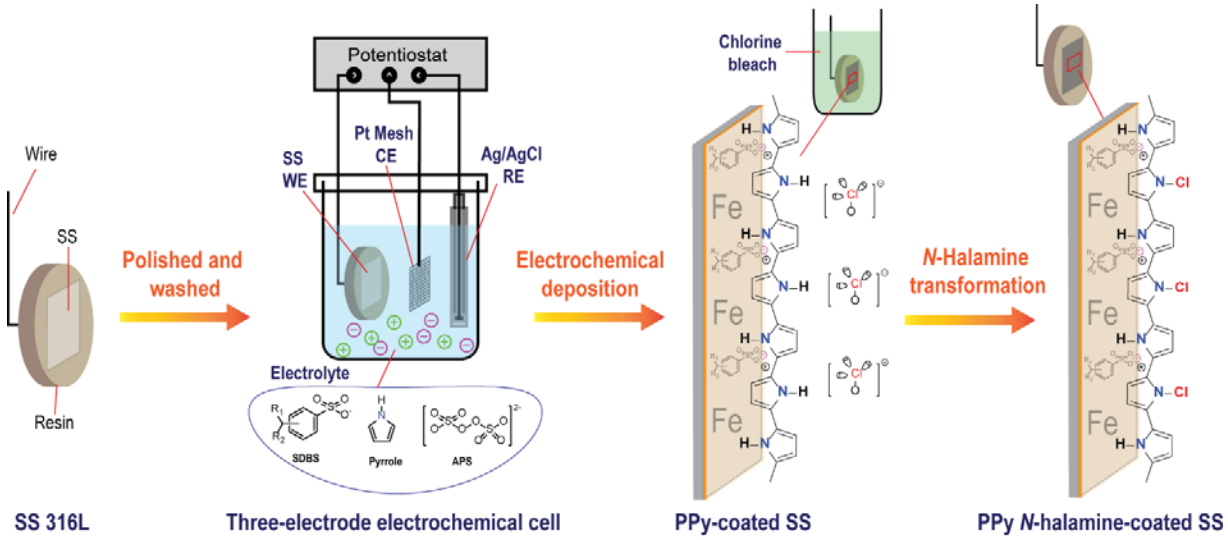
The antimicrobial efficacy of PPy *N*-halamine coated tape films was determined using a "sandwich" method as described previously.(Liu and others 2015) A gram negative bacterium of

E. coli O157:H7 and a gram positive bacterium of *S. aureus* were used in this study. A single colony of each bacteria was transferred into 15 mL of Trypticase soy broth (Becton and Dickinson Co., MD) and incubated at 37 °C for 16 h. The culture was washed twice with Butterfield's phosphate buffer (BPB) through centrifugation and was re-suspended in the BPB buffer. Bacterial population was estimated by the absorbance at $O.D._{640\ nm}$ and the inoculum with the designated population was prepared. An aliquot of 25 µL of the inoculum was added into the center of the square film, and a second identical film was placed on the sample to ensure complete contact with inoculated bacteria. A sterile weight was placed on the top to ensure complete contact with inoculated bacteria. At the contact times of 1, 5, and 10 min, the films were transferred into $Na_2S_2O_3$ solution (0.05 N) and vortexed vigorously to quench any oxidative chlorine residuals and to detach survived bacteria from the sample. Ten-fold serial dilutions were made for all samples and each dilution was plated on Trypticase soy agar plates (Becton and Dickinson Co., MD). The plates were incubated at 37 °C for 48 h and bacterial colonies were enumerated and recorded for antimicrobial efficacy analysis.

3.2.5 Preparation of PPy *N*-halamine-coated stainless steel

PPy was coated onto the surface of Type 316 L stainless steel (SS) using an electrochemical deposition method (Scheme 3.1) (Nautiyal and Parida 2016). SS plate was cut into coupons (1.5×1.5 cm) and a copper wire was welded on one side for electrical connection. Then the SS plate was cold mounted into acrylic resin so that only one surface was exposed to the electrolyte. After drying overnight, the embedded SS sample was polished with 800, 1000 and 1200 grit size sandpapers (3M™ Imperial Wetordry™) and washed thoroughly with

deionized water. PPy coating was electrochemically deposited from an electrolyte solution containing the monomer of pyrrole (0.1 M), oxalic acid (0.3 M), and sodium dodecylbenzenesulfonates (SDBS, 25 mM). The electrochemical cell was a single compartment system with three electrodes: SS plate served as a working electrode, Pt mesh worked as a counter electrode, and Silver/Silver chloride/KCl (3 M) (Ag/Ag^+, $E° = +205$ mV vs. SHE) was used as a reference electrode. Potentiostatic deposition was carried out on CH Instrument potentiostat (CHI 601D) with Electrochemical Analyzer software (Version 15.03) through applying 0.8 V/MSE for 5 min. The formation of a black thin film on the surface of SS plate indicated the successful deposition of PPy coating. For *N*-halamine transformation, PPy-coated SS samples were immersed in 1% chlorine bleach (pH 7.0/HCl, $[Cl^+] = 414±8$ ppm) for 10 min, washed thoroughly with deionized water, and dried overnight in a vacuum oven.



Scheme 3.1 Illustration of the preparation of PPy *N*-halamine-coated stainless steel.

3.2.6 Characterization of PPy *N*-halamine-coated stainless steel

Surface morphology of the coatings was analyzed using a Scanning Electron Microscopy (SEM) on JOEL JSM-7000F at 20 keV with 10 mm working distance. Surface energy of the coating was measured using a ramé-hart Standard Contact Angle Goniometer Model 200 (ramé-hart Inc., Mountain Lakes, NJ) equipped with DROPimage software. The water contact angles were measured using the sessile drop method and three independent measurements were performed for each type of sample. The formation of nitrogen-chlorine bond from nitrogen-hydrogen bond was confirmed with X-ray Photoelectron Spectroscopy (XPS) through analyzing the presence of chlorine element. The thickness of the coating d (cm) was calculated using the previous reported method using the equations below (Nautiyal and Parida 2016):

$$d = \frac{m}{2\rho}$$

$$m = \frac{Q\ (M_m + \gamma\ M_d)}{F(2 + \gamma)A}$$

where, Q was the total Faradic charge consumed in the electropolymerization (1.86 C), M_m was the molar mass of the monomer (pyrrole, 67.09 g/mol), M_d was the molar mass of the dopant (dodecylbenzene sulfonate, 348.48 g/mol), γ was the doping density of polypyrrole (0.33), F was Faradic constant (96,485 C/mol), A was the surface area (cm^2), and ρ was density of the polymer (1.48 g/cm^3).

3.2.7 Antimicrobial efficacy test with PPy *N*-halamine coated stainless steel

Antimicrobial efficacy was determined using the same "sandwich" method as mentioned previously in this study. The PPy-coated and PPy *N*-halamine-coated SS samples were autoclaved at 121 °C for 45 min and dried before use. An aliquot of 10 μL *S. aureus* was inoculated and contact times of 1 and 5 min were performed. After antimicrobial test (experiment 1), all tested samples were recycled and autoclaved. Then, samples were treated with $Na_2S_2O_3$ to reduce the >N-Cl in PPy *N*-halamine to >N-H. This process of quenching chlorine from the PPy *N*-halamine coated stainless steel was called "discharge". The "discharged" PPy coated SS was "recharged" back to PPy *N*-halamine coated SS through rechlorinating in 1% bleach for 10 min. These "recharged" PPy *N*-halamine coated SS were used for the second antimicrobial efficacy test (experiment 2) following the same procedures as in experiment 1.

3.2.8 Anticorrosion test

The PPy *N*-halamine coated SS samples were evaluated for the ability of protection from corrosion by immersing in 3.5% NaCl with linear sweep voltammetry (LSV) at a scan rate of 1 mV/S, scanning ±150 mV from the open circuit potential (OCP). The same single compartment system with three electrodes as mentioned previously in this study was used except that a Mercury/Mercury sulfate/K_2SO_4 (saturated) (MSE, E° = +650 mV vs. SHE) reference electrode was used as instead of Silver/Silver chloride/KCl (3 M) (Ag/Ag^+, E° = +205 mV vs. SHE).

3.2.9 Electrical conductivity measurement

The bulk electrical conductivity of the PPy *N*-halamine coating was measured using a linear four-probe method as previously described (Liu and others 2011). PPy coating and *N*-halamine coating were touched with a linear four probe which was connected to a multifunctional switch/measurement unit (Agilent 34980A) and specific resistivity was recorded. Conductivity of the deposited coatings σ (S/cm) was calculated using the equation (1) and (2):

$$\rho = 4.532 \times t \times R \quad (1)$$

$$\sigma = \frac{1}{\rho} \quad (2)$$

where, t was the thickness of the coating (cm) and R was the resistance (Ω).

3.3 Results and discussion

PPy pure polymer powders were successfully coated on the surface of the tape (Figure 3.1b) and the polymer content was calculated at 0.3 mg/cm^2. After treated with 5% bleach for 5 min, a yellowish color was observed (Figure 3.1d). With the increasing of treatment time and bleach concentration, more PPy was observed to be detached from the tape surface (Figure 3.2a). This could be explained by the attacking of NaClO in bleach solution to adhesive polymers on the tape. Within the treatment time of 5 min, a higher concentration of bleach resulted in higher content of oxidative chlorines on surface of PPy coated tape (Figure 3.2b). Within the range of lower bleach concentrations (1 and 0.2%), longer treatment time resulted in higher oxidative

chlorine content. When the bleach concentration reached 5%, the chlorine content was lower with longer treatment time due to the detachment of PPy polymers from the tape surface. However, the treatment of 5% bleach for 5 min showed the highest oxidative chlorine content on the surfaces ($[Cl^+]$ = 6.7 μg/cm^2). Therefore, this condition was used to chlorinate samples for the following experiments.

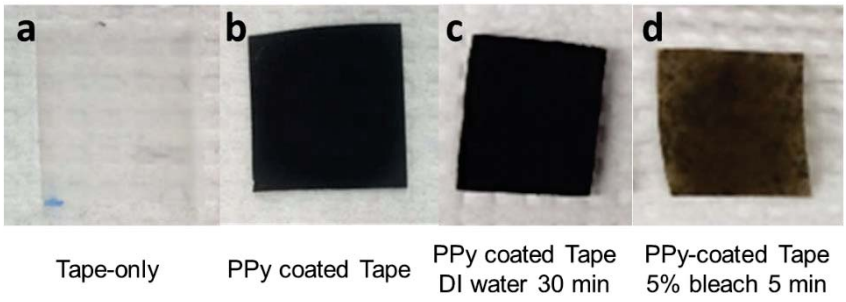


Figure 3.1 Appearance of PPy-coated tape films.

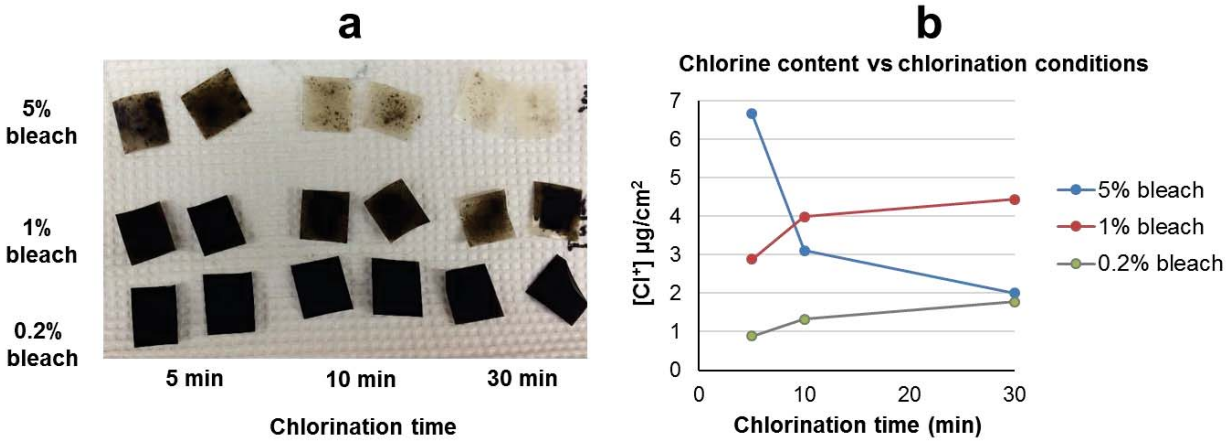


Figure 3.2 (a) Appearance and (b) oxidative chlorine content of PPy *N*-halamine coating on tape after different concentrations and times of chlorine bleach treatment.

The presence of oxidative chlorine on chlorinated PPy coating could be explained by the hypothesis that part of the nitrogen-hydrogen bonds (>N-H) in PPy structure were transformed into nitrogen-chlorine bonds (>N-Cl) through reacting with NaClO and became *N*-halamine polymers (Figure 3.3). The conducting form of PPy with positive charge created empty orbitals in the nitrogen atom of the backbone (approximately 33.3%), therefore the attacking from electron-rich ClO^- was favored and >N-Cl bond was formed. This hypothesis was confirmed based on the results of chlorine titration and spectroscopy. The iodometric reaction ($2I^- + Cl^+ \leftrightarrow I_2 + Cl^-$) indicated that the chlorine element in chlorinated PPy coating was in the oxidative form. After chlorination treatment, the peak of chlorine element was observed on the XPS spectra of PPy coated tape (Figure 3.4). In the FT-IR spectra (Figure 3.5), the band located at 1539.7 cm^{-1} was attributed to the C=C/C-C stretching vibration of the PPy chains.(Tabačiarová and others 2015) After chlorination, the vibrational band shifted to 1548.8 cm^{-1}, due to electron-withdrawing effect of the chlorine atoms in the >N-Cl bond (Jiang and others 2016). These are strong evidences to prove that >N-Cl was formed in PPy after chlorination. Based on these evidences, it was concluded that PPy can be transformed into *N*-halamines after chlorination. The PPy *N*-halamine was recorded as PPy-Cl in this study.

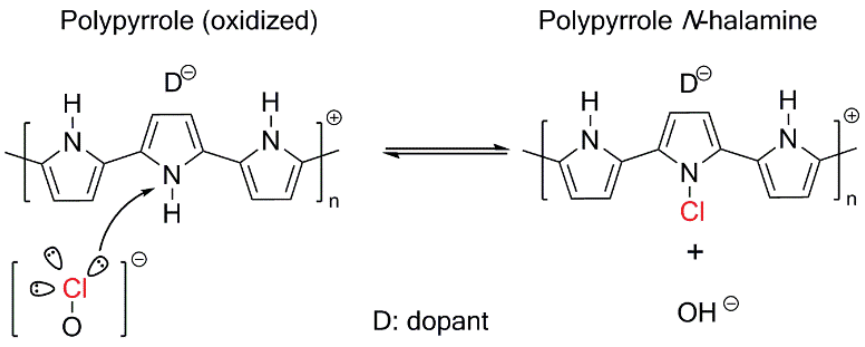


Figure 3.3 *N*-halamine transformation of PPy.

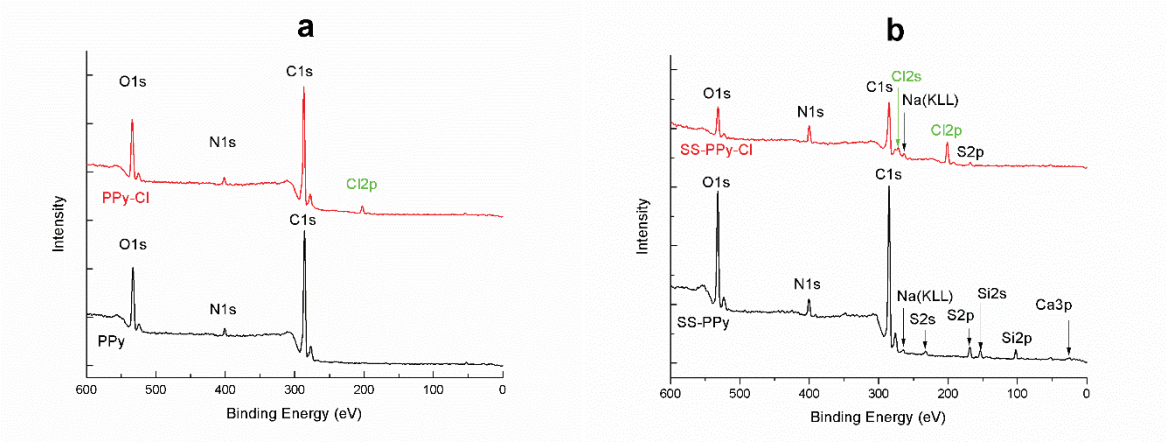


Figure 3.4 XPS spectra of PPy and PPy *N*-halamine coated tape (a) and stainless steel (b).

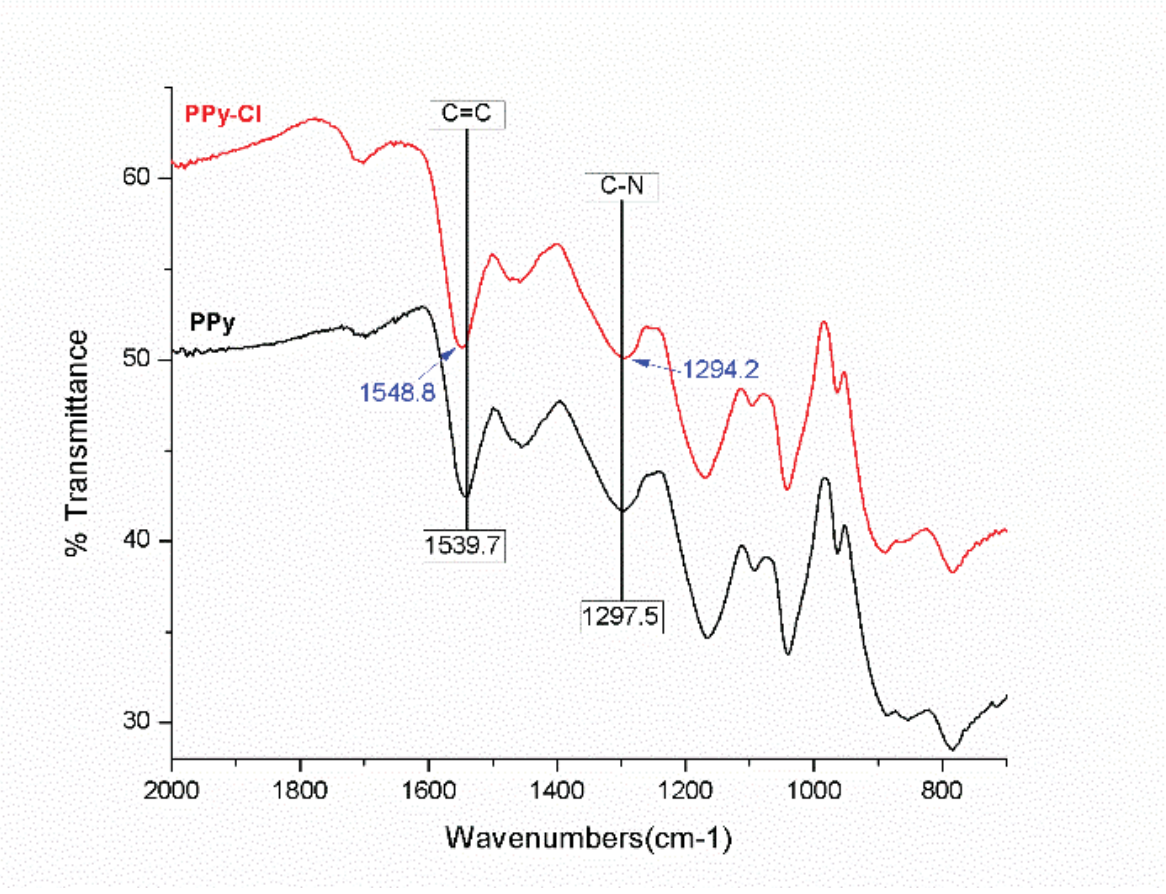


Figure 3.5 FT-IR spectra of PPy and PPy *N*-halamine.

PPy pure polymer coated tape caused 0.93 log CFU reduction of *E. coli* O157:H7 and 1.57 log CFU reduction of *S. aureus* within 10 min of contact (Table 3.1). Both were higher than tape only. This slight increase of antimicrobial activity was due to the weak antimicrobial activity of PPy (da Silva Jr and others 2016). After being transformed into *N*-halamine, the coated tape killed all inoculated bacteria (both *S. aureus* and *E. coli* O157:H7) within one min of contact, which antimicrobial activity was highly potent. This significant improvement of antimicrobial activity was contributed from the oxidative chlorines in >N-Cl of PPy based *N*-halamine. *N*-halamine compounds usually have potent antimicrobial activity based on previous research (Chen and Sun 2006; Hui and Debiemme Chouvy 2013). Compared with all other *N*-halamines found to date, the antimicrobial activity of PPy *N*-halamine was one of the most potent. The superior antimicrobial function of PPy *N*-halamine was ascribed to high density of >N-H bonds in PPy polymers that resulted in the formation of more >N-Cl groups on the surface, and the >N-Cl in PPy *N*-halamine is highly active due to the special structure of pyrrole (Figure 3.6d). Usually, the biocidal activity of *N*-halamine follows the orders of imide > amide > amine (Figure 3.6a, b & c) (Hui and Debiemme Chouvy 2013). To date, cyclic hydantoin based *N*-halamine (Figure 3.6e) containing both amide and imide groups were found to be the most potent *N*-halamine functional structures and their coating on fabrics could inactivate 100% bacteria within 1-5 min using the same testing procedures in this study (Kocer and others 2011). The biocidal activity of PPy *N*-halamine was comparable to the most potent *N*-halamine polymers based on the cyclic hydantoin structure. The potent antimicrobial activity in PPy *N*-halamine may be influenced by the adjacent C=C bond as the similar mechanism of adjacent C=O bond in imide and amide *N*-halamines.

Table 3.1 Antibacterial efficacies of PPy *N*-halamine coated tape.[a]

Samples	Contact time	Bacterial reduction (log CFU)	
		***E. coli* O157:H7**	***S. aureus***
Tape-Cl	10 min	0.78	1.00
Tape-PPy	10 min	0.93	1.57
Tape-PPy-Cl	1 min	6.25*	6.19*
	5 min	6.25*	6.19*
	10 min	6.25*	6.19*

[a] Chlorination condition: 5% bleach for 5 min. Inoculum: 6.25 log CFU/sample of *E. coli* O157:H7, 6.19 log CFU/sample of *S. aureus*. * Total killing of inoculated bacteria under detection limit.

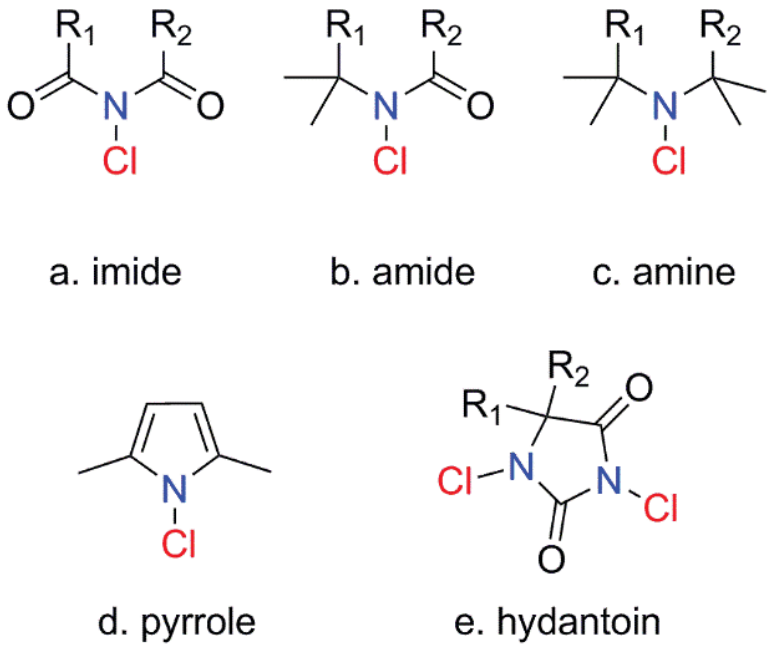


Figure 3.6 Molecular structures of *N*-halamines mentioned in this study.

Based on previous research, the *N*-halamine bond (>N-Cl) was not stable under UV light (Hui and Debiemme Chouvy 2013). For these *N*-halamines with potent antimicrobial activity such as imide and amide, the decrease of stability was often observed (Liu and others 2016b). Therefore, the stability of PPy *N*-halamine coating was also investigated in this study (Table 3.2). The PPy based *N*-halamine lost 58% of oxidative chlorines on coated surface after storage under UV for 24 h. Under fluorescent light, 50% percent of oxidative chlorines were lost after storage for one week while in dark condition for two weeks, only 20% of oxidative chlorine remained on the surface. The stability of *N*-halamine functional group (>N-Cl) in PPy was not as good as cyclic hydantoin based *N*-halamine containing amide group, which retained 67% of oxidative chlorines after 15 days storage under dark (Liu and others 2016a). Usually, the food processing plant performs sanitation daily, which means the recharge process can be repeated within a day. Therefore, PPy *N*-halamine has sufficient stability for supporting the application as antimicrobial equipment coating in the food processing environment. The stability of *N*-halamine can be further improved through the combination with titanium dioxide (TiO_2), which has been routinely used as an antimicrobial agent on stainless steel surface (Liu and others 2016b).

Table 3.2 Stability of PPy *N*-halamine coating on tape under different storage conditions.

UV		**Fluorescent Light**		**Dark**	
Time (h)	$[Cl^+]$ ($\mu g/cm^2$)	Time (d)	$[Cl^+]$ ($\mu g/cm^2$)	Time (d)	$[Cl^+]$ ($\mu g/cm^2$)
0	5.33	0	5.33	0	5.33
1	4.73	1	4.44	1	5.33
3	3.33	3	2.96	3	4.44
12	3.11	5	2.66	7	2.81
24	2.22	7	2.66	14	1.04

A typical chronoamperometry curve was observed during the electrodeposition of polypyrrole on stainless steel surface (Figure 3.7). Initially, there was a sharp increase in current density indicating the diffusion controlled pyrrole oxidation and nucleation of pyrrole on the steel surface. After nucleation, the current density remained constant corresponding to continuous growth of polypyrrole film on stainless steel. PPy was successfully coated on the surface of stainless steel 316 L (Figure 3.8a) and homogenously formed a smooth thin film (Figure 3.8b). After being treated with 1% bleach for 10 min, no damage was observed on the coated surface (Figure 3.8c). Therefore, 1% bleach for 10 min treatment was used to chlorinate the PPy coating on stainless steels and the chlorine content was 2.05±0.15 μg/cm^2. It was also found that there was no damage of the coating after sterilization in 121 °C for 45 min (Figure 3.8d). The SEM pictures (Figure 3.9) confirmed the homogenous deposition of polypyrrole on the surface of stainless steel with some granule particles. The thickness of the PPy coating was calculated to be 2.25 μm. After vortexing in a salt solution, the coating was also unchanged. Based on these observations, PPy *N*-halamine coating was firmly attached to the surface of stainless steel, which had the potential to resist aggressive conditions in food processing environment such as high temperature, high moisture, high salt content solutions, and corrosive sanitation agents. After chlorination (*N*-halamine transformation), the contact angle increased from 42.4±0.8° to 53±1.5°. This phenomenon could be explained by the fact that >N-Cl is more hydrophobic compared with >N-H. The increase of hydrophobicity was desired as an antimicrobial/anticorrosion surface because the hydrophobic surface will prevent the contact of biological or corrosion agents that are usually present in aqueous systems.

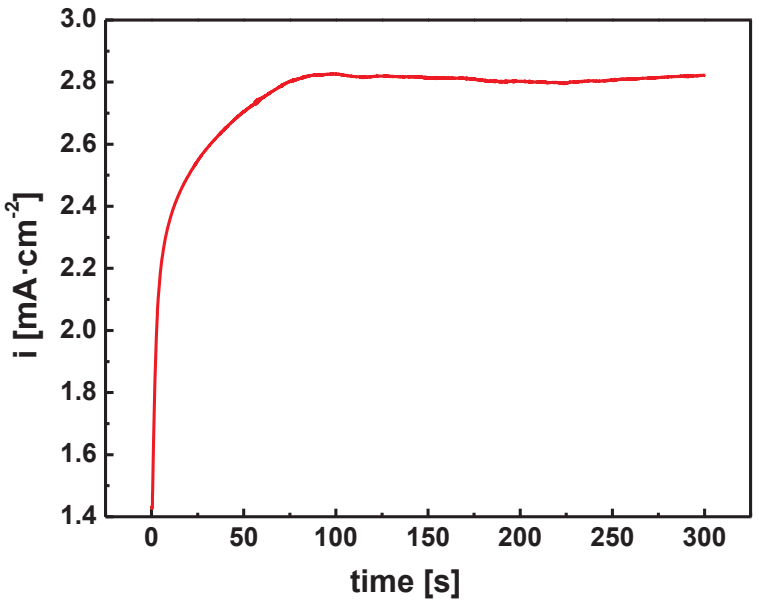


Figure 3.7 Chronoamperometry curve of electropolymerization of polypyrrole on stainless steel.

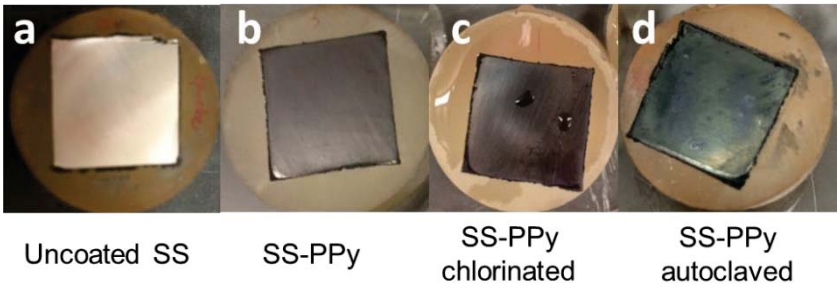


Figure 3.8 Appearance of (a) uncoated stainless steel, (b) PPy coated stainless steel, (c) PPy coated stainless steel after chlorination treatment in 1% bleach for 10 min, and (d) PPy coated stainless steel after autoclave at 121 °C for 40 min.

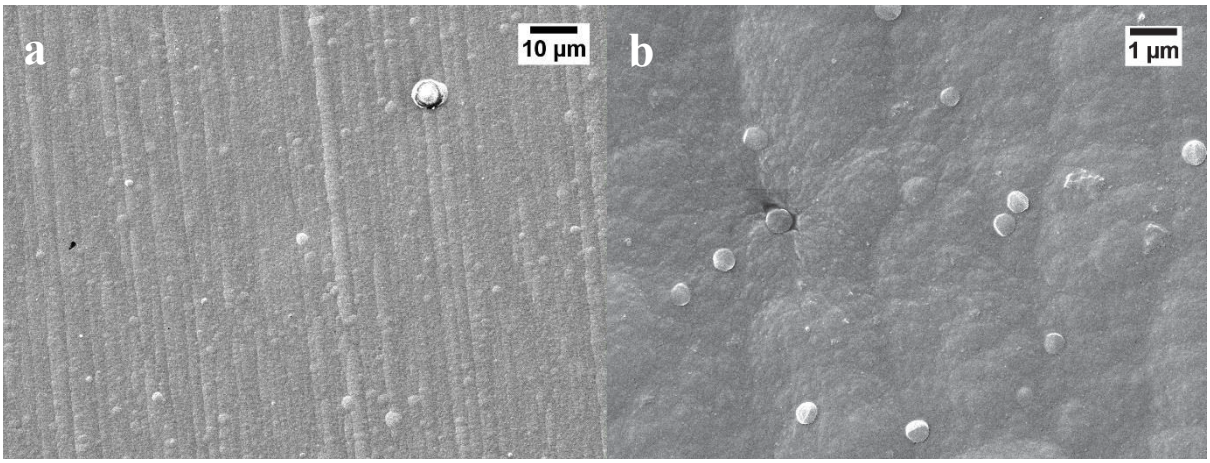


Figure 3.9 Scanning microscopy of PPy coated stainless steels at (a) ×1,000 magnification and (b) ×10,000 magnification.

The antimicrobial activity of PPy *N*-halamine coated stainless steel was shown in Table 3.3. The bare stainless steel caused 0.94 log CFU reduction of *S. aureus* after 5 min of contact; however, PPy *N*-halamine coated stainless steel killed all inoculated bacteria (6.29 log CFU) within one minute of contact time. The antimicrobial activity remained unchanged after being rechlorinated for one time, which indicated that the antimicrobial function of PPy *N*-halamine coating could be recharged. It was unexpected that the unchlorinated PPy coating on stainless steel was also able to have a total kill of bacteria (6.29 log CFU reduction) in experiment 1. This may due to the use of dodecylbenzenesulfonate (SDBS) as dopant, which is also a strong antimicrobial agent (Cords and others 2005). From our preliminary research, SDBS as dopant gave the optimum adhesive and anticorrosion effects of PPy coating on the stainless steel surface. Therefore, the antimicrobial effect of unchlorinated PPy-coated SS was attributed to the synergetic antimicrobial activity of SDBS and PPy. However, the SDBS on the coating surface attached to PPy backbones through ionic attraction, which is easily to be removed after vigorous

vortexing in a buffer solution. Therefore, a decrease of antimicrobial activity was observed in experiment 2 with unchlorinated PPy coated stainless steel. The other fact was that, in the previous section (Table 3.1), when Cl^- was used as the dopant instead of SDBS, the PPy coating on tape caused only one log CFU reduction of bacteria within 10 min of contact. Based on these results, it was confirmed that the potent antimicrobial function of PPy *N*-halamine-coated stainless steel was mainly contributed from *N*-halamine functional groups (>N-Cl). The antimicrobial activity remained unchanged after one cycle of discharge and recharge process, which indicated that the PPy *N*-halamine coating on stainless steel had rechargeable antimicrobial function.

Table 3.3 Rechargeable antimicrobial function of PPy *N*-halamine coating on stainless steel.[a]

Samples	Contact time	Bacterial reduction (log CFU)	
		Experiment 1	Experiment 2
SS	1 min	0.68	0.81
	5 min	0.94	1.00
SS-PPy	1 min	6.29*	3.89
	5 min	6.29*	4.49
SS-PPy-Cl [a]	1 min	6.29*	6.02*
	5 min	6.29*	6.02*

[a] Chlorination conditions: 1% bleach for 10 min. Experiment 1: first-time chlorination, inoculum 6.29 log CFU/sample of *S. aureus*. Experiment 2: discharged and rechlorinating, inoculum 6.02 log CFU/sample of *S. aureus*. * Total killing of inoculated bacteria under detection limit.

Figure 3.10 showed that polypyrrole coating made surface of stainless steel nobler by shifting its corrosion potential (E_{corr}) from -0.12 to +0.15 V vs Ag/Ag^+. Although there was a little decrease (30 mV) in corrosion potential after chlorination, the E_{corr} of +0.12 V vs Ag/Ag^+

was still considered good anticorrosion ability. SDBS was used as both dopant for the polymer and corrosion inhibitor simultaneously.(Free 2002) The mechanism of corrosion inhibition is that functional groups adsorb on the surface and form aggregates, which contributes to additional protection originally from a single layer of polypyrrole thus enhancing the nobleness of the surface. The resistances were 5 and 200 kΩ for unchlorinated and chlorinated polypyrrole coating, respectively. The conductivity was calculated to be dropped from 1.96 S/cm to 0.049 S/cm, which is still within the range of good conductivity. The formation of N-Cl bond to a certain extent blocked the electron transfer in the original conducting structure of oxidized PPy; however, this slight decrease in conductivity did not affect the function of PPy conducting polymer as anti-corrosion coating.

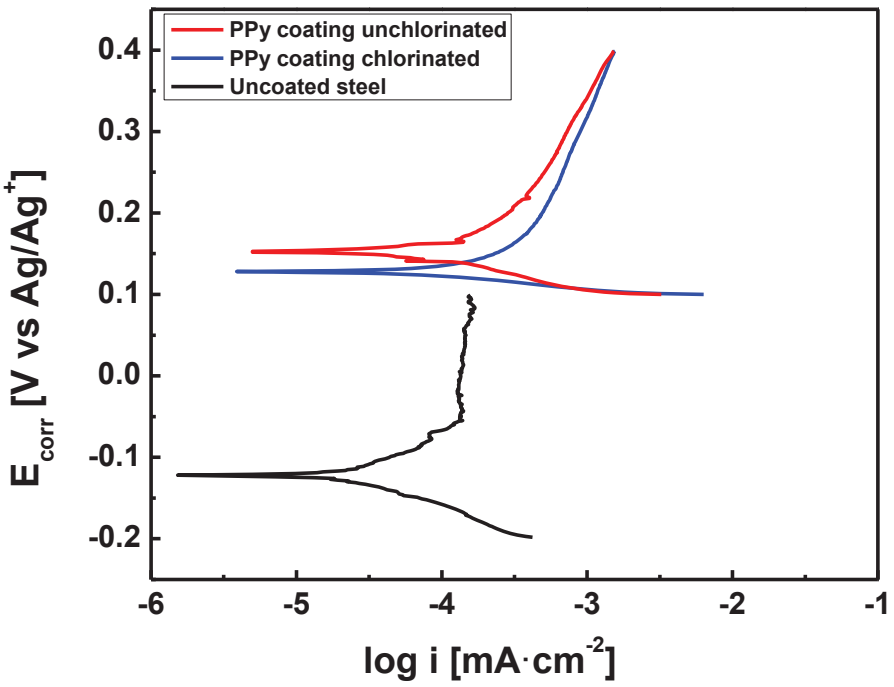


Figure 3.10 Polarization curves of polypyrrole (unchlorinated and chlorinated) coated and uncoated stainless steel in 3.5% wt. NaCl.

3.4 Conclusions

PPy conducting polymer was transformed into *N*-halamines after chlorinating with bleach. This PPy *N*-halamine had superior antimicrobial activity which inactivated 6 log CFU of both *S. aureus* and *E. coli* O157:H7 within one minute of contact time. The PPy *N*-halamine structure was relative stable under fluorescent light and dark. A PPy thin film was successfully coated onto the surface of stainless steel 316 L through electrochemical deposition. The coating has the potential to withstand aggressive conditions such as high pressure, high temperature, vigorous washing with salt and sanitation solutions, etc. The PPy coating on stainless steel was transformed into *N*-halamine after bleach treatment. This *N*-halamine coated stainless steel inactivated 6 log CFU of *S. aureus* with one minute of contact and the antimicrobial function could be recharged. The PPy *N*-halamine coating also significantly improved the anticorrosion effect of stainless steel. This reported method for preparing antimicrobial/anticorrosion coatings on stainless steel is simple, green and highly effective. It has a great potential to apply as low-cost and high-performance multifunctional coatings for protecting steels used in the food and biomedical industries.

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3. 6 Appendices

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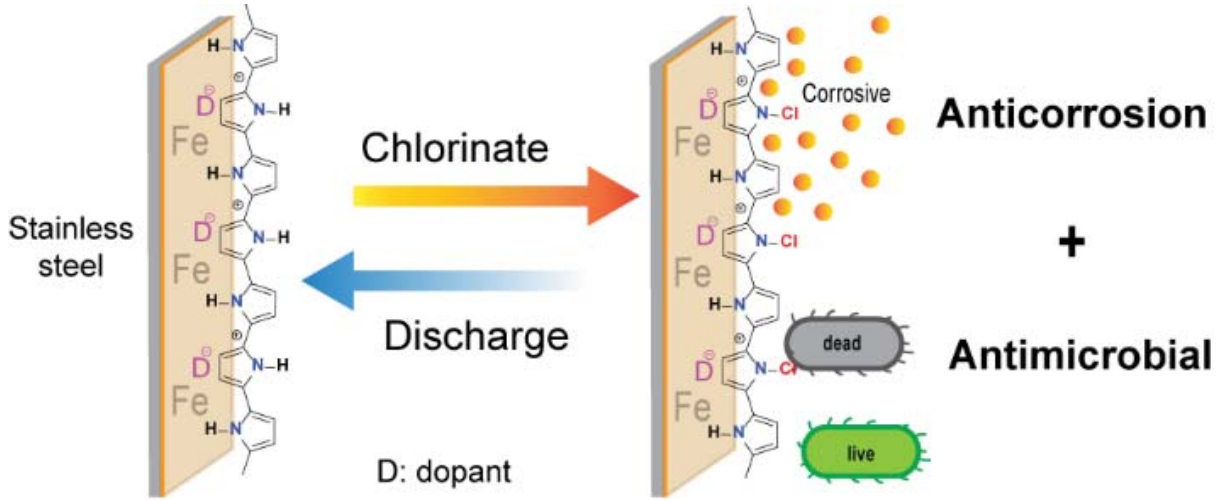


Chapter 4

In Vitro Toxicity Assessment of Antimicrobial *N*-halamine Compounds

Abstract: *N*-halamines, as a group of emerging antimicrobial agents, have drawn more and more interest in recent years. However, the toxicity of *N*-halamines has rarely been examined previously. In this study, the toxicity effects and possible mechanism of one *N*-halamine compound MC (1-chloro-2,2,5,5-tetramethylmidazolidin-4-one) was evaluated through the combination of a basal cytotoxicity assay and a bacterial reverse mutation assay. Results showed that MC has a predicted acute oral LD_{50} of 276±17 mg/kg body weight in rat, which may be potential toxic substances after ingestion. However, its precursor compound TMIO (2,2,5,5-tetramethyl-4-imidazolidione) has an estimated acute oral LD_{50} of 3702±162 mg/kg body weight, which was suitable to be classified as non-toxic. It is highly possible that the mechanism of MC cytotoxicity was contributed solely from the function of oxidative chlorines in its nitrogen-chlorine bond. Compared with bleach, MC has the advantage of higher stability. Based on bacterial reverse mutation assay, no mutagenicity effect was observed for either TMIO or MC at maximum testing doses with or without external enzyme metabolizing system in all tester strains used in this study. Results from this study suggest that *N*-halamine based antimicrobial compounds may have great potential to be applied as safe antimicrobial agents for food, agriculture and biomedical applications.

Keywords: *N*-halamine; *in vitro*; toxicity assessment; cytotoxicity; mutagenicity

4.1 Introduction

N-halamines are a group of antimicrobial agents containing nitrogen-halogen covalent bond (>N-X) which is formed through the halogenation of nitrogen-hydrogen bond (>N-H) in moieties such as amide, imide and amine (Chen and Sun 2006). The biocidal activity of *N*-halamine is contributed from the oxidation effect of halide atoms (e.g. chlorine, bromide or iodine) in the nitrogen-halogen bond. In recent years, *N*-halamines have attracted a great deal of interest due to their superior antimicrobial efficacy against a broad spectrum of microorganisms (bacteria, fungi and viruses), good stability and low cost (Hui and Debiemme-Chouvy 2013; Kenawy and others 2007). A most recent comprehensive review article by Dong and others (2017) in *Chemical Reviews* indicated the great importance of *N*-halamines as emerging antimicrobial agents. *N*-halamines have been researched for both monomers and polymers: the monomers can be used as non-bleaching and long-lasting disinfectants and the polymers can be used as antimicrobial coatings. One feature makes *N*-halamine different from any other existed antimicrobial agents is that its antimicrobial function can be recovered through treating with halogenation agents such as chlorine bleach. Recently several studies have explored the applications of *N*-halamines in various areas including water treatment (Chen and others 2004; Jiang and others 2016), antimicrobial textiles and films (Jiang and others 2014; Du and others 2015; Jiang and others 2017; Liu and others 2016; Cheng and others 2015; Liu and others 2017), antimicrobial paintings (Kocer and others 2011; Cao and Sun 2009; Kocer 2012), biomedical devices (Sun and others 2012; Luo and others 2006; Luo and others 2011), and food packaging and equipment coatings (Qiao and others 2017; Appendini and Hotchkiss 2002; Bastarrachea and

Goddard 2015; Bastarrachea and Goddard 2013; Bastarrachea and others 2014; Bastarrachea and others 2015; Qiao and Huang 2016).

Besides their outstanding antimicrobial properties, another advantage of *N*-halamines which attracts a lot of interest is safety: they are generally considered as low or non-toxic to human health as cited by many previous publications (Luo and others 2011; Hui and Debiemme-Chouvy 2013; Kenawy and others 2007; Demir and others 2015). This advantage makes *N*-halamines especially promising to be applied in the situation where strict requirement of safety is desired such as direct human contact materials (e.g. biomedical equipment, artificial organs) and direct food/water contact materials (e.g. food processing or packaging materials, water purification cartridge). Although some studies claimed the safety of *N*-halamines, there was no supportive study for this "general" statement. A search of literature and databases also revealed very limited toxicological data related to *N*-halamines. Meanwhile some recent studies showed that some small molecular *N*-chloramine products that share the similar >N-Cl structure as *N*-halamines may be toxic or have mutagenic effect to human at very low concentration (Laingam and others 2012). More novel *N*-halamine compounds and polymers with superior antimicrobial activities have been continuously synthesized and studied, systematic toxicity assessments of these *N*-halamine compounds are desired to ensure their safety prior practical application.

MC (1-chloro-2,2,5,5-tetramethylmidazolidin-4-one) is a heterocyclic *N*-halamine compound which has comparable biocidal effect as bleach with additional advantages of long-lasting antimicrobial activity and non-bleaching effect. It was first synthesized in 1991 by Dr. Worley's group (Tsao and others 1991) and has been proposed for wide applications such as antimicrobial agents for facial mask (Demir and others 2015), fish diseases (Bridges and others 2013), and foodborne pathogens (Lauten and others 1992; Worley and others 1992). Our

ongoing projects introduce MC as novel sanitizers for food contact surface applications. However, its toxicity has not been thoroughly investigated previously. The other reason is that the structures of MC and its precursor compound TMIO (2,2,5,5-tetramethyl-4-imidazolidione) are well-defined, which makes it more convenient to study the potential toxicity mechanism of *N*-halamine. In this study, MC was chosen as a representative *N*-halamine compound to investigate the potential toxicity effect of antimicrobial *N*-halamine agents.

Toxicity assessment is usually a multiple-step process requiring screening strategy based on several expensive and time-consuming complementary tests from microbes to mammals, to draw valid conclusion (Eisenbrand et al., 2002). For toxicity assessment, one single study is not expected to answer all questions while complete toxicity study will be extremely expensive and unnecessary for every new chemical or material which is still in research stage. Therefore, the toxicity assessment can be designed based on weight-of-evident and step-wise strategies. A review of current *in vitro* toxicity study methodology and state-of-art recommendations indicates that a combination of optimized *in vitro* basal cytotoxicity and mutagenicity assays can illuminate sufficient important toxicity information with least cost of time, labor and budget. Therefore, the aim of this study is to assess the toxicity and possible mechanism of MC *N*-halamine compound through interpreting the results from *in vitro* toxicity assays.

4.2 Materials and methods

4.2.1 Chemicals and reagents

Sodium azide, 9-aminoacridine and 2-aminoanthracene were purchased from Alfa Aesar (Ward Hill, Massachusetts). D-biotin was purchased from Chem-Implex (Wood Dale, Illinois). Nicotine adenine dinucleotide phosphate (NADP) was supplied by Amresco (Solon, Ohio). 4-Nitro-*o*-phenylenediamine (4-NOPD) and neutral red (NR) were purchased from Sigma (St. Louis, Missouri). L-histidine was purchased from Acros Organics (Morris, New Jersey). Glucose-6-phosphate (G-6-P) sodium salt and rat liver S9 (Aroclor-1254-induced) were purchased from MOLTOX (Boone, North Carolina). Newborn calf serum (NCS) was purchased from HyClone Laboratories (South Logan, Utah). Dulbecco's modification of eagle's medium (DMEM) without L-glutamine (4.5 mg/L glucose) and Dulbecco's phosphate-buffered saline (D-PBS) was purchased from Lonza (Walkerville, Maryland). Chlorine bleach was purchased from Walmart (Great Value^{TM}, 8.25% of NaClO, Bentonville, AR).

4.2.2 Bacterial strains and cell cultures

Five *Salmonella* Typhimurium tester strains (TA98, TA100, TA102, TA1535 and TA1537) were obtained from MOLTOX (Boone, North Carolina) and DNA sequence specificity of the tester strains was shown in Table 4.1. All tester strains were confirmed for their phenotype characteristics and analyzed for their genetic integrity and spontaneous mutation rates before use

(Mortelmans and Zeiger 2000). Mouse embryonic fibroblast cell BALB/c 3T3 clone A31 (ATCC® CCL163™) cell line were purchased from the American Type Culture Collection (ATCC, Manassas, Virginia). 3T3 cells were cultured in DMEM (high glucose at 4.5 g/L) supplemented with 10% of NCS and 4 mM of L-Glutamine. Cells were maintained in 75 cm^2 cell culture flasks under a humidified incubator at 37 °C with 5% CO_2 atmosphere and was routinely passaged every 2-3 day intervals.

Table 4.1 DNA sequence specificity on the *Salmonella* tester strains (Mortelmans and Zeiger 2000).

Strains	DNA target	Mutation event
TA98	-C-G-C-G-C-G-C-G-	Frameshifts
TA100	-G-G-G-	Base-pair substitution
TA102	TAA (ochre)	Transitions/Transversions
TA1535	-G-G-G-	Base-pair substitution
TA1537	+1 frame shit (near -C-C-C- run)	Frameshifts

4.2.3 Preparation of testing samples

N-halamine precursor compound TMIO (2,2,5,5-tetramethyl-4-imidazolidione) and *N*-halamine compound MC (1-chloro-2,2,5,5-tetramethyl-4-imidazolidinone) were synthesized and purified according to previous reported method (Tsao and others 1991). Molecular structures of TMIO and MC were shown in Figure 4.1 and the synthesization procedure was shown in Scheme 4.1. The obtained MC pure compound was white crystal and TMIO compound was white powder. The obtained products were confirmed with Nuclear magnetic resonance (NMR) spectroscopy (Bruker AVANCE™ 250 Hz) and Fourier transform infrared (FT-IR) spectroscopy

(Thermo Scientific Nicolet 6700). MC and TMIO were dissolved at 95% ethanol at 20 mg/mL as stock solutions and stored in dark before use. In addition, 1% (v/v) bleach solution was freshly prepared from house bleach with deionized water and the pH value was determined at pH=10.2. A portion of the 1% bleach solution was adjusted to pH=7.0 using 3 M HCl.

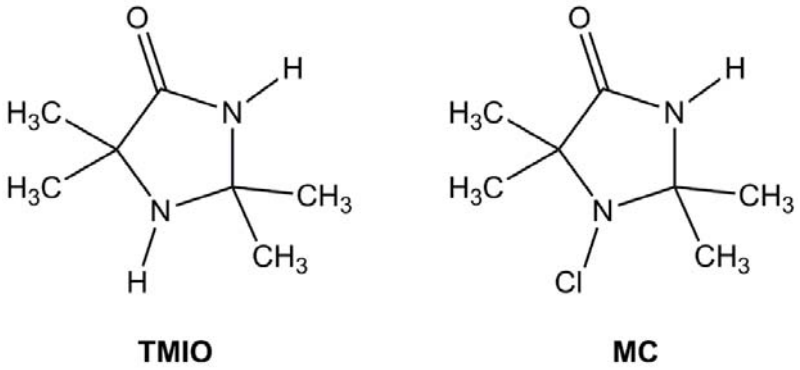
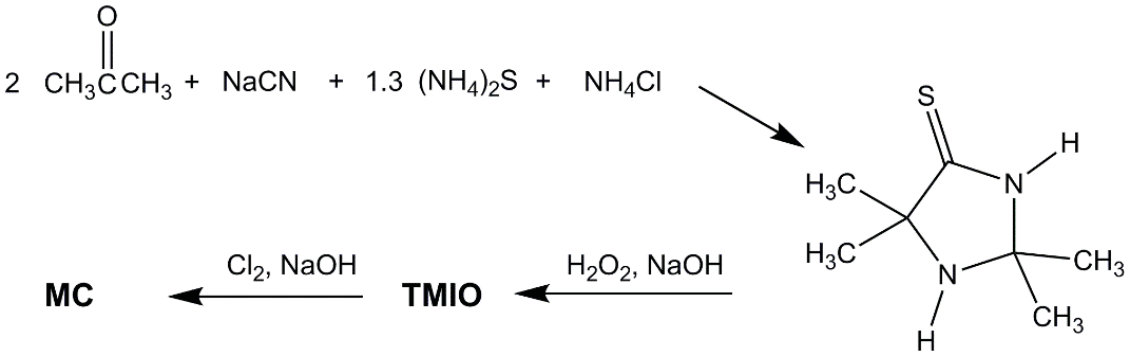


Figure 4.1 Molecular structures of TMIO (2,2,5,5-tetramethyl-4-imidazolidinone) and MC (1-chloro-2,2,5,5-tetramethyl-4imidazolidinone).



Scheme 4.1 Synthesization procedure of TMIO and MC (adapted from Tsao et al., 1991).

4.2.4 Antibacterial efficacy test

The antibacterial efficacy of MC was confirmed using a time-kill test as described previously (Ren 2015). Briefly, a single colony of *S.* Typhimurium was transferred into 15 mL of Trypticase soy broth (Becton and Dickinson Co., MD) and incubated at 37 °C for 16 h. The culture was washed twice with Butterfield's phosphate buffer (BPB) through centrifugation and was re-suspended in the BPB buffer. Bacterial population was estimated by the spectrometer at $O.D._{640\ mm}$ and the inoculum with designated population (8×10^7 CFU/mL) was prepared. An aliquot of *S.* Typhimurium suspension (100 μL) was added into each 4 mL of solutions containing 0.04% TMIO, 0.02% MC and 0.04% MC, respectively. Then the mixtures were vortexed thoroughly and 200 μL of the mixture from each tube were transferred into an empty tube containing 40 μL of sodium thiosulfate at reaction times of 5, 10, 30 and 60 min to quench the oxidative chlorine residuals in solutions to stop further biocidal reaction. After that, each sample was diluted decimally and plated in a Trypticase soy agar plates (Becton and Dickinson Co., MD). The plates were incubated at 37 °C for 48 h and bacterial colonies were enumerated and recorded for antibacterial efficacy analysis.

4.2.5 Titration of available chlorine content

A modified iodometric/thiosulfate titration method was used to determine the available oxidative chlorines in MC and bleach solutions. Briefly, 0.1 mL of each solution was added into a flask containing 20 mL of water and 0.2 g of potassium iodine and stirred at room temperature

for 20 min to form I_2. Then three drops of 0.5% starch solution were added into the solution as indicator and 0.001 N sodium thiosulfate was used to titrate the solution. The available chlorine content [Cl^+] (μg/mL) in each solution was calculated using the following equation:

$$[Cl^+] = \frac{N \times \Delta V \times 35.5}{2V}$$

where N and ΔV are the normality (equiv/L) and volume (L) of the titrant sodium thiosulfate and V is volume of titrated samples (mL).

4.2.6 Basal cytotoxicity test and acute oral toxicity estimation

To evaluate basal cytotoxicity, NRU assay was performed following the protocol recommended by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) for basal cytotoxicity evaluation test using BALB/c 3T3 cells (NIH 2006). Briefly, 100 μl of 3T3 cells (2.7×10^4 cells/mL) were seeded in a 96-well plate and incubated for 24 h at 37 °C under 5% CO_2 atmosphere. After the culture medium was removed, the cells were treated with 50 μL of culture medium and 50 μL of testing compound solutions at eight different concentrations and then incubated for 48 h. Afterward, the medium with the testing compounds was removed, cells were rinsed with D-PBS. Then 200 μL of NR medium (5% serum, 25 μg NR dye/mL) were added and cells were incubated in dark for another 3 hours. After that, the NR medium was removed and 100 μL of ethanol/acetic acid (49:1) desorb solution was added into all wells after rinsing with D-PBS and shaking rapidly for 20 min to extract NR. Then, samples were set to stand still for 5 min, and absorbance at $O.D._{540\ nm}$ of samples were measured by a

microtiter plate reader (Multiskan Ascent, Thermo Fisher Scientific). Sodium lauryl sulfate (SLS) was used as the positive control through the whole process.

After the range finding test, TMIO at 100 mg/mL and MC at 100 µg/mL were determined to be used as the starting concentrations. Eight consecutive concentrations were applied for each solution. For each concentration, cytotoxicity was expressed as the mean percentage of cell viability from three replicates. Dose response curves were constructed and fitted using non-linear regression Hill function (GraphPad PRISM® 6 Demo, GraphPad Software, La Jolla, CA) with $R^2 > 95\%$. IC_{50} values (50% cell viability inhibition concentrations) were calculated using the fitted Hill equation and were presented as mean±SD from three independent experiments. For bleach solution, 1% bleach solutions with different pH values (pH 10.2 and pH 7.0) were used as the starting concentrations. The available chlorine content in each stock solution of bleach was titrated and seven consecutive concentrations were prepared and tested for NRU assay. The available chlorine concentration in solution which inhibits 50% cell viability ($[Cl^+]_{IC50}$) was estimated from the constructed dose response curves and Hill function as described previously. For MC, the same method was used to estimate the $[Cl^+]_{IC50}$.

4.2.7 Bacterial reverse mutation assay

This assay was designed following OECD TG471 instructions (OECD 1997), and standard plate incorporation procedure was chosen (Maron and Ames 1983; Mortelmans and Zeiger 2000). Briefly, tester strain cultures were incubated in nutrient broth (EMD Millipore) overnight at 37 °C to achieve a population of $1\text{-}2\times10^9$ CFU/mL. Test samples consisted of 100 µL overnight bacterial suspension (~10^8 bacteria), 50 µL chemical compound and 500 µL

phosphate buffer (0.1 mM, pH 7.4, for the tests without external metabolizing system) or S9 mix (for the tests with an external metabolizing enzyme system) were added to 2 mL of top agar containing limited quantities of histidine and biotin (0.5% agar with 0.05 M biotin/histidine) at 45 °C, vortexed and poured onto a pre-poured minimal agar plate after vortex. Sodium azide (NaN_3, 5 μg/plate), 9-aminoacridine (9-AAC, 50 μg/plate), 4-nitro-o-phenylendiamine (4-NOP, 2.5 μg/plate) and Mitomycin C (MMC, 5 μg/plate) were used as positive controls for those tests without S9 mix while 2-aminoanthracene (2-AAN, 10 μg/plate) was used as the positive control for tests with the S9 mix. Samples containing only water or ethanol solvent were used as negative controls for all tests. All plates were incubated at 37° C for two days, and bacterial colonies were counted. Triplicate plating was used at each dose level. The tests without S9 mix and with S9 mix were repeated twice. Data were presented as the mean±SD of revertants for each treatment. Mutagenicity potential was suspected if the chemical treated bacteria have twice number of revertants compared with negative controls and a dose-response phenomenon was observed (Li and others 2013).

4.3 Results and discussion

For toxicological evaluation, the information of testing substances is crucial to ensure that correct and consistent toxicity information is reported. Since the compounds used in this study are newly synthesized, detailed characterization tests were warranted to confirm that correct chemicals were used for the toxicological evaluation. Since the testing compound (MC) was proposed to have antimicrobial activity, both chemical (NMR and IR) and biological (antimicrobial assay) methods were used to confirm the TMIO and MC compounds prior to

toxicity assessment. The ^{1}H NMR spectra of TMIO (DMSO-d_6) and MC ($CDCl_3$) and their chemical shifts were shown in Figure 4.2. This result confirmed that the >N-H bond in secondary amine moiety of TMIO (δ = 2.50 ppm) was substituted with >N-Cl bond of MC. The FT-IR spectra of TMIO and MC and their characteristic absorbance peaks was shown in Figure 4.3. The absorbent peak at 3293 cm^{-1} in IR spectra of TMIO was assigned to nitrogen-hydrogen bond (N-H) in secondary amine, therefore it was not observed in MC. The strong absorbance of carbonyl bond (C=O) in amide groups was observed in IR spectra of both compounds, which was 1677 cm^{-1} in TIMO and 1673 cm^{-1} in MC, respectively. These chemical characterization results were also consistent with previously reports (Tsao and others 1991; Demir and others 2015). The biocidal effects of MC and TMIO were also validated by antimicrobial test in solution (Figure 4.4). MC (0.02%) inactivated 100% of *S.* Typhimurium (6.3 log CFU reduction) within 10 min of reaction while TMIO only caused less than 0.3 log CFU reduction within one hour of reaction. This result was also consistent with previous report (Ren 2015). Therefore, correct TMIO and MC compounds were obtained and used for the toxicity assessment assays in this study.

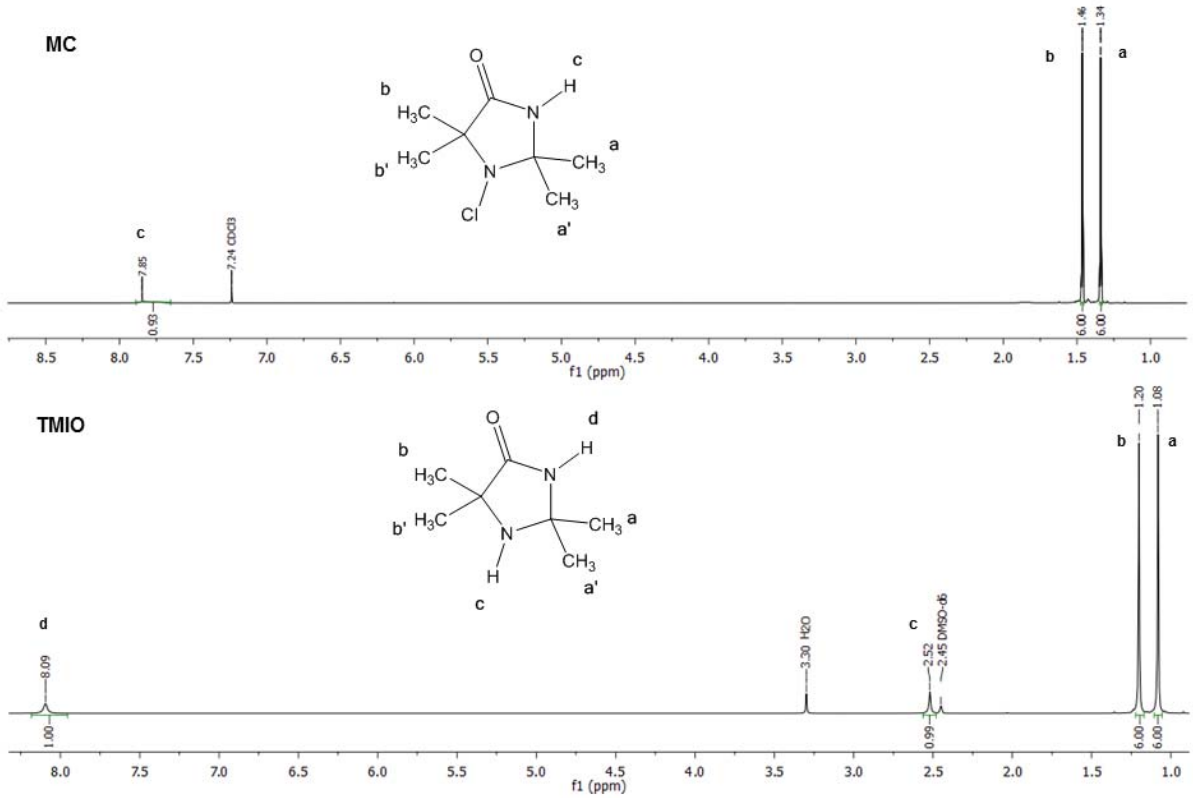


Figure 4.2 ^{1}H NMR spectra of TMIO (DMSO-d_6) and MC ($CDCl_3$).

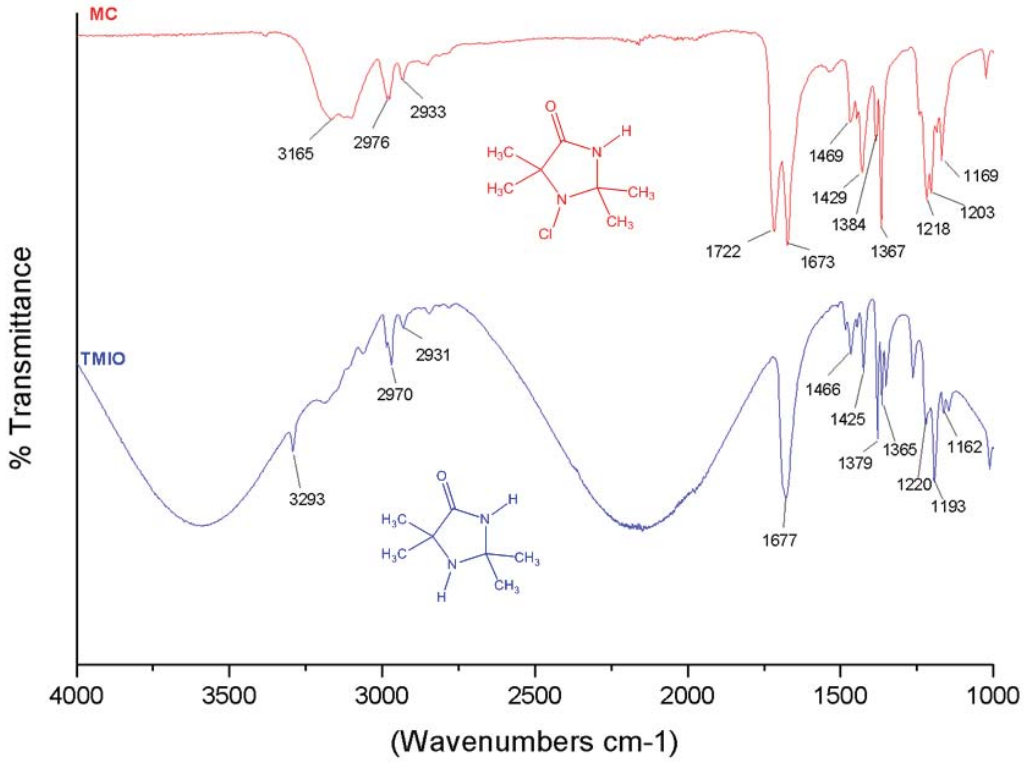


Figure 4.3 FT-IR spectra of TMIO and MC.

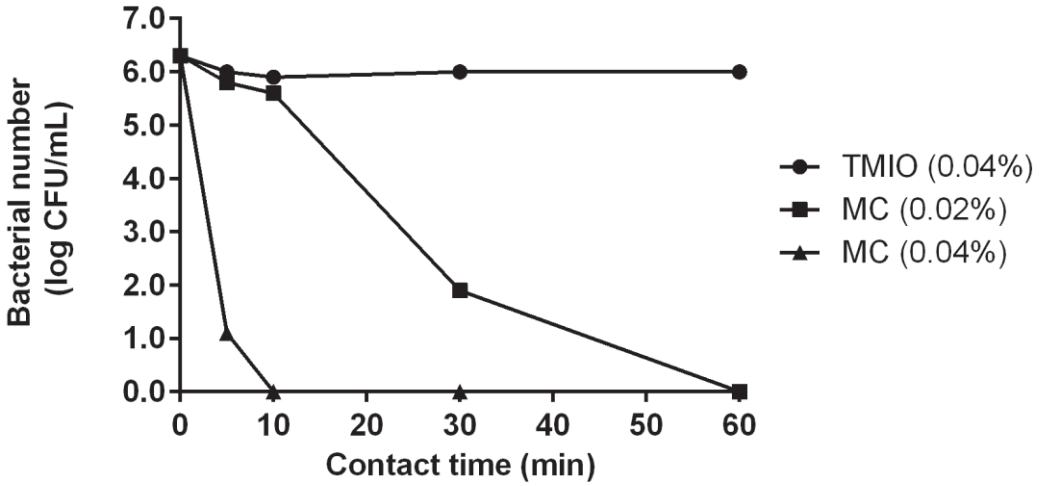


Figure 4.4 Antibacterial efficacies of MC and TMIO against *S.* Typhimurium. Inoculum: 1.9×10^6 CFU/mL (6.3 log CFU/mL).

In recent years, several large national and international projects evaluated the correlation between *in vitro* cytotoxicity and *in vivo* lethality (OECD 2010). Standardized cytotoxicity test was recommended as the initial step for systematic toxicity evaluation of unknown chemicals and was reliable for predicting acute oral lethality in rodent (ECVAM 2013). Therefore, basal cytotoxicity effects of MC and TMIO were investigated in this study using a standardized 3T3 NRU assay and the result was shown in Table 4.2. TMIO has the calculated IC_{50} value of 14,220 ± 1633 μg/mL after 48 h of exposure and MC has IC_{50} value of 13.30 ± 2.15 μg/mL in the same condition. The acute oral LD_{50} for TMIO was estimated to be 3,702±162 mg/kg b.w. (body weight) in rat, which was higher than the threshold value of 2,000 mg/kg b.w. to be categorized as non-toxic substance (ECVAM 2013). Therefore, combined with its structural information, TMIO was suitable to be classified as non-acute oral toxic substance. However, for MC the LD_{50} value was estimated at 276±17 mg/kg b.w., which was lower than cut-off value of 2,000 mg/kg b.w.. Therefore, MC was classified as potential positive for acute oral toxicity.

Table 4.2 Basal cytotoxicity and estimated acute oral toxicity of TMIO and MC.

Chemicals	**IC_{50} after 48 h of exposure (μg/mL)**	**Estimated acute oral LD_{50}* (mg/kg body weight)**
TMIO	14220±1633	3702±162
MC	13.30±2.15	276±17

Data were reported as mean ± SD, n=3. ***** Estimated acute oral LD_{50} was calculated based on IC_{50} (μg/mL) from 3T3 NRU assay using an established RC rat-only weight regression: $\log LD_{50}$ (mg/kg) = 0.372×$\log IC_{50}$ (μg/mL) + 2.024 (ICCVAM, 2006).

To date, there was only one *in vivo* study to investigate the toxicity effect of MC, which result showed the acute oral LD_{50} was 338 mg/kg b.w. in rat (Parent 2000). It was of interest to

find that the LD_{50} value (338 mg/kg b.w.) from the previous *in vivo* study was close to the estimated LD_{50} value (276±17 mg/kg b.w.) obtained from current *in vitro* study using prediction model. However, limitations of this *in vitro* prediction model should also be considered: the cell line used in this study does not have metabolizing system, the action in human or animal body may be different from *in vitro* studies. Therefore, further *in vivo* toxicity studies are needed in the future to validate systematic toxicity of MC.

This *in vitro* toxicity test method could provide additional advantage to disclose potential toxicity mechanism information of unknow chemicals. MC became cytotoxic after an oxidative-chlorine bond (>N-Cl) was transformed from the nitrogen-hydrogen bond (>N-H) of TMIO. The increase of toxicity was highly possible due to the action of >N-Cl. It was well-known that, in physiological pH (7.0-7.4), the cytotoxicity effect of bleach only derived from oxidative chlorines in solution (Hidalgo and others 2002). If oxidative chlorine was the only factor contributing to MC cytotoxicity, MC and its bleach counterpart that cause the same cell inhibition effect should have the same amount of available oxidative chlorines in each solution. To prove this, we also tested the cytotoxicity effects of bleach solution using the same method as in MC. To exempt the cytotoxicity effect from pH, bleach solutions at both pH 10.2 and pH 7.0 were tested. The oxidative chlorine content of each stock solution was titrated and the result was shown in Table 4.3. The available oxidative chlorine concentration in 0.02% MC solution was calculated to be 36±4 μg/mL, and the available oxidative chlorines in 1% bleach solution was titrated as 219±8 μg/mL (pH 10.2) and 207±22 μg/mL (pH 7.0), respectively. The estimated available oxidative chlorine content which inhibited 50% of the 3T3 cells was between 2.0-2.8 μg/mL for MC and between 3.0-4.0 μg/mL for bleach. The values were close to each other.

Table 4.3 Available oxidative chlorine concentrations in stock solutions of MC and bleach and estimated available $[Cl^+]$ at IC_{50}.

Samples	Estimated available $[Cl^+]_{IC50}$ (μg/mL)
MC	2.40±0.38
Bleach pH 10.2	3.34±0.59
Bleach pH 7.0	3.11±0.16

Data were presented as mean ± SD, n=3. $[Cl^+]_{IC50}$: available chlorine concentration in testing compound solutions that produce 50% inhibition of the cell viability.

The other fact was that chlorines in bleach solution evaporated quickly under 37 °C incubation. As shown in Figure 4.5, the available chlorine decreased about 30-40% after 4 h incubation, and was not detectable after 24 h incaution. However, the available chlorine in MC was almost unchanged through the 48-h incubation. Therefore, the actual $[Cl^+]_{IC50}$ value for bleach should be lower than 3.0-4.0 μg/mL, which will be even closer to the value of MC (2.0-2.8 μg/mL). Therefore, NaClO and MC may have same cell inhibition effect provided same amount of oxidative chlorines were available in each solution. In other words, the cytotoxicity effect of MC may be solely contributed from the function of oxidative chlorines. Since it was difficult to determine the real-time oxidative chlorine content in the cell culture medium and the cell killing process is also dynamic, it was unrealistic to determine the accurate $[Cl^+]_{IC50}$ value of MC and bleach solutions. The method used here was simple but effective to disclose the potential toxicity mechanism of MC. This was also in accordance with the step-wise toxicity testing strategy: using minimum test to disclose maximum toxicity information in initial stage. MC may cause adverse health effects after acute exposure through ingestion and the mechanism may be due to the oxidative chlorine bond in its structure.

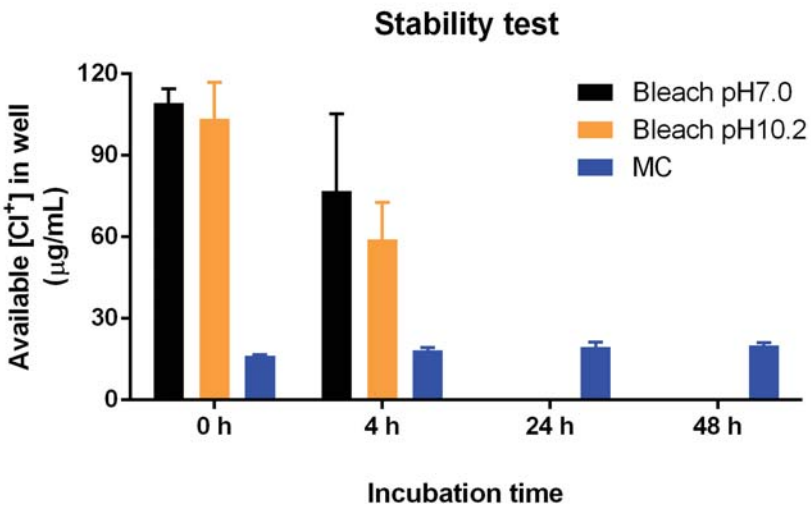


Figure 4.5 Stability of MC and bleach solutions.

Substantial evidences have proved the correlation between gene mutation with many human genetic diseases and cancer (Erickson 2010; Mortelmans and Zeiger 2000). The bacterial reverse mutation assay provides a rapid, inexpensive and easy method to predict mutagenetic and carcinogenic potency of unknown chemicals in mammals (OECD 1997). Therefore, a standardized bacterial reverse mutation assay was performed for MC and TMIO, and their mutagenicity effects were shown in Table 4.4. Results showed that, without external metabolizing enzyme system, no increase of revertant number was observed in either strain of TA98, TA100, TA102, TA1535 and TA1537 within the range of 50-5,000 μg/plate. The result was similar to those external metabolizing enzyme system (rat liver S9) was added. Since MC has very potent antimicrobial activity, four out of five testing strains were totally killed at the concentration of 100 μg/plate, therefore 100 μg/plate of MC was used as the top concentration for Ames assay. Within the range of 6.25-100 μg/plate of MC, no indication of mutagenicity was

observed with and without external metabolizing enzyme system. It was of interest to find that 100 μg/plate of MC had no inhibition effect on the bacteria growth on the plates after metabolizing enzyme system was added. This might be because the *N*-halamine functional group (>N-Cl) in MC was compromised or metabolized by the organic components or enzyme system in rat liver. Ames test can only detect the adverse effect on the gene level, and more *in vitro* and *in vivo* tests on the chromosome level (e.g. micronucleus test) are needed in the future.

Chlorine bleach is currently the most widely used disinfectants due to high-effectiveness and low-cost. It is especially useful as food contact sanitizers and disinfectant for water treatment. However, the chlorine bleach also has several limitations including poor stability, which it will cause irritation and inhalation hazard and has explosion potential. Although chlorine based salts such as calcium chlorite has better stability, but they are still irritating. Additionally, the free chlorines in solutions will react with organic components to form toxic byproducts such as chloramines with mutagenicity (Laingam and others 2012). However, MC is quite stable in water solution and the free chlorine concentration was very low. Because MC has extremely low hydrolysis constant in water solution, which could not readily be measured (Tsao and others 1991). Therefore, MC will provide comparable biocidal effect as bleach; however, this antimicrobial activity can last for longer time than bleach. The potential inhalation and irritation hazard of MC will also be much lowered. Previous reports showed that MC has no

Table 4.4. Mutagenicity effects of TMIO and MC on different *S.* Typhimurium strains in Ames assay.

Treatments (µg/plate)		*S.* Typhimurium strains									
		TA 98		TA 100		TA 102		TA1535		TA1537	
		-S9[a]	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
PC[b]		301±8	1851±51	1699±66	2669±483	1340±111	931±154	909±77	380±43	758±128	595±92
EtOH		20±5	23±2	129±10	113±16	326±17	531±27	9±3	10±3	10±2	11±2
H_2O		22±7	27±7	156±12	118±6	318±11	536±35	16±2	9±3	12±4	12±1
TMIO	5000	22±7	31±10	101±13	99±13	352±34	504±58	9±0	13±2	7±1	13±1
	1582	16±4	27±2	132±10	107±10	340±15	531±27	11±1	11±2	9±1	9±3
	500	18±5	31±1	119±10	123±6	381±14	451±26	8±2	10±3	13±2	11±2
	158	20±1	25±1	127±8	111±10	345±11	525±55	7±2	9±2	10±1	8±1
	50	19±10	27±5	108±9	115±11	346±16	509±26	8±2	16±3	13±4	13±1
MC	100	0	23±3	0	98±7	0	535±90	0	19±2	7±3	6±4
	50	23±6	25±3	143±17	101±11	313±2	477±23	5±2	15±2	13±4	11±4
	25	23±2	28±7	124±3	93±10	341±10	445±34	7±2	18±3	9±2	9±2
	12.5	19±5	31±3	123±21	103±2	351±20	445±16	7±1	12±3	9±4	11±2
	6.25	23±1	27±1	114±5	98±13	344±12	419±56	6±0	12±4	7±2	7±1

a. Tests were carried out in the presence (+S9) or absence (-S9) of an external metabolizing enzyme system (rat liver S9 mix). All tests were repeated with triplicated plating. Data are presented as number of revertants (mean ± SD).

b. PC, positive control chemicals (1) -S9: TA98 and TA1537, 4-NOP (10 µg/plate); TA100, NaN_3 (5 µg/plate); TA1535, 9-AAC (50 µg/plate); TA102 MMC (5 µg/plate); (2) +S9: all tester strains, 2-AA (5 µg/plate).

irritation to animal skin (Parent 2000). In another experiment, MC was coated on fabrics and tested for the irritation in a 3D reconstructed human skin model and no irritation was found (data were not shown here). Another important thing is the MC will be decomposed into TMIO after a certain period (as shown in Figure 4.6). TMIO is not mutagenetic or cytotoxic, which is good to pass through the regulations of environmental concerns.

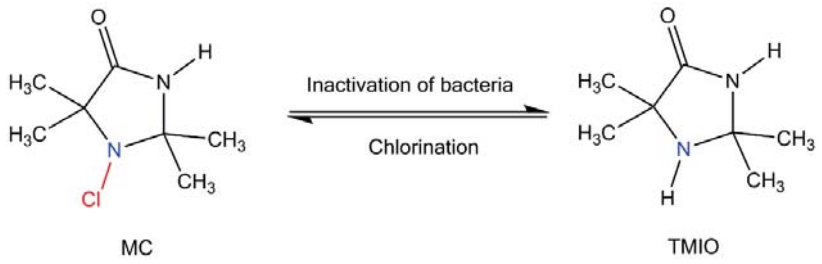


Figure 4.6 Transformation of MC to TMIO.

4.4 Conclusions

This study, for the first time, disclosed important information about the toxicity effects of antimicrobial *N*-halamine compounds. Results showed that MC might have cytotoxic effect; however, this cytotoxicity may be solely caused by the oxidative chlorines in the structure. TMIO, as the precursor compound of MC, was suitable to be classified as non-toxic or non-classified. Neither MC and TMIO was found to cause mutagenicity effect at the maximum testing concentrations. Oxidative chlorines in MC were more stable compared with these in chlorine bleach, which minimized the hazard of inhalation. Although more thoroughly toxicity studies including *in vivo* assays are warranted in the future, results from this study suggest MC,

together with other *N*-halamine compounds that share the same functional group, may be safe for the application as novel, non-bleaching and long-lasting antimicrobials in the food, agricultural and biomedical industries. The regulation of *N*-halamine may be suitable to refer as existed chlorine-based antimicrobial pesticide products.

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Chapter 5

Conclusion and Outlook

This study systematically examined the application methods and efficacies and toxicity information of *N*-halamine antimicrobials for applications in the food industry. This PhD work has laid foundations for future research about applying *N*-halamines in the food industry through proving the following results:

1. *N*-halamine antimicrobial coatings were successfully developed on the surfaces of TPU and stainless steel 316 through bulk modification and electroplating methods
2. These newly developed *N*-halamine coatings showed potent and rechargeable antimicrobial functions
3. *N*-halamine compound had no mutagenicity and its cytotoxicity mechanism might be the same as other chlorine based agents

Currently, the research of *N*-halamines for food industry application just started and is insufficient. More research about engineering and safety assessment of *N*-halamines are still needed for wide applications in the food industry. *N*-halamines may have big impact in the future as high-effective, low-cost and safe antimicrobial technology for food safety controls in the food industry.

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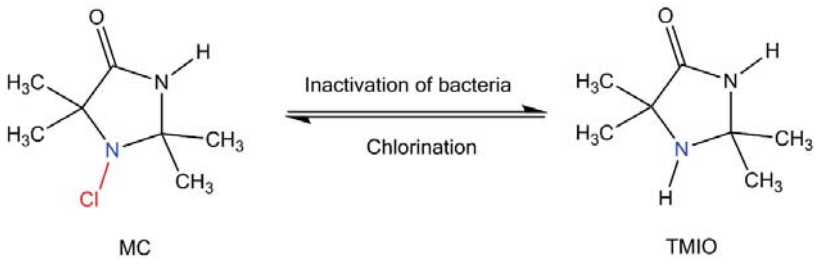

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2. These newly developed *N*-halamine coatings showed potent and rechargeable antimicrobial functions
3. *N*-halamine compound had no mutagenicity and its cytotoxicity mechanism might be the same as other chlorine based agents

Currently, the research of *N*-halamines for food industry application just started and is insufficient. More research about engineering and safety assessment of *N*-halamines are still needed for wide applications in the food industry. *N*-halamines may have big impact in the future as high-effective, low-cost and safe antimicrobial technology for food safety controls in the food industry.

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