ANTIMIBROBIAL PROPERTIES OF CITRIC ACID BASED POLYMERS

by

LEE-CHUN SU

Presented to the Faculty of the Graduate School of

The University of Texas at Arlington in Partial Fulfillment

of the Requirements

for the Degree of

MASTER OF SCIENCE IN BIOENGINEERING

THE UNIVERSITY OF TEXAS AT ARLINGTON

December 2012

ACKNOWLEDGEMENTS

I would like to express her sincere appreciation to her major professors, Dr. Kytai T. Nguyen and Dr. Jian Yang, for their guidance and encouragement throughout this work. I would also like to thank Dr. Liping Tang for many valuable suggestions and Dr. Yi Hong for his participation in this research work as a member of her thesis examination committee.

I wishes to thank Dr. Yi Zang for providing the needed polymeric materials during research and Sonia Santimano for her friendship and advice.

The author also wishes to express her appreciation of support and encouragement she received from her family.

October 15, 2012

ABSTRACT

ANTIMICROBIAL PROPERTIES OF CITRIC ACID BASED POLYMERS

Lee-Chun Su, M.S. of Bioengineering

The University of Texas at Arlington, 2012

Supervising Professors: Kytai T. Nguyen, Jian Yang

A series of citric acid derived polymers synthesized in our lab were investigated and compared for their physiochemical and antimicrobial properties for use in various biomedical applications. We selected citric acid based polymers since citric acid is a product of natural metabolism. These polymers are not only biocompatible but also possess an antimicrobial effect. In addition, citric acid (CA) provides profound functional groups available for binding and crosslinking to allow the controllable crosslinking degrees of polymers. Since bacterial infections became a major issue in medical devices, it is mandatory to determine the antimicrobial properties of materials used for these systems. Our results of investigations concluded that citric acid derived polymers, although did not perform a comprehensive inhibition to bacterial survival, certainly reduced bacterial growth on the materials. Among studied samples, Poly(diol-citrate) (POC) showed relatively superior suppression. The rationale could be the higher ratio of CA in POC. Further studies are needed to evaluate in this respect. Furthermore, peptides or surface modifications with antimicrobial peptides and/or antibiotics can be done to enhance the antimicrobial properties of these polymers for future use.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

Figure Page

LIST OF TABLES

Table Page

CHAPTER 1

INTRODUCTION

1.1 Wound healing

Skin is the largest organ in the integumentary system and provides the most effective protection for our bodies against pathogen invasions. The skin tissue possesses three layers including epidermis, dermis and hypodermis. The outer most epidermis works as a layer of barrier for external environment. It is covered by tightly bound keratinocytes. The differentiated form of these epithelial cells, called stratum conium, mainly provides the barrier function. Dermis, the middle layer of skin tissue, is composed of connective tissue, blood vessels, hair follicles and sweat glands [1]. When damages occur on the skin, cutaneous tissue spontaneously activates reactions for wound repairing. Wound healing is a complex biological reaction involving a variety of cellular activities. Basically, three stages are included during the normal healing process (Figure 1.1):

1. Inflammatory Phase – Immediately after injury, platelet formed clotting at the wound site soon retards bleeding and the system reaches hemostasis state. Inflammatory cells such as neutrophils, macrophages, and red blood cells migrate to the wound site to scavenge bacteria and cell debris, called phagocytosis, therefore, to prepare the injured tissue for healing. At inflammatory phase, tissues are usually red, swollen and painful. The symptoms will be subsided after 2 to 4 days.
2. Proliferation Phase – This phase overlaps with the ending of the inflammatory phase by a day or so. During proliferation phase, fibroblasts appear at the wound site and reconstruct connective tissues by synthesizing collagen. In addition, they also secrete endothelial cells. Fibroblasts and endothelial cells together form granulation tissue
3. growth factors to induce angiogenesis by promoting proliferation and migration of

which serves as a foundation of scar tissue. The last step of proliferation phase is Epithelialization which includes the regeneration, migration, proliferation, and differentiation of keratinocytes. This takes approximately 4 to 8 weeks before the next phase takes place.

4. Remodeling and Maturation Phase – This stage can last from 3 weeks to 6 months. It is a process of collagen fiber remodeling. Specifically, scar tissues reform themselves by simultaneous synthesis and lysis of collagen. Nerve endings are growing and the tissue can be finally remodeled. A tugging feeling from a deeper area might still continue at this stage before the new tissue completely stabilizes [2].

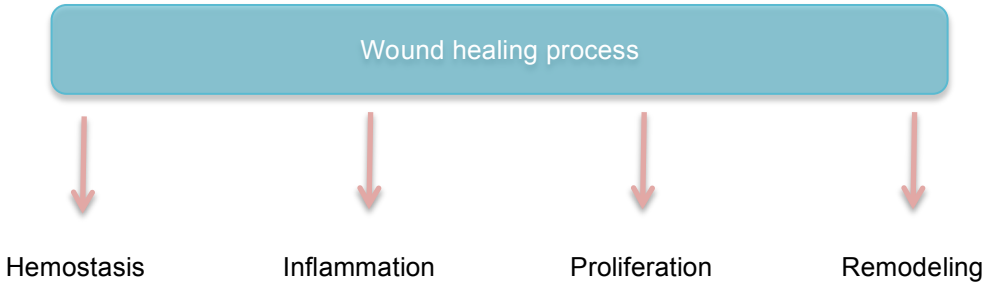


Figure 1.1 Scheme of wound healing process for skin injury

1.2 Bacterial morphology and infection

Escherichia coli and Staphylococcus aureus are two of the common contaminants in skin infection. They are Gram positive and Gram negative bacteria respectively identified by their characteristic staining properties during Gram's methods of staining. The two types of microbes are similar internally but very different externally. Structurally, a thin layer of peptidoglycan and an additional outer membrane can be observed in a Gram negative bacterium. However, a gram-positive bacterium has only a single thick layer of peptidoglycan

[3]. The important difference stands on the membrane characteristics of these two bacterial types. A Gram negative cell wall is more complicated than a Gram positive one both in structure and in chemical compounds. Structurally, an external peptidoglycan is layered on cytoplasmic membrane which is unique to Gram negative bacteria. The area between external surface of cytoplasmic membrane and internal surface of outer membrane is referred to as the periplasmic space [3]. This space is actually a compartment containing a variety of hydrolytic enzymes including proteases, phosphatases, lipases, and so on, which are important to breakdown large macromolecules for cell metabolism. There are no teichoic or lipoteichoic acids in the Gram negative bacterial wall. In the case of pathogenic Gram negative cells, many of the lytic virulence factors are stored in this space [4].

On the other hand, a Gram positive bacterium has a thick, multilayered cell wall consisting mainly of peptidoglycan surrounding bacterial cytoplasmic membrane. The peptidoglycan is essential for the structure, replication and survival during bacterial growth. During infection, the peptidoglycan can interfere with phagocytosis and has pyrogenic activity. Furthermore, other components like teichoic and lipoteichoic acids, and complex polysaccharides are also included in a Gram positive cell wall. These are common surface antigens that distinguish bacterial serotypes and promote attachment to mammalian cells [5].

Pathogens enter the human body through several ways including from mucous membranes and open wounds on skin. Normally, a large number of bacterial invasions are required to cause diseases, as they could be easily defeated by the body host defenses. Even when large numbers of bacteria invade the body, a latent phase is still needed before a pathologic reaction can take place. A severe infection reaction overwhelms the immune defensive system, thus inflammation phase becomes chronic. The prolonged inflammatory not only postpones the process for wound repair but also possibly damages host cells [6].

Generally, many microbes entering a host can be destroyed in lysosomes by phagocytosis. Only when microbes manage to escape from this mechanism do they become harmful to the bodies. Basically, they invade in four ways [7],

1. By using host's nutrients (mainly iron): some pathogens produce proteins called siderophores, which help to steal iron from transport proteins and make it available to bacteria.
2. By causing direct damage in the immediate area of the invasion: pathogens can multiply and kill host cells. Some bacteria can induce engulfment by host cells and be brought to deeper tissue for further damages.
3. By producing toxins: pathogenic toxins may be transported by blood and lymph to damage sites far from the original invasion, called toxigenicity. This is the main mechanism for bacterial infections.
4. By causing the host to react with a hypersensitivity reaction.

1.3 Wound dressing and materials

As we know an opening wound interrupts the integrity of skin structure which allows pathogen invasion and causes infections. Therefore, proper wound care is essential to avoid infectious complications. Wound dressings and other wound products designed to protect skin defects from infections and further recover the healthy physiological functions of skin tissues have been developed for decades. An ideal material for wound healing evolvement should be biocompatible, allowing water containment, and resistance to secondary infections [8]. Record of covering wounds can be tracked back in human history as Egyptians treated wounds with natural biomaterials such as plasters of honey and plant fibers [9]. Since then, materials used for wound care have been slowly evolved from readily available materials in nature to more customized man-made materials (Table 1.1).

Table 1.1 Examples of commercial antimicrobial dressings

Product Name	Dressing format	Manufacturer
Acticoat absorbent	Silver in calcium alginate	Smith & Nephew, Inc, Largo, FL, USA
Iodosorb	Iodine in gel or paste	HealthPoint Ltd, Ft. Worth, TX, USA
Kerlix AMD Gauze	PHMB in Gauze	Tyco Healthcare/Kendall, Mansfield, MA, USA
Aquacel AG	Ionic silver in Hydrofiber	Convatee, Skillman, NJ, USA
Contreet H	Hydrocolloid	Coloplast Corp, Marietta, GA, USA
Contreet F	Foam	Coloplast Corp, Marietta, GA, USA
Silvasorb Antimicrobial Silver Dressing	Ionic silver in Kydrogel sheet or amorphous gel	Medline Industries, Inc, Mundelein, IL, USA

Generally, biomaterials approved by the United States Food and Drug Administration (FDA) to use in wound dressings include naturally derived materials (e.g., collagen and alginate) and synthetic polymers (e.g., polyglycolic acid (PGA), polylactic acid (PLA), and poly(lactic-co-glycolic acid) (PLGA)). Applying synthetic materials is relatively beneficial because they can be manufactured reproducibly on a large scale with controlled properties as well as their flexibility of being tailored chemically, structurally and mechanically [10-12]. However, the universal challenge of using these devices is the infection occurred by microbial-contamination.

It is well-known that infection is a major medical issue. Serious complications, which may result from these infections, include tissue destruction, premature device failure, and the spread of the infection to other areas [13]. The proliferation of microorganism is the stimulus to the cascade of body defensive response; for example, our immune response can be modulated by the presence of some forms of bacterial biofilms [14]. To prevent and control secondary biomaterial related infections, many trials including the incorporation of antimicrobial additives into the device matrix or a coating of antibiotics on the device surface were studied. A list of current commercialized antimicrobial products is shown in Table 1.1. Their effectiveness is usually limited because of rapid loss of antibiotic and confined duration of device functionalities [15]. For example, silver atoms are frequently contained in dressing matrix due to the fact that they are toxic against a broad range of microbes [16]. Ionized silver prevents microbe proliferation because its positive charge would bind to negatively charged particles like proteins, DNA, RNA, and chloride ions [17]. However, the highly reactive positive charge is also responsible to readily bind silver ion to protein and chloride in the wound bed fluid which hinders silver delivery efficiency and limits its active duration [18]. In addition, other issues including leaking of antimicrobial agents (triclosan or iodine) from the system impede the functions of wound dressings.

Antimicrobial polymers have been taken into account to be feasible alternatives for antibacterial applications because of their " non-leaching" potential. In this research, we studied a series of citric acid-based biodegradable polymers for their microbe growth suppression. Citric acid occurs naturally in the body and is an important intermediate in the metabolism. It exists closely in human life from being added as additives in food/drinks, to being used as a filler in dental treatments [19]. Besides its biocompatibility/hemo-compatibility, citric acid is also highly germicidal effective [20, 21]. Therefore, incorporating citric acids in polymer synthesis is a reasonable attempt to promote the antimicrobial applications of biomaterials.

Five different citric acid derived polymers synthesized in our lab are the focus of this

research. These polymers, poly(*1,8*-octanediol-co-citric acid) (POC) [22], biodegradable photoluminescent polymer (BPLP) [23], crosslinked urethane-doped polyester (CUPE) [24], poly(octamethylene maleate (anhydride citrate) (POMC) [25], and poly(ethylene glycol) maleate citrate (PEGMC) [26], were all elastomers and have been intensely studied for their tissue engineering applications. This group of polymers was composed with different ratios of citric acid and other non-toxic monomers through poly-condensation reactions. Hereby, we tried to evaluate their properties, including physical and biological properties. The antimicrobial activity of these citric acid incorporated polymers against the gram-positive S. aureus bacterium and gram-negative E. coli was specifically investigated to understand their potential to resist microbial-induced infection.

CHAPTER 2

OBJECTIVES

A group of citric acid polymers were synthesized in our laboratory. Applications of these polymers mainly contribute to Tissue Engineering such as fabrication of vascular grafts, bone tissue regeneration scaffolds, and diagnostic purposes [23-27]. Our goal in this project is to explore the antimicrobial properties of these citric acid-based polymers. To achieve that, two aims are involved. Specifically, the first aim was focused on the polymer synthesis and characterization to study their potential as wound dressing materials. In the second aim, experiments were performed to investigate their anti-bacterial effects against both gram-positive (S. aureus) and gram-negative (E. coli) pathogens. As we know, citric acid solution creates a low pH environment and is effective in inhibition of microbial proliferation. By incorporating them into polymer synthesis, we potentially developed a system with readily antimicrobial effective properties, which overcomes many limitations of current commercial products. The significance of this research is the possible contribution of citric based polymers in wound dressing evolution.

CHAPTER 3

EXPERIMENTAL PROCEDURES – MATERIALS AND METHODS

3.1 Materials

All chemicals for polymer synthesis were purchased from Sigma-Aldrich (Milwaukee, WI). Escherichia coli (E. coli 25922) and Staphylococcus aureus subsp. Aureus (S. aureus 25923) were purchased from ATCC. LB Broth was from Sigma-Aldrich (Milwaukee, WI). Bacteriostatic wound dressing (4”×4”) was kindly donated by Hydrofera, LLC.

3.2 Methods

3.2.1 Polymer Synthesis

Poly (diol-citrate) (POC), Biodegradable photoluminescent polymer (BPLP), Cross-linked urethane-doped polyester (CUPE), Poly(octamethylene maleate (anhydride) citrate) (POMaC), and poly(ethylene glycol) maleate citrate (PEGMC) were synthesized according to our previous work [23-27]. Generally, different monomers were added to a 250 ml three-necked round bottom flask fitted with an inlet and outlet adapter. The mixture was melted under a flow of nitrogen gas by stirring at 160–165 °C in a silicon oil bath. The temperature of the system was subsequently lowered to 140 °C under Nitrogen purge and allowed to react to get different pre-polymers. The molar ratios of citric acid to monomers are listed in Table 3.1. Next, POC, BPLP, CUPE, and POMaC pre-polymers were dissolved in 1,4-dioxane and precipitated in DI water for purification. Of these polymers, only PEGMC was purified by dialysis (with 500 Dalton molecular weight cut off membrane for 2 days). All pre-polymers were dried by lyophilization. For CUPE, an additional step was needed to conduct urethane dope process. The pre-polymer was re-dissolved to make a 3% (w/w) solution in 1,4-dioxane. 1,6-Hexamethylene diisocyanate (HDI) was added to the pre-polymer solution (1:0.9, citric acid: HDI molar ratio). Stannous actuate was used as catalyst. Further, to prepare thermal crosslinked polymer films, all pre-

polymers were heated at 120 °C under vacuum (2 Pa) for 1 week. Photocrosslinked POMaC and PEGMC were prepared by using 2-hydroxy-1-4(hydroxyethoxy)phenyl-2-methyl-1 propanone (Irgacure 2959) as a photo initiator and a 365 nm ultraviolet light (UVP, Upland, CA) at room temperature. A scheme showing synthesis procedure is presented in Figure 3.1.

Table 3.1. Monomer ratios and crosslinking methods of polymer fabrication

Polymer	Molar ratio of citric acid	Type and molar ratio of diol	Type and molar ration of third compound	Crosslinking method
POC	1.0	1,8-octanediol: 1.0	N/A	Oven Heat
BPLP	1.0	1,8-octanediol: 1.0	L-Cysteine: 0.2	Oven Heat
CUPE	1.0	1,8-octanediol: 1.1	HDI: 0.9	Oven Heat
POMC	0.6	1,8-octanediol: 1.0	Maleic anhydride: 0.4	Oven Heat and UV
PEGMC	0.6	PEG200: 1.0	Maleic anhydride: 0.4	Oven Heat and UV

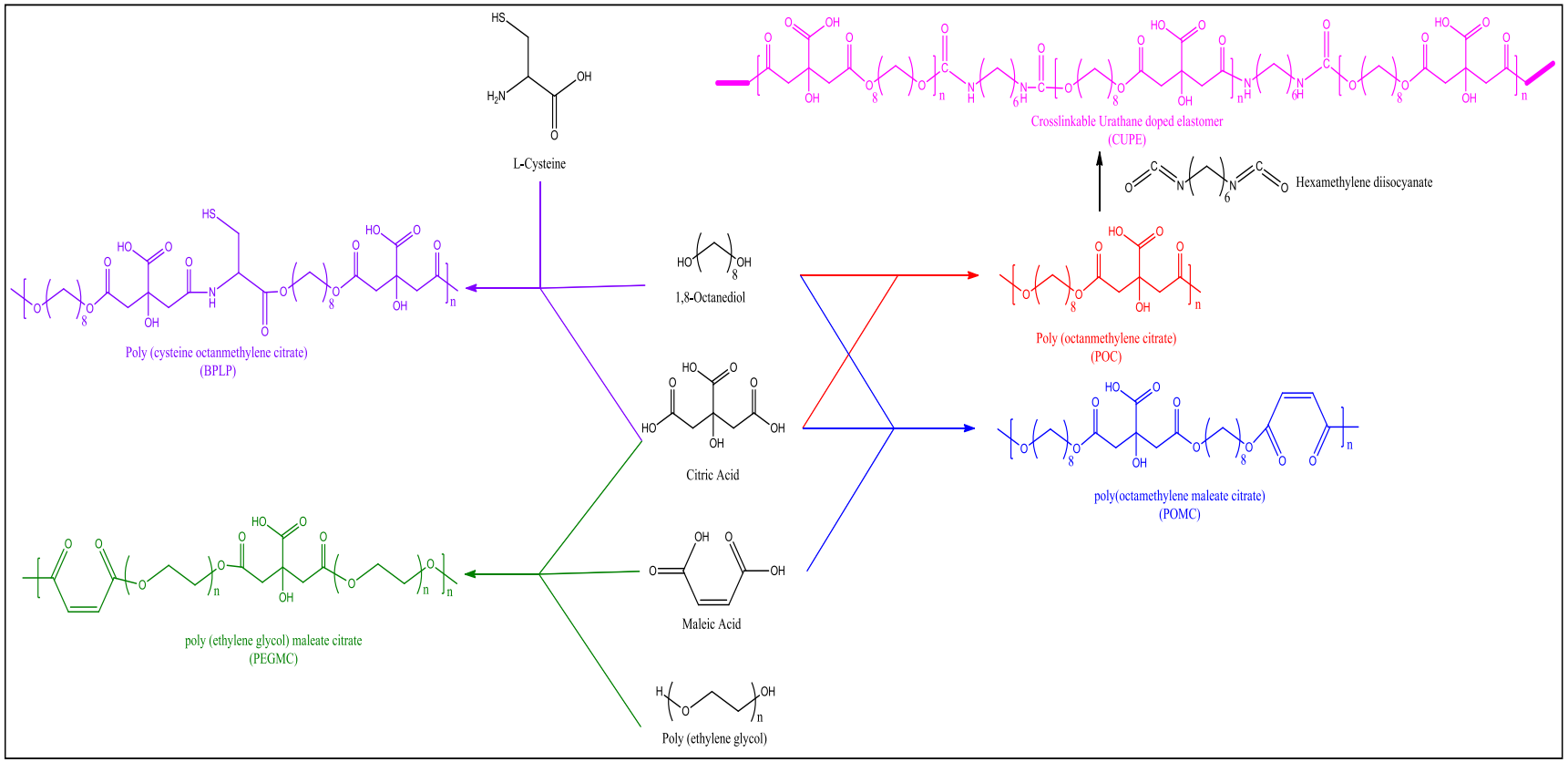


Figure 3.1 Chemical structure and synthesis routes of citric acid derived polymers.

3.2.2 Water Uptake

Polymer discs prepared as above were incubated in deionized water until the equilibrium state was achieved (up to 50 hours). The surface of the swollen discs was gently blotted with filter papers to remove any excess swelling agent. The samples were then weighed (M_w). The discs were again lyophilized for 3 days and weighed to determine the dry weight (M_d). Equation (1) calculated the equilibrium-swelling ratio.

$$\text{Swelling (\%)} = (M_w - M_d) / M_d \times 100 \quad (1)$$

3.2.3 In Vitro Degradation

Degradation studies were conducted in both PBS (pH 7.4). 6 cylindrical disc specimens (7 mm in diameter; 2 mm thick) were cut from crosslinked, purified, and lyophilized polymers using a cork borer. The samples were weighed, placed in a tube containing PBS (10 ml) for up to 60 days, and incubated at 37 °C. At the predetermined time, samples were thoroughly washed with deionized water, lyophilized for 1 week, and weighed. The mass loss was calculated by comparing the initial mass ($\mathbf{W}_0$) with the mass measured at the pre-determined time point ($\mathbf{W}_t$), as shown in Equation (2). The results are presented as the means $\pm$ standard deviation (**n=**6).

$$\text{Mass loss (\%)} = (W_0 - W_t) / W_0 \times 100 \quad (2)$$

3.2.4 Microbial Culture And Optical Density

Gram-negative E. coli and Gram-positive S. aureus were reconstituted based on product instructions and incubated on an orbital shaker at 37°C overnight for cell expansion. Before the study, optical density (OD) of bacteria suspension was adjusted to 0.07 at 600 nm (measured by UV-vis spectrophotometer), which corresponds to the approximate cell density of

McFarland Standard solution #1 (3×10^8 CFU/ml), and diluted 100 folds to reach an optimized concentration as previously described [28]. Polymer scaffolds (50mg) were then added to bacterial suspension and incubated with constant shaking for 0 to 28 hours at 37°C. Scaffolds incubated with broth only were prepared as background. 2.5μg/ml ampicillin and 29mg/ml citric acid (based on the average amount used for polymer synthesis) were prepared separately in broth and served as positive controls in the study. All operation was done in aseptic conditions.

3.2.5 Antimicrobial Assay

Scaffolds were soaked with sterile PBS for 1 hour and then cut into 0.8 mm round discs. 10 μl bacterial suspension was pipetted onto CA scaffolds (N=3) in 48 well plates and incubated for 1 hour at 37°C. Here, Hydrofera Blue scaffold serves as positive control and PLGA serves as negative. 1 ml broth was added to the well and vigorously shaked for 3 minutes. Serial 10-fold-dilutions of cell suspensions were then seeded on agar plates which were incubated for 48 hours at 37°C. The antimicrobial effect of scaffolds was calculated by the % reduction of counted colony forming units (CFU) before and after incubating with samples.

3.2.6 Microbial Morphology Observation With SEM

10 μl bacterial suspension was sprayed on 8mm-diameter scaffolds and incubated for 2 hours at 37°C. The scaffolds were then immediately fixed with 2.5% glutaraldehyde for 20 min and washed with PBS at least three times. Samples were then dehydrated in a graded ethanol series (50%, 75%, 95%, and 100%). Final drying process was done over night before samples were subjected for imaging.

CHAPTER 4

RESULTS AND DISCUSSION

4.1Water Uptake of CA Polymer Scaffolds

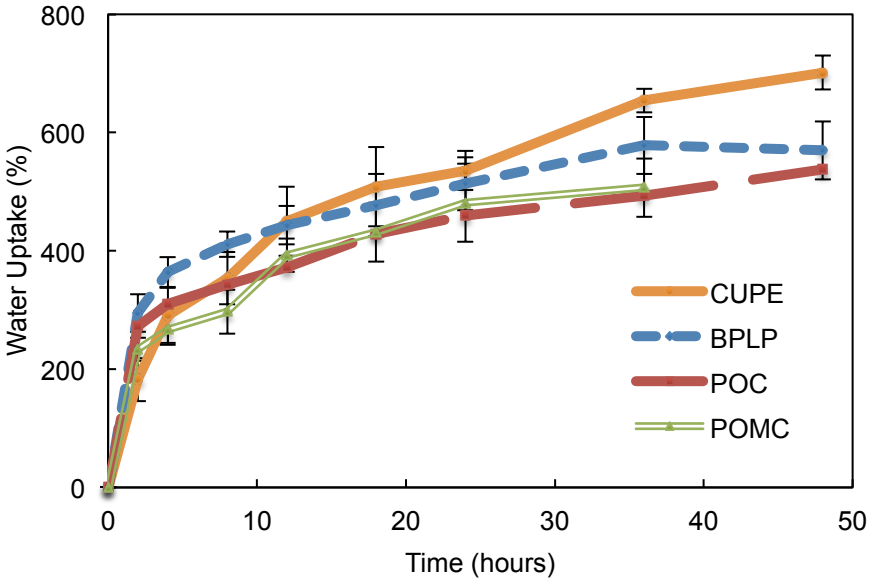


Figure 4.1 Water uptake/swelling of citric acid based polymer scaffolds for up to 50 hours. Samples were weighted at each time point. Amount all, CUPE showed the highest water uptake; whereas POC and POMC had the least water uptake.

Figure 4.1 showed the sol content of a series of CA polymeric scaffolds. All scaffolds are able to uptake water. Result showed poly(vinyl acetate) (PVA) based Hydrofera Blue had ~ 1000% water uptake which was significantly higher comparing to other polymers (data not shown). The water content increase during the study of CUPE, BPLP, POC, and POMC was in

the range of 400%-600% in which CUPE had the highest uptake; POC and POMC had the least. In addition, UV crosslinked POMC scaffolds showed higher water uptake than oven cross-linked samples, suggesting a lower crosslinking density for UV treatment (data not shown).

4. 2 Degradation of CA Polymers

Degradation rate varies with polymer chemical, physical structure as well as crosslinking approaches. The data for degradation characterization of CA scaffolds are presented in Figure 4.2, which shows all CA based polymers are completely degradable. Difference in degradation rates was observed among polymers which might be due to different crosslinking degrees and rigidity of polymer chains. CUPE degrades especially slower than the rest of samples due to urethane bonding cannot be hydrolyzed. We also noticed that polymers crosslinked with heating by oven sustained longer than those with UV exposure (data not shown). This result is consistent with the swelling data indicating a low crosslinking density for

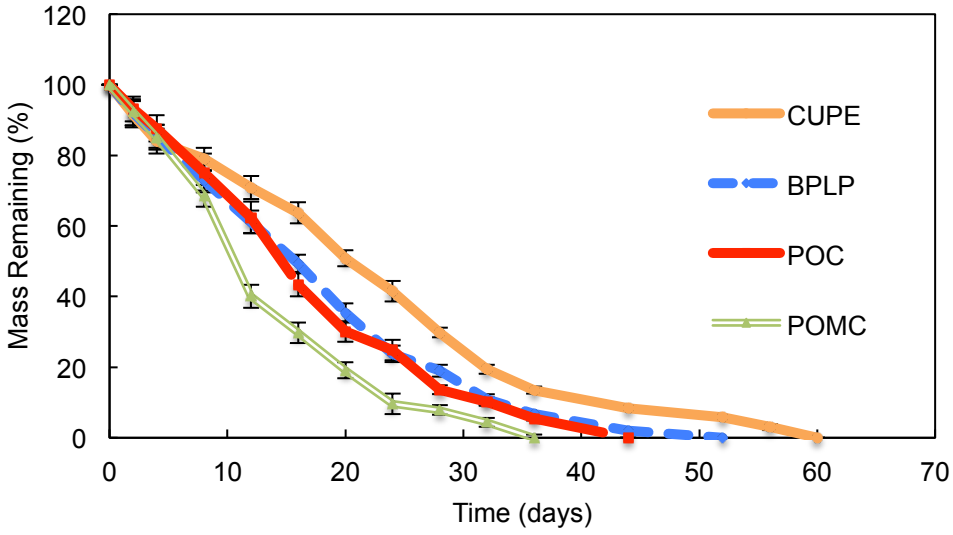


Figure 4.2 Degradation study of citric acid-based polymers up to 60 days. Samples were freeze-dried and weighted at each time point. Our results demonstrated that POMC consisted of the highest degradation and CUPE maintained of lowest degradation.

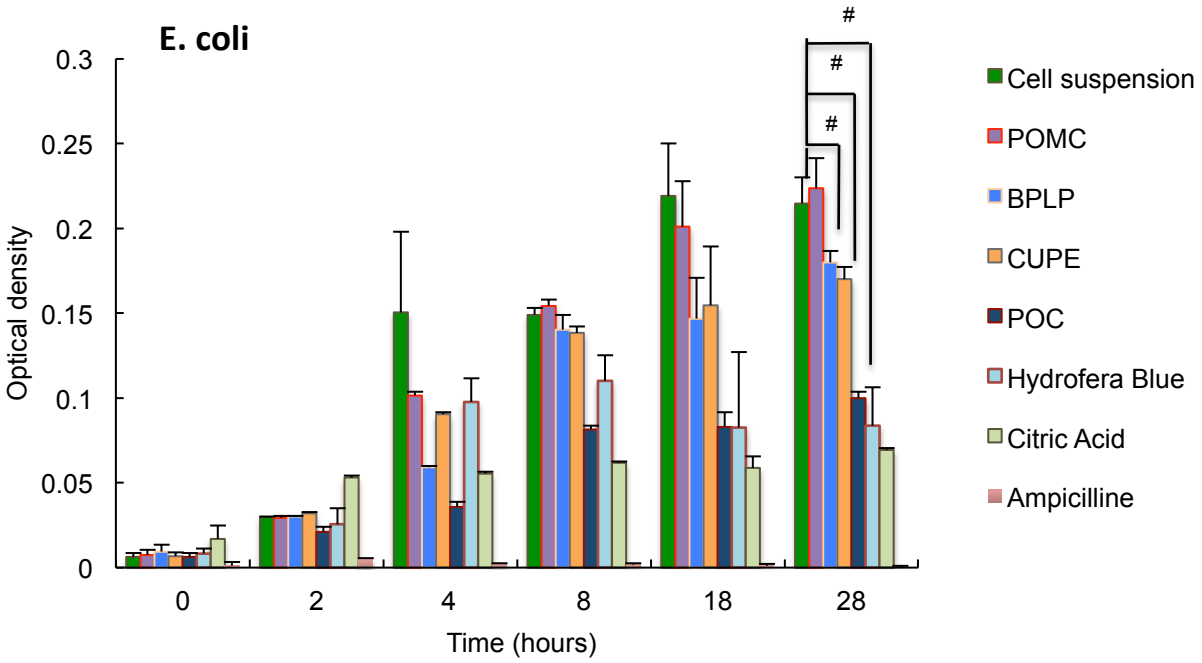
UV crosslinking.

4. 3 Bacteria Growth

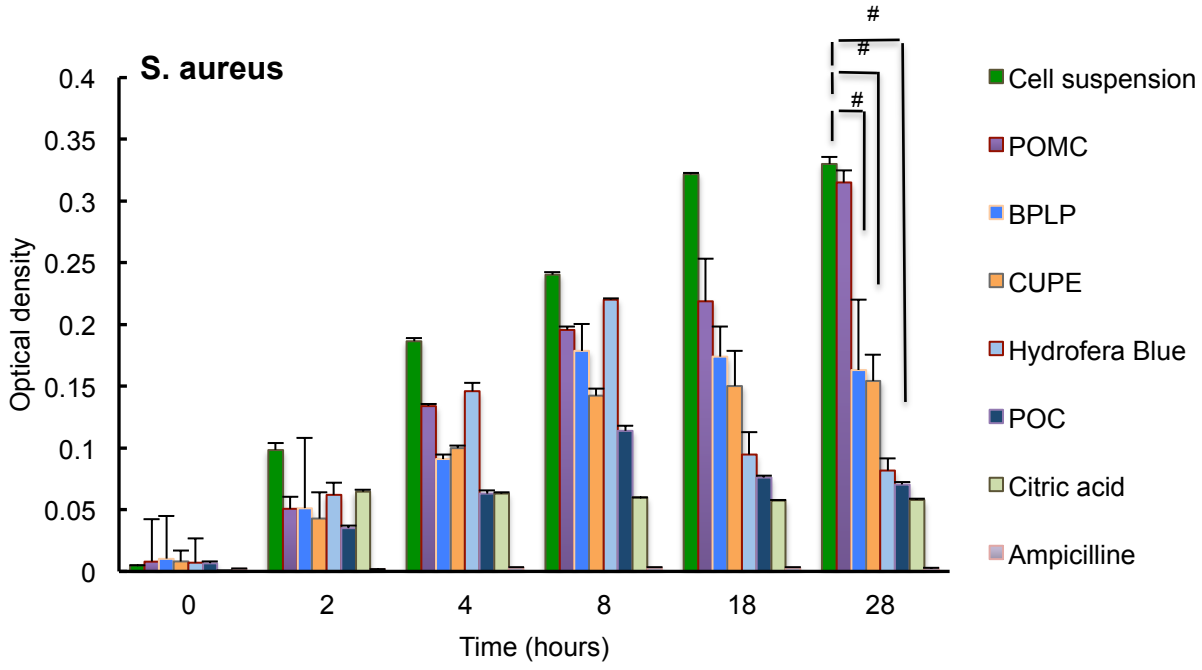
The antimicrobial properties of polymers were evaluated against determining the growth of two bacterial strains, namely E. coli (Gram negative) and S. aureus (Gram positive) via optical/turbulence measurement. Figure 4.3 (a) and (b) show the UV-via spectra of many citric acid based polymers with positive (bacteria/cell suspension) and negative (ampicillin solution) controls as comparisons. For both bacteria strains, POC consistently possesses better suppression for bacterial proliferation whereas POMC shows poor antimicrobial property, having a similar cell growth profile as the positive control (cell suspension only). Other polymers perform intermediate strength against microorganism. The commercial wound dressing, Hydrofera Blue, interestingly showed non-antimicrobial property at the early study time points but gradually activated its germicidal function at the later time points. POC has the anti-microbial activity comparable to that of citric acid and commercial Hydrofera Blue samples.

Polymeric biomaterials became major players in medical implants since decades ago. With attentions arising, their performance is expected to meet multiple requirements in a wide range of applications in tissue engineering scaffolding, drug delivery, wound dressing, diagnostic imaging, and medical device coatings. Since the bacterial infection is a major medical complication when using polymeric biomaterials for these biomedical applications, in this paper, we especially investigated antimicrobial properties of various citric-acid based polymers developed in our lab, which are BPLP, CUPE, POC, POMC, and PEGMC [23-27]. Crosslinking processes of these polymers create ester bonds, which allows hydrolysis during degradation. Moreover, the introduction of maleic acids also provides another approach for crosslinking. Adding maleic acids on backbones, polymers like POMC and PEGMC can be crosslinked through either UV exposure or high temperature. By tuning chemical structure of these citric based polymers, different swelling ratio and degradation rate can be achieved for different applications as various tissue engineering scaffolds. Besides excellent physical

properties as listed above, citric acid usually serves as important additives for antibacterial functions. Ana Allende *et al.* compared and concluded that E. coli proliferation was suppressed in fresh-cut cilantro after treating the cilantro with citric acid containing solution [30]. Xiaorong Fu and his group mixed citric acid as crosslinker with chitosan derivatives in cotton fabrics to create antimicrobial properties. They found 99% of S. aureus and 96% of E. coli was killed when 14% of citric acid was used in the system [31]. The advantages of incorporating citric acid in polymers attribute to two reasons. Firstly, citric acid (CA) structurally provides multiple functional groups, which permits the pre-polymer formation via poly-condensation reaction with –COOH groups, as well as allowing the controllable degrees of ester-crosslinking with –OH groups [25]. Secondly, citric acid is a non-toxic metabolic product of the Krebs cycle and has been approved to use by the Food and Drug Administration in many perspectives [26, 32]. Furthermore, CA is one of the organic polycarboxylic acids. Based on the mechanism of microbes attack, CA contributes to pH reduction, which depresses the internal pH of bacteria by ionizing the un-dissociated acid molecules, therefore altering the permeability of microbial membrane by disrupting their substrate transport [30][33].



(a)



(b)

Figure 4.3 Turbidity change of (a) E. coli and (b) S. aureus contained medium incubated with citric acid based polymers for 28 hours. Bacteria suspension was collected at each time point from each group to measure and compare turbulence. The results indicated that significant suppression of cell proliferation when bacteria incubated with POC scaffolds. And POC group was also the only specimen showed comparative result as the commercial product, Hydrofera Blue. #: $p<0.01$

4. 4 Antimicrobial Assay

The result of antimicrobial assay was presented as a percentage of reduced CFUs before and after incubating with samples shown in Figure 4.4. Hydrofera Blue served as positive control and PLGA scaffolds were negative controls. Among polymers, POC and BPLP scaffolds had more than 80% kill for both grand negative (E. coli) and grand positive (S. aureus) cells, especially POCs whose antimicrobial effect is competitive to the positive control group, Hydrofera Blue scaffolds. Subsequently, around CUPE and POMC scaffolds showed 60%-80% effective reductions of CFU, whereas PLGAs had the least effect of killing, about 10%-20% kill was concluded.

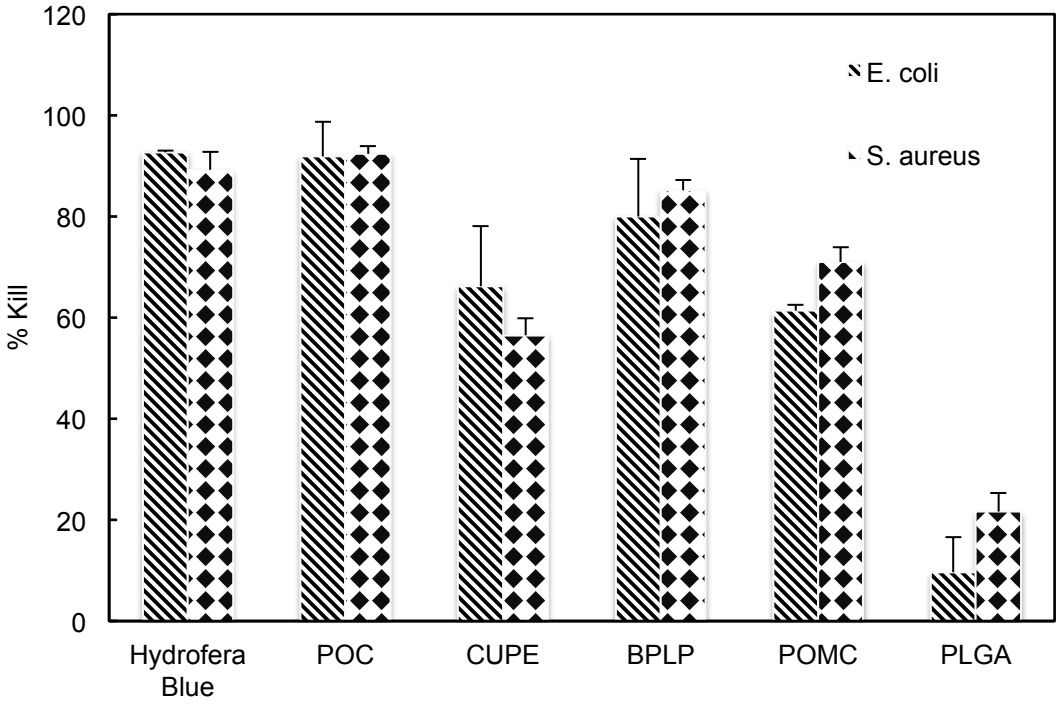


Figure 4.4 Quantification of % Kill of two pathogens by seeding cell suspensions on agar plates after incubating with studied polymer scaffolds. The results were compared as the reduction of CFUs (data was normalized based on CFUs before and after the incubations). Hydrofera Blue served as positive control and PLGA Scaffold were taken as negative control in this study.

4. 5 Morphology of Bacteria

Bacteria seeded on scaffolds were observed with SEM. The results were presented as 10X and 50X images from center and edge areas of the scaffolds. Hydrofera Blue, a highly hydrophilic porous scaffold, was used as the control. E. coli on Hydrofera blue seemed to mainly accumulate and aggregate at the edge of pores. Whereas, on surface of POC scaffold the morphology of cells appears relatively intact but wrinkled; less aggregation was found. Those on BPLP exhibit severe deformation and activation. At the edge of BPLP scaffold, we found a net-like pattern indicating the beginning of biofilm formation [29]. Many cell aggregations were observed on both POMC and CUPE scaffolds. Later stage of biofilm formation was greatly seen to distribute on CUPE scaffold surface. For both strains of microbes, we have also explored similar behaviors among the studied specimens.

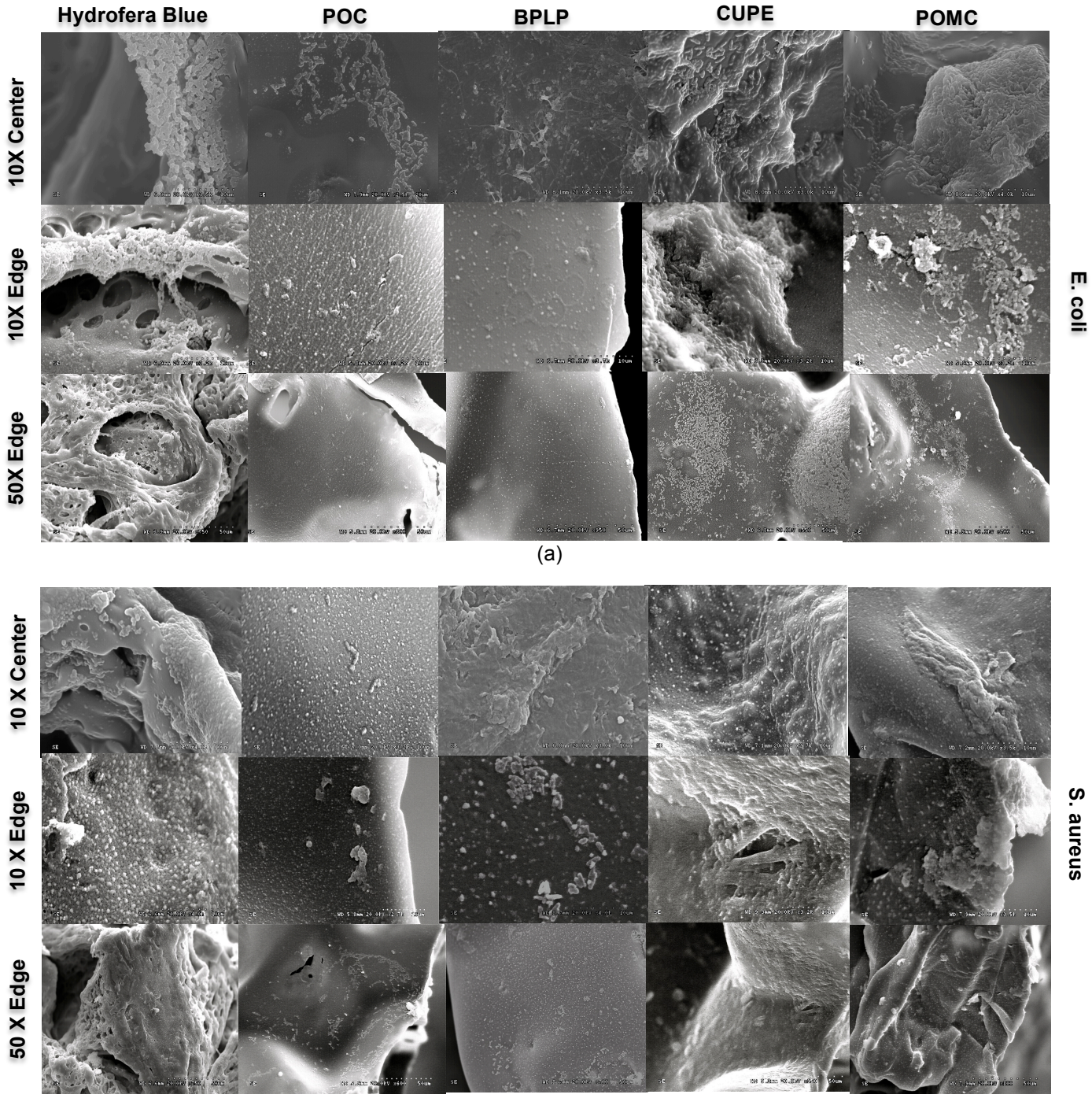


Figure 4.5 Morphology of microbes at the center and edge of scaffold surface through SEM images showing different degrees of (a) E.coli and (b) S. aureus attachment and proliferating behaviors. On highly hydrophilic Hydorfera Blue, microbes tend to accumulate and aggregate at the edge of pores; Less cell growth was observed on POC and BPLP; however, bacteria seemed to be activated on BPLB scaffolds; early stage of biofilm formation was seen on BPLP as well. Large aggregations and abundant bacterial activities were formed on CUPE and POMC scaffold. Especially, late stage of biofilm formation was found on CUPE.

Our investigations indicated different degrees of suppression to bacteria growth among CA-scaffolds. Particularly, POC was the most resistant to cell proliferation when bacteria were presented in a planktonic state. In optical density of cell suspension and CFU reduction, results of POC scaffolds also appeared the most compatible to pure CA solutions toward both E. coli and S. aureus. In fact, POC contains relatively higher ratio of citric acids comparing to other polymers, owing to only two monomers (1,8-octanediol and citric acid with 1:1 ratio) were involved in synthesis.

Based on *in vitro* observation, four steps are usually concluded for patterns of bacterial proliferation: (1) cells adhere on material surface; (2) cells accumulate and aggregate in multiple layers; (3) biofilm formation and maturation; (4) cells detach from the biofilm to a planktonic state for a new cycle of bacterial proliferation [34]. Chuncai Zhou *et. al.* described bacteria surfaces as becoming wrinkled and withered after incubating with their antimicrobial hydrogel coating [35]. In addition, Caitlin C Otto observed E. coli lyse, representing cell death, when samples were treated with mineral leachates [36]. Our SEM images reveal various morphology of bacterial activities including cell attachment, cell aggregation, and biofilm formation on the surface of studied CA scaffolds; as well as membrane surface roughness and deformation. Though we did not see any microbe lyse during our observations, these citric acid contained scaffolds surely disturbed normal microbe survival patterns. A more aggressive antimicrobial mechanism is definitely the next step.

CHAPTER 5

CONCLUSION AND FUTURE WORK

Five different citric acid contained polymers were evolved in my laboratory and their contributions in the field of Tissue Engineering had been published individually [23-27]. These polymers can be tailored to have various physical and chemical properties by varying monomer selection, ratio and crosslinking approaches. Two main aims in this research are, first, *in vitro* characterizations of these polymers as scaffold forms. Moreover, in second aim, we explored their potential antimicrobial properties. Our purpose was to develop a system of "non-leaching" antimicrobial materials as candidates for wound healing products. Two microbes, E. coli and S. aureus, the common pathogens for skin infection, were used in this investigation. From results we concluded CUPE could absorb the most amount of water and withstand the longest for degradation; however, it did not show many antimicrobial properties. Instead, POC scaffolds showed the highest antimicrobial tendency among the studied citric-acid based polymers (BPLP, CUPE, POC, POMC, and PEGMC). Citric acid molecules in the film created an acidic environment which might suppress microbe proliferation [23-27]. POC was synthesized by mixing *1,8*-octanediol and citric acid as one to one ratio. It possesses a higher percentage of citric acid monomers comparing to other involved polymers. Our result also indicated that POC was as anti-bacterially effective as Hydrofera Blue, the commercial patch. Therefore, continuous studies can be carried on with POC for wound healing patches. Future studies will be emphasized on optimization of wound dressing systems developed with citric acid polymers, especially POC. Our goals include to provide and maintain a moist environment for optimal healing, have appreciative mechanical strength, further enhance effectiveness against gram positive and gram negative bacteria, and finally, to be able to promote tissue reconstruction processes while providing the antimicrobial environment to prevent infection *in vivo* [37].

REFERENCES

[1] Rawlings AV, Harding CR. Moisturization and skin barrier function. Dermatol Ther. 2004;17 Suppl 1:43-8.

[2] Morton LM, Phillips TJ. Wound healing update. Semin Cutan Med Surg. 2012;31:33-7.

[3] L.M. Prescott JPH, D.A. Klein. Microbiology. 5 ed: McGraw-Hill; 2002.

[4] Weidenmaier C, Peschel A. Teichoic acids and related cell-wall glycopolymers in Gram-positive physiology and host interactions. Nat Rev Microbiol. 2008;6:276-87.

[5] De Kimpe SJ, Kengatharan M, Thiemermann C, Vane JR. The cell wall components peptidoglycan and lipoteichoic acid from Staphylococcus aureus act in synergy to cause shock and multiple organ failure. Proc Natl Acad Sci U S A. 1995;92:10359-63.

[6] Rojas IG, Padgett DA, Sheridan JF, Marucha PT. Stress-induced susceptibility to bacterial infection during cutaneous wound healing. Brain, behavior, and immunity. 2002;16:74-84.

[7] Bacterial Pathogenesis: Molecular and Cellular Mechanisms: Caister Academic Press; 2012.

[8] Akturk O, Tezcaner A, Bilgili H, Deveci MS, Gecit MR, Keskin D. Evaluation of sericin/collagen membranes as prospective wound dressing biomaterial. J Biosci Bioeng. 2011;112:279-88.

[9] G. M. The healing hand: man and wound in the ancient world. Cambridge: Harvard University press; 1975.

[10] Wiegand C, Abel M, Ruth P, Hipler UC. Superabsorbent polymer-containing wound dressings have a beneficial effect on wound healing by reducing PMN elastase concentration and inhibiting microbial growth. J Mater Sci Mater Med. 2011;22:2583-90.

[11] Costache MC, Qu H, Ducheyne P, Devore DI. Polymer-xerogel composites for controlled release wound dressings. Biomaterials. 2010;31:6336-43.

[12] Leipziger LS, Glushko V, DiBernardo B, Shafaie F, Noble J, Nichols J, et al. Dermal wound repair: role of collagen matrix implants and synthetic polymer dressings. J Am Acad Dermatol. 1985;12:409-19.

[13] Woo GL, Mittelman MW, Santerre JP. Synthesis and characterization of a novel biodegradable antimicrobial polymer. Biomaterials. 2000;21:1235-46.

[14] Christensen GD, Barker LP, Mawhinney TP, Baddour LM, Simpson WA. Identification of an antigenic marker of slime production for Staphylococcus epidermidis. Infect Immun. 1990;58:2906-11.

[15] Bach A, Bohrer H, Motsch J, Martin E, Geiss HK, Sonntag HG. Prevention of bacterial colonization of intravenous catheters by antiseptic impregnation of polyurethane polymers. J Antimicrob Chemother. 1994;33:969-78.

[16] Murphy PS, Evans GR. Advances in wound healing: a review of current wound healing products. Plast Surg Int. 2012;2012:190436.

[17] Ip M, Lui SL, Poon VK, Lung I, Burd A. Antimicrobial activities of silver dressings: an in vitro comparison. J Med Microbiol. 2006;55:59-63.

[18] Wiwanitkit V. Silver dressings. Int Wound J. 2010;7:544.

[19] Scelza MF, Pierro V, Scelza P, Pereira M. Effect of three different time periods of irrigation with EDTA-T, EDTA, and citric acid on smear layer removal. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2004;98:499-503.

[20] Georgopoulou M, Kontakiotis E, Nakou M. Evaluation of the antimicrobial effectiveness of citric acid and sodium hypochlorite on the anaerobic flora of the infected root canal. Int Endod J. 1994;27:139-43.

[21] Smith JJ, Wayman BE. An evaluation of the antimicrobial effectiveness of citric acid as a root canal irrigant. J Endod. 1986;12:54-8.

[22] J. Yang, A. R. Webb, Ameer GA. Novel Citric Acid-Based Biodegradable Elastomers for Tissue Engineering. ADVANCED MATERIALS. 2004;16:6.

[23] Yang J, Zhang Y, Gautam S, Liu L, Dey J, Chen W, et al. Development of aliphatic biodegradable photoluminescent polymers. Proc Natl Acad Sci U S A. 2009;106:10086-91.

[24] Dey J, Xu H, Shen J, Thevenot P, Gondi SR, Nguyen KT, et al. Development of biodegradable crosslinked urethane-doped polyester elastomers. Biomaterials. 2008;29:4637-49.

[25] Tran RT, Thevenot P, Gyawali D, Chiao JC, Tang L, Yang J. Synthesis and characterization of a biodegradable elastomer featuring a dual crosslinking mechanism. Soft Matter. 2010;6:2449-61.

[26] Gyawali D, Nair P, Zhang Y, Tran RT, Zhang C, Samchukov M, et al. Citric acid-derived in situ crosslinkable biodegradable polymers for cell delivery. Biomaterials. 2010;31:9092-105.

[27] Yang J, Webb AR, Pickerill SJ, Hageman G, Ameer GA. Synthesis and evaluation of poly(diol citrate) biodegradable elastomers. Biomaterials. 2006;27:1889-98.

[28] Morgana Eli Vianna D, MSa,, Brenda P.F.A Gomes D, MS, PhDb, Vanessa Bellocchio Berber D, Alexandre Augusto Zaia D, MS, PhDb, Caio Cezar Randi Ferraz D, MS, PhDc,, Francisco José de Souza-Filho D, MS, PhDb. In vitro evaluation of the antimicrobial activity of chlorhexidine and sodium hypochlorite. Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology. 2004.

[29] El Abed SKI, Hassan Latrache, Fatima Hamadi. Scanning Electron Microscopy (SEM) and Environmental SEM: Suitable Tools for Study of Adhesion Stage and Biofilm Formation. In: Kazmiruk V, editor. Physics2012.

[30] Ana Allende JM, Yang Tao, Yaguang Luo. Antimicrobial effect of acidified sodium chlorite, sodium chlorite, sodium hypochlorite, and citric acid on Escherichia coli O157:H7 and natural microflora of fresh-cut cilantro. Food Control. 2009;20:5.

[31] Xiaorong Fu YS, Xue Shen, Xue Jiang, Dan Huang, Yiqi Yan. Chitosan derivatives with dual-antibacterial functional groups for antimicrobail finishing of cotton fabrics. Carbohydrate Polymers. 2011;85:7.

[32] Gyawali D, Tran RT, Guleserian KJ, Tang L, Yang J. Citric-acid-derived photo-cross-linked biodegradable elastomers. J Biomater Sci Polym Ed. 2010;21:1761-82.

[33] Bischof Vukusic S, Flincec Grgac S, Budimir A, Kalenic S. Cotton textiles modified with citric acid as efficient anti-bacterial agent for prevention of nosocomial infections. Croat Med J. 2011;52:68-75.

[34] Mack D, Becker P, Chatterjee I, Dobinsky S, Knobloch JK, Peters G, et al. Mechanisms of biofilm formation in Staphylococcus epidermidis and Staphylococcus aureus: functional molecules, regulatory circuits, and adaptive responses. Int J Med Microbiol. 2004;294:203-12.

[35] Zhou C, Li P, Qi X, Sharif AR, Poon YF, Cao Y, et al. A photopolymerized antimicrobial hydrogel coating derived from epsilon-poly-L-lysine. Biomaterials. 2011;32:2704-12.

[36] Otto CC, Cunningham TM, Hansen MR, Haydel SE. Effects of antibacterial mineral leachates on the cellular ultrastructure, morphology, and membrane integrity of Escherichia coli and methicillin-resistant Staphylococcus aureus. Ann Clin Microbiol Antimicrob. 2010;9:26.

[37] Wittaya-areekul S, Prahsarn C. Development and in vitro evaluation of chitosan-polysaccharides composite wound dressings. Int J Pharm. 2006;313:123-8.

BIOGRAPHICAL INFORMATION

Lee-Chun Su was born in Pescadores, Taiwan on April 20th, 1980. In 2000, she left for Taichung to study in Feng Chia University of Fiber and Composite Materials and received her bachelor degree in 2004. She then pursued graduate work in the Department of Material Science and Engineering at The University of Texas at Arlington, USA. She obtained her Master's degree in Fall 2007 for Master of Science in Material Science and Engineering. She joined Bioengineering department in Summer 2009. Currently, Lee-Chun is a candidate for the degree of Master of Science in Bioengineering to be awarded at Fall commencement in 2012.